



ELECTRON PARAMAGNETIC RESONANCE STUDIES OF ARTIFICIAL SUPRAMOLECULAR STRUCTURES AND BIOLOGICAL SYSTEMS

by

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ABSTRACT

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The research described in this thesis employs a variety of Electron Paramagnetic Resonance (EPR) techniques for the study of the electronic and structural properties of artificial supramolecular porphyrin systems and of protein complexes of biological relevance.

The electron delocalisation in the cationic radical and photoexcited triplet states of linear and cyclic π -conjugated multiporphyrin arrays was investigated. In the radical cations, information on the extent of delocalisation can be inferred from the measurement of hyperfine couplings, either indirectly from the continuous wave EPR spectrum or directly using pulsed hyperfine EPR techniques. The results of room temperature EPR experiments showed complete delocalisation of the electron on the timescale of the EPR experiments, but frozen solution EPR measurements revealed localisation onto mainly two to three porphyrin units in the larger porphyrin systems.

Information on the delocalisation of the triplet state in the same porphyrin systems is contained both in the hyperfine couplings and in the zero-field splitting (ZFS) interaction. The results outlined in this thesis show that the hyperfine couplings provide a much more accurate estimate of the extent of delocalisation. The trends in proton and nitrogen hyperfine couplings with the size of the porphyrin systems indicate uneven spin density distributions over the linear arrays, but complete delocalisation in the cyclic systems. Time-resolved EPR and magnetophotoselection experiments have shown a re-orientation of the zero-field splitting tensor between a single porphyrin unit and longer linear arrays, resulting in alignment of the main optical transition moment and the Z axis of the ZFS tensor.

Continuous wave and pulsed dipolar EPR techniques were employed for the determination of the structure of two different protein complexes, the homomultimeric twin-arginine translocase A (TatA) protein channel and the ferredoxin-P450 complex of the electron transport chain in *Novosphingobium aromaticivorans*. The interaction between nitroxide spin labels introduced at different positions of the TatA monomer was investigated in the complex reconstituted in detergent micelles by analysing the dipolar broadening of the EPR spectra and the results of three- and four-pulse Double Electron-Electron Resonance (DEER) measurements. In combination with results from NMR and molecular dynamics calculations, a structure for the channel complex was proposed.

The structure of the ferredoxin-cytochrome P450 complex was investigated by orientation-selective DEER between nitroxide labels introduced on the cytochrome P450 protein and the iron-sulfur cluster of the ferredoxin. The distance and orientation information contained in the experimental DEER data was interpreted in terms of a structural model of the protein complex by orientation-selective DEER simulations combined with a modelling approach based on protein-protein docking.

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1 INTRODUCTION TO EPR

Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR) spectroscopy studies the interactions of electron spins with an external magnetic field, the interactions between electron spins and the interactions between electron and nuclear spins. This is achieved with a wide range of different continuous wave and pulsed techniques. EPR can be applied to any paramagnetic system, for example organic radicals, triplet states, transition metal ions, defects in solids etc. Typical applications include the characterisation of electronic structure, the study of spin dynamics, the determination of the spatial distribution of paramagnetic species and the characterisation of conformational dynamics of macromolecules.

In this thesis, EPR spectroscopy is used for the investigation of a series of artificial and natural macromolecular systems. Supramolecular porphyrin structures were characterised in terms of electron delocalisation in their radical cation and photoexcited triplet states. Information on the distribution of the unpaired electrons in linear and cyclic multiporphyrin arrays was obtained from measurements of the electron-electron interactions, i.e. the zero-field splitting interaction, in the case of the triplet states, and from measurements of electron-nuclear interactions for both radical cations and triplet states. The measurement of electron-electron interactions was also used for the structure resolution of two biologically relevant protein complexes: the twin-arginine translocase A (TatA) protein channel and the P450-ferredoxin complex of the electron transport chain in *Novosphingobium aromaticivorans*. In the case of the homomultimeric TatA protein complex, nitroxide spin labels were inserted at different positions and the distances between them were measured. In the case of the P450-ferredoxin complex, the interactions between nitroxide labels on the P450 and the iron-sulfur cluster of the ferredoxin were determined. The orientation selection introduced by the use of the iron-sulfur cluster as an intrinsic spin label was taken into account in the analysis to provide orientation as well as distance constraints for the structure determination.

The theory of EPR relevant to the projects described above is summarised in Chapter 1, more detailed information can be found for example in references [1–3]. The EPR techniques used for the analysis of hyperfine couplings between electron and nuclear spins are introduced in Chapter 2 and their use in the determination of the extent of electron delocalisation in radical cations and triplet states of linear and cyclic porphyrin arrays is discussed in Chapters 3 and 4, respectively. The EPR techniques used for structure determination by distance measurements based on electron-electron dipolar couplings

are described in Chapter 5 and their application to the multimeric TatA complex and a ferredoxin-cytochrome P450 complex are discussed in Chapters 6 and 7, respectively.

1.1 ELECTRON SPIN

EPR spectroscopy is centred on the concept of electron spin, a quantum mechanical property, evidence of which was first discovered in the early 1920s by O. Stern and W. Gerlach. The electron spin has an intrinsic angular momentum characterised by a quantum number $s = \frac{1}{2}$. Due to space quantisation it exists in two states: an α or spin-up state with $m_s = +\frac{1}{2}$ and a β or spin-down state with $m_s = -\frac{1}{2}$. A magnetic moment is associated with the spin angular momentum of the electron due to its charge and is defined as

$$\boldsymbol{\mu} = -g \frac{e\hbar}{2m_e} \hat{\mathbf{S}} = -g\beta_e \hat{\mathbf{S}} \quad (1.1)$$

where β_e is the Bohr magneton, $\hat{\mathbf{S}}$ is the spin angular momentum and the factor g accounts for the deviations from a classical behaviour and can be obtained based on Dirac's relativistic quantum mechanics. The free electron g value is $g_e = 2.00231930436153(53)$. The significance of the g -factor for electrons in different atomic and molecular frameworks will be discussed below.

An analogous description can also be applied to nuclear spins. The associated magnetic moments are significantly smaller than the magnetic moment of the electron due to the increased mass of nuclei with respect to electrons.

1.2 SPIN HAMILTONIAN

A system of unpaired electrons and nuclei in a magnetic field is characterised by the interactions of the magnetic moments with the external magnetic field and by their mutual interactions. These interactions can be expressed just in terms of the electron and nuclear spin angular momentum operators and of phenomenological parameters; hence the Hamiltonian describing the system is referred to as a spin Hamiltonian. The contributions of the orbital angular momenta, which are important for electrons, are approximated and absorbed into the magnetic parameters, expressed as tensors. The advantage of a Hamiltonian containing only spin operators and no spatial operators is that the EPR experiments can be described by considering just the spin part of the electronic wavefunction, greatly simplifying the theoretical treatment.

The spin Hamiltonian for the general case of an effective electron spin S interacting with N nuclei with spins I consists of the following terms:

$$\begin{aligned} \hat{H}_0 &= \hat{H}_{eZ} + \hat{H}_{ZFS} + \hat{H}_{nZ} + \hat{H}_{hf} + \hat{H}_{nq} \\ &= \frac{\beta_e}{\hbar} B_0 g \hat{\mathbf{S}} + \hat{\mathbf{S}} \mathbf{D} \hat{\mathbf{S}} - \beta_n \sum_{k=1}^N \frac{g_{n,k}}{\hbar} B_0 \hat{I}_k + \sum_{k=1}^N \hat{\mathbf{S}} \mathbf{A}_k \hat{I}_k + \sum_{I_k > \frac{1}{2}} \hat{I}_k \mathbf{P}_k \hat{I}_k \end{aligned} \quad (1.2)$$

where the symbol $\hat{}$ denotes an operator and vectors and matrices are written in bold.

The different types of interactions will be described in the following sections. In general, each type of interaction can be described by a second-rank tensor (denoted as $\mathbf{g}$, $\mathbf{D}$, $\mathbf{A}_k$ and $\mathbf{P}_k$ in equation 1.2), which is diagonal in an appropriate coordinate system. In this principal axis system the interaction is completely characterised by the three principal components. In general, the principal axis systems of tensors relative to different types of interactions do not coincide.

In this thesis, the spin Hamiltonian will be expressed in angular frequency units.

1.2.1 ELECTRON ZEEMAN INTERACTION

The electron Zeeman interaction describes the interaction between the electron spin magnetic moment, $\hat{\mathbf{S}}$, and the externally applied magnetic field, $\mathbf{B}_0$, and the corresponding spin Hamiltonian is

$$\hat{H}_{eZ} = \frac{\beta_e}{\hbar} \mathbf{B}_0 \mathbf{g} \hat{\mathbf{S}} \quad (1.3)$$

The $\mathbf{g}$ -tensor can be diagonalised in an appropriate principal axis system and the electron Zeeman interaction can then be described by three principal g -values, g_x , g_y and g_z , and three Euler angles defining the orientation of the tensor in the molecular frame.

In liquid solutions of low viscosity the electron Zeeman interaction is typically averaged to a single isotropic value, $g_{iso} = \frac{1}{3}(g_x + g_y + g_z)$. In frozen solutions or powders the principal components of the $\mathbf{g}$ -tensor can be determined from the turning points in the EPR spectrum. The lineshape of an EPR spectrum dominated by the electron Zeeman interaction (e.g. of a transition metal ion or of certain organic radicals at high fields) depends on the symmetry of the $\mathbf{g}$ -tensor. The absorption and derivative EPR lineshapes for electron spins described by cubic symmetry ($g_x = g_y = g_z$), axial symmetry ($g_x = g_y = g_{\perp}$, $g_z = g_{\parallel}$) and orthorhombic symmetry ($g_x \neq g_y \neq g_z$), in the absence of any other resolved interactions, are shown in figure 1.1.

The electron Zeeman interaction is described by a $\mathbf{g}$ -tensor rather than the free electron g -value due to the spin-orbit interaction, which causes an admixture of the orbital angular momentum of excited states into the ground state. The Hamiltonian thus becomes

$$\hat{H}_Z + \hat{H}_{LS} = \frac{\beta_e}{\hbar} \mathbf{B}_0 (\hat{\mathbf{L}} + g_e \hat{\mathbf{S}}) + \lambda \hat{\mathbf{L}} \hat{\mathbf{S}} \quad (1.4)$$

where an orbital angular momentum electron Zeeman term as well as a spin-orbit interaction term with the spin-orbit constant λ have been added to equation 1.3. In order to remain within the formalism of the spin Hamiltonian, the spin-orbit term is treated as a second-order perturbation to the g -value, yielding the $\mathbf{g}$ -tensor

$$\mathbf{g} = g_e \mathbf{1} + 2\lambda \mathbf{\Lambda} \quad (1.5)$$

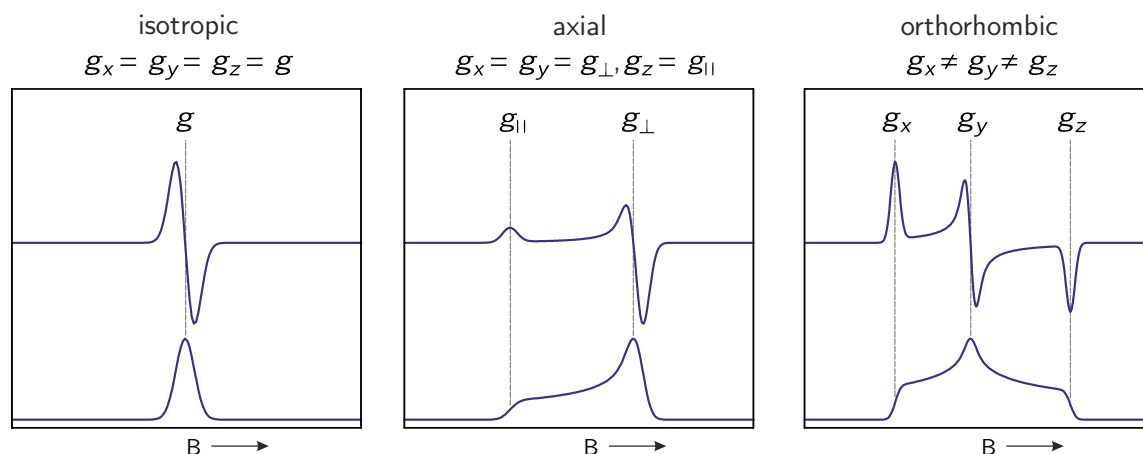


FIGURE 1.1: Simulated derivative (top) and absorption (bottom) EPR spectra for isotropic, axial and orthorhombic g -tensors. A convolutional spectral broadening was also considered in the simulations.

where the elements of the symmetric tensor Λ are defined as

$$\Lambda_{ij} = \sum_{n \neq 0} \frac{\langle \psi_0 | L_i | \psi_n \rangle \langle \psi_n | L_j | \psi_0 \rangle}{E_0 - E_n} \quad (1.6)$$

and ψ_0 and ψ_n are the ground state and n^{th} excited state wavefunctions with the associated energies E_0 and E_n , respectively. Equations 1.5 and 1.6 show that the deviation from the free electron g -value will be larger in the presence of significant spin-orbit coupling and in systems with excited states close to the ground state. The g -anisotropy is thus generally small for organic radicals with high energy excited states and can only be easily resolved in their EPR spectra at high fields. The g -anisotropy of transition metal ions on the other hand is generally large and usually dominates the corresponding EPR spectra.

1.2.2 NUCLEAR ZEEMAN INTERACTION

The nuclear Zeeman interaction of a spin I in an external magnetic field B_0 can similarly be described by the spin Hamiltonian

$$\hat{H}_{nZ} = -\frac{g_n \beta_n}{\hbar} B_0 \hat{I} \quad (1.7)$$

where the spin quantum number I and the nuclear g_n factor depend on the type of nucleus. ^1H and ^{13}C nuclei have nuclear spins with $I = \frac{1}{2}$ and nuclear g_n factors of 5.59 and 1.40, ^{14}N is a $I = 1$ nucleus with $g_n = 0.40$. The term $g_n \beta_n B_0 \hbar^{-1}$ is often referred to as the nuclear Larmor frequency, describing the precession frequency of the nuclear spin around the applied magnetic field. Due to its dependence on the inverse of the mass, the nuclear Bohr magneton is three orders of magnitude smaller than the Bohr magneton of the electron. The nuclear Zeeman interaction thus generally does not contribute to the EPR spectrum; it becomes however important in the hyperfine techniques discussed in Chapter 2.

1.2.3 HYPERFINE INTERACTION

The Hamiltonian describing the hyperfine interaction, the interaction between the electron spin and coupled nuclei of arbitrary spin I , is given by

$$\hat{H}_{hf} = \hat{\mathbf{S}}\hat{\mathbf{A}}\hat{\mathbf{I}} = a_{iso}\hat{\mathbf{S}}\hat{\mathbf{I}} + \hat{\mathbf{S}}\hat{\mathbf{T}}\hat{\mathbf{I}} \quad (1.8)$$

The hyperfine tensor $\mathbf{A}$ is determined by two different contributions: the isotropic Fermi contact interaction described by the constant a_{iso} and the anisotropic electron-nuclear dipole-dipole interaction described by the dipolar coupling tensor $\mathbf{T}$. The anisotropic hyperfine interaction is averaged out by molecular motions in liquid solution, where only the isotropic hyperfine coupling constant a_{iso} can be determined.

The isotropic hyperfine coupling constant a_{iso} is defined as

$$a_{iso} = \frac{2}{3} \frac{\mu_0}{\hbar} g_e \beta_e g_n \beta_n |\psi_0(0)|^2 \quad (1.9)$$

The isotropic contribution to the hyperfine coupling arises from finite electron spin density at the nucleus ($|\psi_0(0)|^2$). The spin population of the s -orbital of the nucleus clearly contributes to this interaction, but the spin populations in p -, d - and f -orbitals can also contribute through spin polarisation mechanisms [1].

The anisotropic hyperfine interaction can be described in terms of the classical dipole-dipole interaction and the elements of the traceless and symmetric tensor $\mathbf{T}$ can be written as

$$T_{ij} = \frac{\mu_0}{4\pi\hbar} g_e \beta_e g_n \beta_n \left\langle \psi_0 \left| \frac{3r_i r_j - \delta_{ij} r^2}{r^5} \right| \psi_0 \right\rangle \quad (1.10)$$

where ψ_0 is the ground-state electronic wavefunction and $\mathbf{r}$ is the distance vector between the nuclear and the electron spin.

The dipolar hyperfine coupling can be directly related to the spin distribution in a paramagnetic system by using the distributed point-dipole approximation. In this case, the anisotropic hyperfine tensor $\mathbf{T}$ for the nucleus N can be calculated as

$$\mathbf{T} = \frac{\mu_0}{4\pi\hbar} g_e \beta_e g_n \beta_n \sum_{k \neq N} \rho_k \frac{(3\mathbf{n}_k \tilde{\mathbf{n}}_k - \mathbf{1})}{R_k^3} \quad (1.11)$$

where the sum is over all nuclei k , bearing a spin density ρ_k and located at a distance R_k from the nucleus N . $\mathbf{n}_k$ is the unit vector defining the orientation of $\mathbf{R}_k$ in the molecular frame. This approximation can be used to determine the positions of specific nuclei from measurements of their hyperfine couplings or to test spin density distributions predicted by computational methods.

In systems with large g -anisotropies, a correction due to the electron orbital angular momentum contribution has to be considered [3].

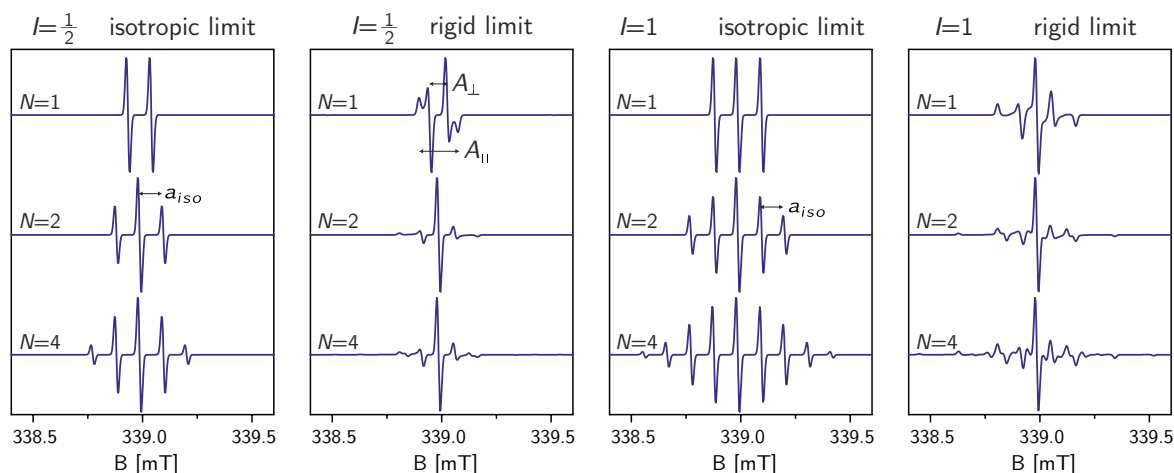


FIGURE 1.2: Isotropic (liquid solution) and rigid limit (frozen solution) X-band EPR simulations at 9.5 GHz for an electron spin with $g=2.0023$ coupled to N equivalent nuclei with nuclear spin $I = \frac{1}{2}$ or $I = 1$ with an isotropic hyperfine coupling of 3 MHz and an axial anisotropic hyperfine coupling with $T_{\perp} = -1$ MHz and $T_{\parallel} = 2$ MHz. An intrinsic linewidth of 0.015 mT was used in the simulations.

The hyperfine interactions cause splitting of the EPR line. In solution EPR spectra the isotropic hyperfine coupling can be determined from the distance between peaks of the hyperfine structure, if the couplings are large enough compared to the intrinsic EPR linewidth. The splitting pattern of an EPR spectrum depends on the number and type ($I = \frac{1}{2}$, $I = 1$, etc.) of coupled nuclei and can be used for the characterisation of molecules. The interaction of an electron spin with N nuclei of nuclear spin I causes a splitting into $2NI+1$ lines with separations determined by the corresponding isotropic hyperfine coupling and relative intensities that can be predicted using Pascal's triangles. Simulated spectra obtained for different numbers of equivalent nuclei with nuclear spins $I = \frac{1}{2}$ and $I = 1$ are shown in figure 1.2.

In frozen solutions and powders, the anisotropic hyperfine couplings also contribute to the EPR spectrum (see figure 1.2), often complicating the analysis significantly. In this case it is often necessary to study the hyperfine interaction using pulse techniques such as Electron Nuclear DOuble Resonance (ENDOR) or Electron Spin Echo Envelope Modulation (ESEEM).

1.2.4 NUCLEAR QUADRUPOLE INTERACTION

The non-spherical charge distribution in nuclei with a spin $I > \frac{1}{2}$ gives rise to a nuclear electrical quadrupole moment Q , which interacts with the electric field gradient according to the following Hamiltonian

$$\hat{H}_{nq} = \hat{I} \mathbf{P} \hat{I} \quad (1.12)$$

where $\mathbf{P}$ is the traceless nuclear quadrupole tensor. In the eigenframe of the nuclear quadrupole tensor, the Hamiltonian can be written as a function of just two terms: the nuclear quadrupole coupling

constant $e^2qQ\hbar^{-1}$ (eq is the electric field gradient) and an asymmetry parameter $\eta = (P_x - P_y)P_z^{-1}$.

$$\hat{H}_{nq} = \frac{e^2qQ}{4I(2I-1)\hbar} \left[(3\hat{I}_z^2 - I(I+1)) + \eta(\hat{I}_x^2 - \hat{I}_y^2) \right] \quad (1.13)$$

The magnitude of the nuclear quadrupole coupling depends on the size and charge of the nucleus, determining its nuclear quadrupole moment Q (e.g. $Q=0.0028$ b for ^2H , $Q=0.0204$ b for ^{14}N [4]), and on the electron distribution in its surroundings, determining the electric field gradient eq . The nuclear quadrupole interaction generally only has a negligible effect on frozen solution EPR spectra, but becomes important for ENDOR and ESEEM of $I > \frac{1}{2}$ nuclei.

1.2.5 ZERO-FIELD SPLITTING INTERACTION

The zero-field splitting (ZFS) interaction is the dipolar interaction between electron spins in systems with $S > \frac{1}{2}$, which removes the degeneracy of the different m_S levels in the absence of an external magnetic field. This interaction is described by the Hamiltonian

$$\hat{H}_{ZFS} = \hat{\mathbf{S}}\mathbf{D}\hat{\mathbf{S}} \quad (1.14)$$

where $\mathbf{D}$ is the symmetric, traceless zero-field splitting interaction tensor. In the principal axis system, the Hamiltonian is usually written in terms of just two zero-field splitting parameters, D and E , in analogy to equation 1.13 for the nuclear quadrupole interaction.

$$\hat{H}_{ZFS} = D \left[\hat{S}_z^2 - \frac{1}{3}S(S+1) \right] + E(\hat{S}_x^2 - \hat{S}_y^2) \quad (1.15)$$

where the zero-field splitting parameters D and E are defined in terms of the principal values of the $\mathbf{D}$ tensor as: $D = \frac{3}{2}D_z$ and $E = \frac{1}{2}(D_x - D_y)$.

The zero-field splitting interaction generally consists of different contributions: a first-order contribution arising from the spin-spin dipolar interaction and second-order contributions arising from the spin-orbit interaction [5]. The spin-spin contribution usually dominates for organic molecules, while the second-order contributions become important for transition metal ions and other systems with large spin-orbit coupling constants. The zero-field splitting interaction can range from the order of MHz to THz and usually dominates the EPR spectrum in systems with $S > \frac{1}{2}$.

In triplet states of organic molecules, the spin-orbit contribution can usually be safely neglected and the spin-spin contribution to the Hamiltonian can be expressed in analogy to the classical dipole-dipole interaction as

$$\hat{H}_{spin-spin} = \frac{\mu_0}{4\pi\hbar} g_1 g_2 \beta_e^2 \left[\frac{\hat{\mathbf{S}}_1 \hat{\mathbf{S}}_2}{r^3} - \frac{3(\hat{\mathbf{S}}_1 \mathbf{r})(\hat{\mathbf{S}}_2 \mathbf{r})}{r^5} \right] \quad (1.16)$$

where g_1 and g_2 are the g -values for the two unpaired electrons and $\mathbf{r}$ is the vector connecting the electron spins. Equation 1.16 can be rewritten in terms of the total spin angular momentum $\hat{\mathbf{S}}$ to give an expression of the form of equation 1.14; diagonalisation of the corresponding ZFS tensor $\mathbf{D}$ yields

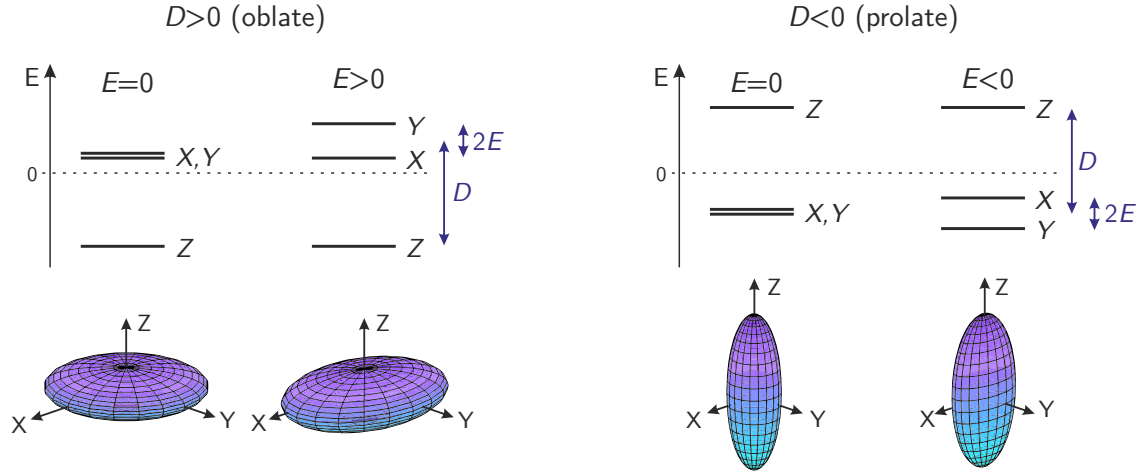


FIGURE 1.3: Energy level ordering for zero-field triplet state sublevels for oblate (\$D > 0\$) and prolate (\$D < 0\$) spin density distributions with or without axial symmetry.

the following expressions for the ZFS parameters \$D\$ and \$E\$:

$$\begin{aligned} D &= 3 \frac{\mu_0 g_1 g_2 \beta_e^2}{16\pi\hbar} \left\langle \frac{r^2 - 3z^2}{r^5} \right\rangle \\ E &= 3 \frac{\mu_0 g_1 g_2 \beta_e^2}{16\pi\hbar} \left\langle \frac{x^2 - y^2}{r^5} \right\rangle \end{aligned} \quad (1.17)$$

The expressions in equation 1.17 illustrate the physical interpretation of the zero-field splitting parameters \$D\$ and \$E\$. The \$D\$-value is related to the average distance between the two electron spins, while \$E\$ is a measure of the rhombicity of the \$\mathbf{D}\$ tensor. The sign of the \$D\$-value depends on the shape of the spin density distribution; it is positive for an oblate spin distribution and negative for a prolate spin distribution. The ZFS \$Z\$ axis corresponds to the axis of maximum dipolar coupling and is directed along the out-of-plane axis of oblate spin distributions and along the principal symmetry axis of prolate spin distributions (see figure 1.3). The sign of the \$E\$-value depends on the assignment of the \$X\$ and \$Y\$ axes of the ZFS tensor and has no physical meaning.

The principal axes of the ZFS tensor are generally chosen in order to satisfy the condition \$|D| > |3E|\$ and accordingly \$|D_z| > |D_x|, |D_y|\$. The energies of the triplet state sublevels at zero-field can be expressed as functions of \$D\$ and \$E\$:

$$\begin{aligned} X = -D_x &= \frac{1}{3}D - E \\ Y = -D_y &= \frac{1}{3}D + E \\ Z = -D_z &= -\frac{2}{3}D \end{aligned} \quad (1.18)$$

The relative ordering of the triplet state sublevels at zero-field depends on the sign of \$D\$ and \$E\$; schematic energy level plots for \$S = 1\$ are shown in figure 1.3.

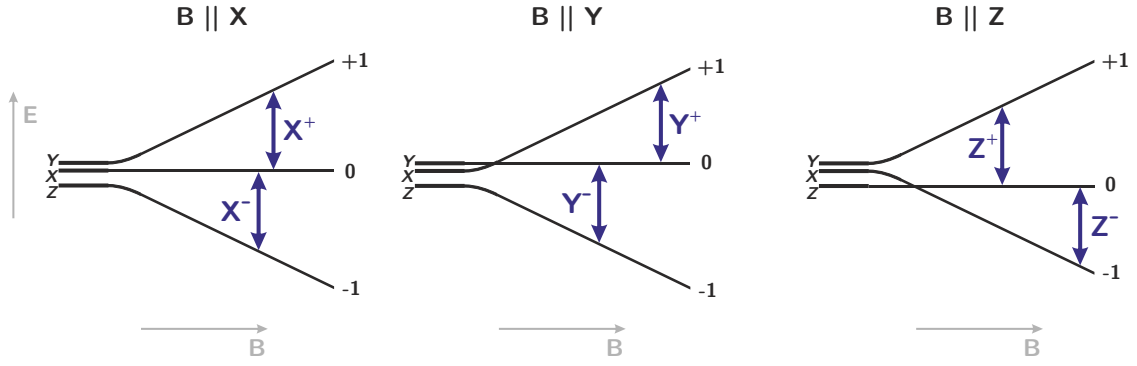


FIGURE 1.4: Energy levels of an $S = 1$ spin system with positive D and E as a function of applied magnetic field for orientations of the molecule with the field parallel to one of the ZFS tensor axes. The allowed EPR transitions are highlighted and labelled.

The spin Hamiltonian of a triplet state in an EPR experiment is determined by the electron Zeeman term in addition to the ZFS term. The preceding discussion considered the ZFS interaction in the zero-field basis $|X\rangle$, $|Y\rangle$ and $|Z\rangle$, but in the presence of the electron Zeeman interaction, which generally exceeds the ZFS interaction, the Hamiltonian is most conveniently expressed in the basis $|+1\rangle$, $|0\rangle$ and $|-1\rangle$. The energies of the triplet state sublevels in a magnetic field directed along the three principal axes of the ZFS tensor are depicted in figure 1.4. There are two allowed transitions with $\Delta m_S = \pm 1$ for each orientation of the magnetic field with respect to the ZFS tensor axes: a low-field transition $|-1\rangle \rightarrow |0\rangle$ (B_i^-) and a high-field transition $|0\rangle \rightarrow |+1\rangle$ (B_i^+).

In the absence of significant g -anisotropy, the powder triplet state EPR spectrum is characterised by four or six turning points, depending on symmetry, at field positions corresponding to orientations where the magnetic field is aligned with the ZFS tensor axes. The zero-field splitting parameters D and E can be determined from the distances between the turning points as illustrated in figure 1.5.

The intensity of the EPR signal at each field position is determined by the population difference between the electron spin energy levels connected by the corresponding transition. The populations of the triplet state sublevels in the presence of an external magnetic field can be expressed as linear combinations of the zero-field sublevel populations as follows

$$P_i = \sum_k |c_{ik}|^2 P_k \quad \text{with} \quad i = +1, 0, -1 \quad \text{and} \quad k = X, Y, Z \quad (1.19)$$

where c_{ik} are the coefficients of the high-field basis, expressed as linear combinations of the zero-field basis functions. In the high-field approximation, the sublevel populations for $B||Z$ can be written as:

$$P_0 = P_Z \quad P_{\pm 1} = \frac{1}{2} (P_X + P_Y) \quad (1.20)$$

with analogous expressions for magnetic fields directed along the other principal axes of the ZFS tensor. The intensity of a transition is then proportional to

$$I_{0 \rightarrow +1} \propto P_Z - \frac{1}{2} (P_X + P_Y) \quad (1.21)$$

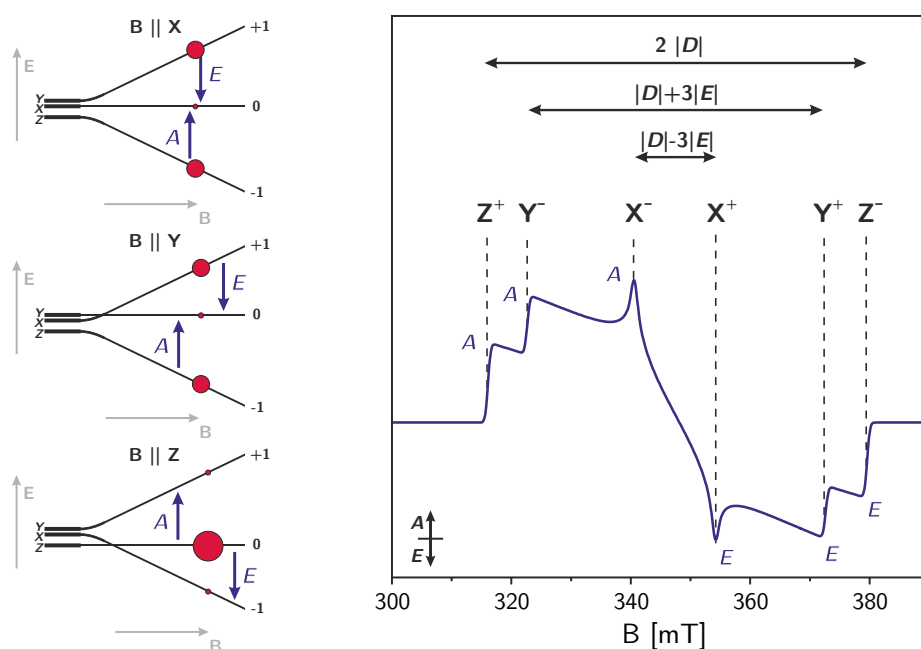


FIGURE 1.5: Simulation of a triplet state EPR spectrum for $D > 0$ and $E > 0$ and relative sublevel populations $P_X : P_Y : P_Z = 0 : 0 : 1$. The relationship between the sublevel populations and the spin polarisation observed in the spectrum is explained on the left, where the spheres represent the relative sublevel populations in the presence of a magnetic field, calculated from the zero-field populations according to equation 1.20, and the arrows indicate whether the transition connecting two sublevels is absorptive (A) or emissive (E). The spectrum was simulated for $g=2.0023$, $D=890$ MHz and $E=170$ MHz at a frequency of 9.75 GHz.

As a consequence of this, the line corresponding to the $|0\rangle \rightarrow |+1\rangle$ transition will be absorptive if $P_Z > \frac{1}{2}(P_X + P_Y)$ and emissive otherwise. At thermal equilibrium, the populations of the triplet state sublevels are given by the Boltzmann distribution and all the triplet transitions are absorptive. In a photoexcited triplet state, each triplet sublevel is populated at a different rate by the anisotropic Intersystem Crossing (ISC) mechanism, hence the triplet state is formed in a spin polarised state and the triplet EPR spectrum is partly in absorption and partly in emission. The spin polarisation of the EPR spectrum can be related to the relative sublevel populations of the triplet state, providing information on the mechanism of triplet state formation. An example for a polarised triplet state spectrum, arising from a triplet state mainly populated in the Z sublevel, is shown in figure 1.5 and has an AAAEEE polarisation (A = absorptive, E = emissive). After the formation of a spin polarised triplet state, spin-lattice relaxation processes tend to restore the equilibrium Boltzmann populations of the sublevels and a thermal equilibrium spectrum is observed at long times after the laser pulse generating the triplet state, if the triplet state lifetime is sufficiently long.

1.2.6 ELECTRON-ELECTRON DIPOLE INTERACTION

In some cases the interaction between several electron spins in a specific spin system is weak and a description in terms of a total spin $S > \frac{1}{2}$ with a ZFS interaction as discussed in the previous section is not convenient. In the case of a two-spin system, the Hamiltonian consists of the Hamiltonians for

the single spins, potentially including all the contributions mentioned in sections 1.2.1 to 1.2.4, as well as a dipolar coupling and an exchange coupling Hamiltonian:

$$\hat{H}(S_1, S_2) = \hat{H}(S_1) + \hat{H}(S_2) + \hat{H}_{dd} + \hat{H}_{exchange} \quad (1.22)$$

The dipolar coupling Hamiltonian $\hat{H}_{dd}$ is equivalent to the Hamiltonian in equation 1.16, however, in this case it is not convenient to rewrite it in terms of the total spin operator $\hat{S}$, and expansion of the scalar products leads to the following Hamiltonian, written in terms of the dipolar alphabet:

$$\hat{H}_{dd} = \frac{\mu_0}{4\pi\hbar} \frac{g_1 g_2 \beta_e^2}{r_{12}^3} (\hat{A} + \hat{B} + \hat{C} + \hat{D} + \hat{E} + \hat{F}) \quad (1.23)$$

where

$$\begin{aligned} \hat{A} &= \hat{S}_{1z} \hat{S}_{2z} (1 - 3 \cos^2 \theta) \\ \hat{B} &= -\frac{1}{4} (\hat{S}_{1+} \hat{S}_{2-} + \hat{S}_{1-} \hat{S}_{2+}) (1 - 3 \cos^2 \theta) \\ \hat{C} &= -\frac{3}{2} (\hat{S}_{1+} \hat{S}_{2z} + \hat{S}_{1z} \hat{S}_{2+}) \sin \theta \cos \theta e^{-i\phi} \\ \hat{D} &= -\frac{3}{2} (\hat{S}_{1-} \hat{S}_{2z} + \hat{S}_{1z} \hat{S}_{2-}) \sin \theta \cos \theta e^{+i\phi} \\ \hat{E} &= -\frac{3}{4} \hat{S}_{1+} \hat{S}_{2+} \sin^2 \theta \cos \theta e^{-2i\phi} \\ \hat{F} &= -\frac{3}{4} \hat{S}_{1-} \hat{S}_{2-} \sin^2 \theta \cos \theta e^{+2i\phi} \end{aligned} \quad (1.24)$$

In the high-field approximation, i.e. when the electron Zeeman interaction is much larger than the dipolar interaction, the non-secular terms $\hat{C}$ - $\hat{F}$ can be neglected, the pseudo-secular term $\hat{B}$ can also be neglected if the difference in resonance frequency of the two spins is larger than the dipolar coupling constant ω_{dd} . In that case the Hamiltonian of equation 1.23 simplifies to:

$$\hat{H}_{dd} = \frac{\mu_0}{4\pi\hbar} \frac{g_1 g_2 \beta_e^2}{r_{12}^3} (1 - 3 \cos^2 \theta) \hat{S}_{1z} \hat{S}_{2z} = \omega_{dd} (1 - 3 \cos^2 \theta) \hat{S}_{1z} \hat{S}_{2z} \quad (1.25)$$

The exchange coupling Hamiltonian is given by

$$\hat{H}_{exchange} = \hat{S}_1 \mathbf{J} \hat{S}_2 \quad (1.26)$$

where $\mathbf{J}$ is the Heisenberg exchange coupling tensor consisting of an isotropic exchange coupling constant J_{12} , related to through-bond coupling and expressed as an exchange integral over the orbitals occupied by the two electrons, and an anisotropic part, determined by spin-orbit coupling. The Heisenberg exchange coupling requires an overlap of the orbitals populated by the two electron spins. The sign of the isotropic exchange coupling constant depends on whether the coupling describes a weak bonding (antiferromagnetic coupling, $J_{12} > 0$) or anti-bonding situation (ferromagnetic coupling, $J_{12} < 0$). A detailed discussion of exchange coupling can be found in reference [6].

The dipolar and exchange couplings lead to splitting of the EPR signal or more commonly to broadening of the EPR spectra, depending on their size compared to the other interactions present in the spin system and to the intrinsic EPR linewidth. The dipolar coupling discussed here is at the basis of Double Electron Electron Resonance (DEER) and other dipolar EPR techniques discussed in Chapter 5.

1.3 CONTINUOUS WAVE AND PULSE EPR

Spin systems characterised by the spin Hamiltonians described in the previous section can be studied using continuous wave and pulse EPR techniques. In most cases the fundamental interaction for EPR is the electron Zeeman interaction: the presence of a static external magnetic field causes a splitting of the electron spin energy levels with different values of m_S and microwave radiation can typically be used to induce transitions between these energy levels.

Continuous wave EPR (cw-EPR) is based on the measurement of the resonance frequencies of a spin system; the EPR sample is continuously irradiated by microwaves at a fixed frequency during a sweep of the external magnetic field and the absorption of microwaves at magnetic field positions in resonance with the energy level splitting is detected.

Pulse EPR has later been developed to overcome the limitations in spectral and time resolution of cw-EPR. In pulse EPR, the continuous wave irradiation is replaced by high intensity microwave pulses with lengths of the order of nanoseconds. A wide range of pulse sequences has been developed to probe specific properties of the spin system [3], such as for example hyperfine couplings, dipolar couplings or relaxation times.

EPR can be performed at different microwave frequencies, corresponding also to different strengths of the applied magnetic fields. The most commonly used EPR frequencies today are X-band (ca. 9.5 GHz), Q-band (ca. 34 GHz) and W-band (ca. 95 GHz), but spectrometers operating at both lower and higher frequencies/fields are used in many laboratories. At high frequencies the spectral resolution for field-dependent interactions as well as the sensitivity, determined by the population difference of the energy levels connected by an EPR transition, increase. The advantage of a multi-frequency approach is that the sensitivity for specific types of magnetic interactions, introduced in the previous section, can be modulated, considerably aiding the identification and interpretation of the different contributions to the spin Hamiltonian.

1.3.1 CONTINUOUS WAVE EPR (cw-EPR)

The principal components of a continuous wave (cw) EPR spectrometer are a microwave source, a magnet generating the external field B_0 , a resonator cavity used for amplification of the input and the output EPR signal and a detection system. During operation, the external field causes a splitting of the electron spin energy levels in the sample placed at the centre of the resonator.

For most of the typically used frequency bands, e.g. X-band and Q-band, the microwave radiation is transmitted to the resonator through a waveguide. The coupling of the microwave source to the resonator occurs through an isolator, preventing back-reflections into the microwave source, an attenuator for adjustment of the incident microwave power and a circulator that directs the microwaves from the source to the resonator and the microwaves reflected from the cavity to the detector.

The microwave radiation generates a standing wave inside the resonator. The resonators for EPR are typically designed such that the magnetic field component of the electromagnetic radiation is maximised at its centre, the sample position, while the electric field component is minimised at the same position, so as to reduce interference. Further, the magnetic field component of the microwave radiation, B_1 , is generally perpendicular to the static magnetic field B_0 . Typically a tuning procedure is required to critically couple the resonator, which occurs through fine-adjustment of the frequency and of the coupling of the microwaves into the cavity through a coupling element, called an iris (small hole with adjustable screw). The optimal operating conditions are reached when the reflection of microwaves from the resonator is minimal, i.e. when the impedance of the resonator corresponds to the impedance of the line. The efficiency of the resonator under operating conditions is measured by the quality factor Q , indicating how efficiently the resonator stores microwave energy [7].

When the resonance condition $\Delta E = h\nu$ is met, B_1 induces a transition between the electron spin energy levels, the absorption of the microwave radiation causes a change in impedance, which is detected and constitutes the EPR signal. The detector is typically a diode, and in order to reduce the effect of random frequency noise and increase sensitivity, phase-sensitive detection is employed. The magnetic field B_0 is modulated by generating a small additional field modulated at a frequency of typically 100 kHz using Helmholtz coils outside the resonator. Only the component of the absorption signal at this modulation frequency is detected. As a consequence of this field modulation, the first derivative of the absorption line is detected. The modulation amplitude has to be adjusted based on the linewidth of the EPR signal in order to obtain the intrinsic lineshape [7].

The detector diode exhibits optimal performance at a constant incident microwave power and to achieve this, a reference arm is often present, which branches off part of the microwave radiation from the source and is used, after power and phase adjustments, for biasing of the power level at the detector.

Further explanations, technical details and procedures for cw-EPR tuning and measurements can be found for example in references [2, 7, 8].

1.3.2 EPR LINESHAPES AND LINEWIDTHS

EPR spectra can exhibit lines characterised by different linewidths and lineshapes, depending on the mechanism of line broadening. The minimum linewidth is determined by lifetime broadening, i.e. the excited state has a finite lifetime τ and according to Heisenberg's uncertainty principle ($\tau\Delta E \geq \hbar$) the

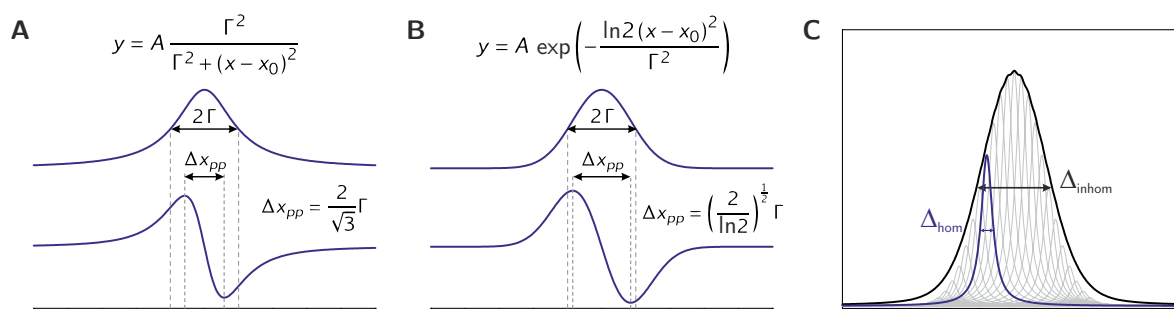


FIGURE 1.6: Absorption and first derivative Lorentzian (A) and Gaussian (B) lineshapes with the corresponding absorption equations and with indicated full width at half maximum (FWHM) 2Γ and peak-to-peak linewidth Δx_{pp} . (C) Inhomogeneously broadened line obtained as the superposition of a series of homogeneously broadened lines with a distribution of resonance positions. The single homogeneously broadened lines are Lorentzians and the envelope is approximately Gaussian.

transition has a certain spectral width. Additional broadening mechanisms also come into play and EPR lines are usually classified as homogeneously or inhomogeneously broadened.

A homogeneously broadened EPR line is obtained if all the spins are characterised by the same spin Hamiltonian and experience the same time-averaged field. An ensemble of spins fulfilling these conditions is defined as a spin packet. Homogeneously broadened lines are characterised by a Lorentzian lineshape with a width of $2T_m^{-1}$ (see section 1.4.2 for a definition of T_m). The homogeneous broadening can occur in the presence of fluctuating fields due, for example, to electron and nuclear spin flips.

Inhomogeneously broadened lines arise from spin ensembles with resonance frequencies distributed over an unresolved lineshape. The inhomogeneously broadened line is formed from the superposition of a series of homogeneous lines with slightly different resonance positions, as shown in figure 1.6. As a result of this, the lineshape is typically Gaussian. This type of broadening can occur in the presence of an inhomogeneous external field B_0 , of unresolved hyperfine structure or generally of contributions from anisotropic interactions. Inhomogeneous broadening due to the presence of a dipolar interaction between two electron spins will be discussed in detail in Chapter 5. Even in the presence of such mechanisms, a homogeneous line can be obtained, if their effects are averaged out by e.g. molecular motion on an appropriate timescale.

1.3.3 PULSE EPR

The basic components of pulse EPR spectrometers are similar to the cw-EPR spectrometers described in section 1.3.1, however some modifications and additional elements are required. The fundamental difference between the two types of spectrometers concerns the microwave excitation. For pulse EPR applications, the microwave radiation from the source passes through a pulse forming unit with several channels with individual attenuators and phase shifters, allowing independent amplitude, pulse

length and phase adjustments, the latter being crucial whenever phase cycling is required to eliminate unwanted echoes. The pulse forming unit also contains fast microwave switches generating the microwave pulses with lengths of the order of several nanoseconds.

Many pulse EPR experiments are based on the simultaneous excitation of several electron spin transitions or of spins with a distribution of resonance frequencies and often require short pulse durations and high microwave powers. Consequently, the pulses from the pulse forming unit are amplified in a TWT (travelling wave tube) amplifier. For X-band applications, TWT amplifiers with an output power of 1 kW are typically used, for Q-band measurements, an output power of about 100 W is usually recommended [3], however solid state amplifiers with lower output powers are often also used.

The microwave pulses are transmitted to the resonator after passing through an additional variable attenuator and a circulator. The resonator design for pulse EPR applications somewhat differs from that used for cw-EPR and several types of resonators, often developed for specialised applications, are available (see [3] and references therein). The resonators used for the work described in this thesis were a dielectric resonator (Bruker EN 4118X-MD4) for ENDOR measurements and a split-ring resonator (Bruker ER 4118X-MS3) for the DEER measurements. For the Q- and W-band measurements, the Bruker pulsed ENDOR resonators, EN 5107D2 and EN 600-1021H, respectively, were used. A fundamental problem of pulse EPR is the dead-time, the time after the microwave pulses during which no signal can be detected due to the ringing of the high power microwave pulses in the resonator, preventing measurement of the much smaller sample signals. This problem is reduced by over-coupling of the resonator, which decreases the quality factor of the cavity, decreasing the sensitivity. Additionally, this also increases the excitation bandwidth of the resonator.

The output signal is directed to the pulse detection unit, equipped with a gated limiter, protecting the detector from the reflected high-power microwave pulses. The signal is usually acquired using quadrature detection, which separates the signal into a real and an imaginary part. For pulse experiments a boxcar integrator is often used to obtain the experimental spectrum.

Additional equipment is required for more specialised applications. For example, Electron Nuclear DOuble Resonance (ENDOR) measurements require an additional radiofrequency (rf) sequence generator and an rf amplifier, as well as a probehead with rf coils. Double Electron Electron Resonance (DEER) measurements require an additional microwave source and channel (ELDOR channel) for excitation at a second microwave frequency.

1.3.4 SEMI-CLASSICAL DESCRIPTION OF PULSE EPR

The effect of a sequence of microwave pulses on the magnetisation of an ensemble of spins can most easily be understood using the semi-classical vector model. This very approximate intuitive approach generally describes single spin packets and simple pulse sequences quite well, but is often not sufficient for the description of more sophisticated experiments and in the presence of multiple spins.

In the presence of an external magnetic field, the electron spin magnetic moment experiences a torque, which leads to precession of the magnetic moment around the magnetic field axis at an angular frequency called the Larmor frequency. An EPR sample usually contains an ensemble of spins and hence a net magnetisation vector, the vector sum of all the spin magnetic moments of the sample, can be defined. At thermal equilibrium there is an excess of β spins and no phase correlation of the magnetic moments in the plane perpendicular to the field axis, usually assigned as the z-axis of the laboratory reference frame, and hence the magnetisation is aligned with the z-axis and is stationary in the laboratory reference frame.

The linearly polarised microwaves of the time-dependent field B_1 can be described as the sum of two counter-rotating circularly polarised components. The effect of B_1 during a microwave pulse is most conveniently described in a frame rotating around the laboratory z-axis at the frequency of this time-dependent field. The transformation into the rotating frame allows to consider one of the components as stationary and to neglect the other component, rotating at twice the rotating frame frequency.

Application of a microwave pulse causes the magnetisation to precess around the axis of the B_1 field. At the end of a microwave pulse of duration t_p , the magnetisation is rotated by an angle $\beta = \omega_1 t_p$, where β is called the flip angle of the pulse and ω_1 is the amplitude of the B_1 field in angular frequency units. A single $\frac{\pi}{2}$ pulse applied along the x-axis of the rotating frame coordinate system rotates the magnetisation from the z-axis to the -y-axis as illustrated in figure 1.7. After the microwave pulse, the components of the magnetisation rotated into the plane perpendicular to the static magnetic field B_0 start to precess around the z-axis of the laboratory frame.

For spins satisfying the resonance condition, the angular frequency of the rotating frame corresponds to the Larmor frequency and hence components of the magnetisation precessing at the Larmor frequency will appear stationary. In this case the external field B_0 disappears in the rotating frame. Off-resonance spins precess at frequencies smaller or larger than the rotating frame frequency and the transformation to the rotating frame leads to the corresponding magnetisation rotating in a clockwise or counter-clockwise fashion, respectively. Due to the precession of the magnetisation in the rotating frame, the external field B_0 does not disappear for off-resonance spins and during the microwave pulse the magnetisation is rotated around the effective field, determined by the vector sum

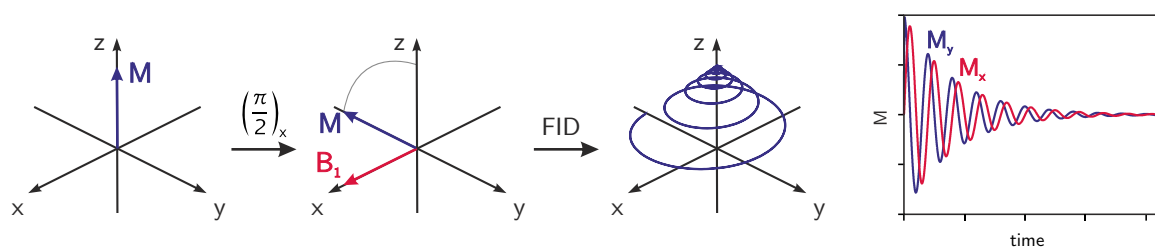


FIGURE 1.7: Schematic representation of the generation of an FID (free induction decay) using the vector model: the equilibrium magnetisation is rotated into the xy-plane by a $\frac{\pi}{2}$ pulse along the x-axis of the rotating frame and then decays back to equilibrium. The time evolution of the magnetisation along the x- and y-axes is shown on the right.

of B_0 and B_1 in the rotating frame. After a $\frac{\pi}{2}$ pulse applied along the x-axis the magnetisation vectors of off-resonance spins are not exactly aligned with the -y-axis and usually have residual out-of-plane components. The exact amount of transverse magnetisation after the pulse depends on the offset of the resonance frequency of the spins and can be described by a sinc function ($\sin x x^{-1}$ with $x = \beta \omega_{eff} \omega_1^{-1}$ and the effective rotation frequency $\omega_{eff} = \sqrt{\omega_1^2 + \Delta\omega^2}$, determined by the frequency offset $\Delta\omega$).

The precession of the magnetisation vector around B_0 after the microwave pulse is accompanied by a decay of the magnetisation through the relaxation mechanisms described in section 1.4. Measurement of the components of the magnetisation vector rotating in the xy-plane as a function of time after the microwave pulse yields the free induction decay (FID); the x and y components correspond to the real and imaginary part of the signal. The quadrature detection typically employed corresponds to detection of the transverse magnetisation in the rotating frame.

In most EPR experiments the spin system is investigated by detecting a spin echo rather than the FID. This is mainly due to the fact that the FID generally decays so quickly that most of the information is lost in the dead-time. Refocusing of the magnetisation to an echo solves this problem. The basic sequence for the generation of a spin echo is the two-pulse or Hahn echo sequence consisting of a $\frac{\pi}{2}$ pulse followed by a π pulse after a delay τ (see figure 1.8). The $\frac{\pi}{2}$ pulse flips the magnetisation vector into the xy-plane as described above. During the free evolution time τ , the different spin packets constituting the sample precess around the z-axis at different frequencies and this causes a defocusing of the magnetisation. The effect of the π microwave pulse is to rotate all magnetisation vectors around the x-axis by 180° . After the pulse, the spin packets continue to precess at their characteristic frequencies, refocusing after a time τ in the absence of spin relaxation and forming the electron spin echo. In the presence of spin relaxation, only a fraction of spin packets will contribute to the echo signal.

The length of a microwave pulse, at constant flip angle, determines the frequency range simultaneously excited by the pulse. The excitation profile of the pulse is approximately described by the Fourier transform of the pulse shape; the excitation profile for an ideal rectangular pulse is a sinc function ($\sin x x^{-1}$) with a linewidth of $1.207 t_p^{-1}$ and first zero-crossings at $\pm t_p^{-1}$ [3]. A 16 ns pulse can be assumed to uniformly excite a region of 62.5 MHz ($\nu \pm \frac{1}{2} t_p$), corresponding to about 2.2 mT.

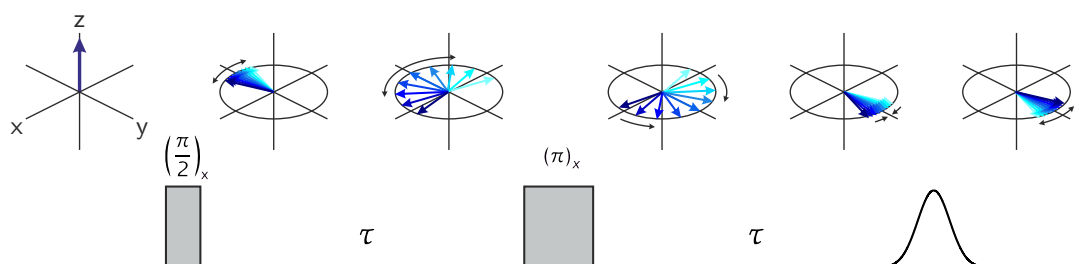


FIGURE 1.8: Two-pulse or Hahn echo sequence and magnetisation vector representation explaining the formation of an electron spin echo.

The selectivity of the microwave pulse can be changed by varying the pulse length. However, in most cases, excitation of the whole spectrum is not possible. Selection of only specific resonance positions, corresponding to different orientations of the molecule with respect to the magnetic field in powder samples, can be achieved in many cases and this orientation selection can often provide additional or more detailed information on the spin system (see section 1.3.6).

In pulse EPR, the spectra can be recorded using either FID or spin-echo detected field sweep experiments. The obtained EPR spectrum is in absorption mode and should roughly correspond to the cw-EPR spectrum. In some cases spin-echo detected field sweeps are affected by modulation due to hyperfine couplings, leading to distorted EPR spectra. In such cases FID detection, if possible on the grounds of sensitivity, is more convenient.

1.3.5 DENSITY MATRIX DESCRIPTION OF PULSE EPR

The vector model approach is adequate for the description of simple experiments, generally considering just a single or, at most, two coupled spins, but a more rigorous quantum mechanical approach is needed to explain more sophisticated pulse experiments. This is provided by the density matrix formalism [3, 9].

An ensemble of spin states can be described by a density matrix $\hat{\rho}$, a matrix of $n \times n$ elements, where $n = (2S + 1)(2I + 1)$ and S is the electron spin quantum number and I the quantum number of a nuclear or a second electron spin. The diagonal elements of the density matrix correspond to the populations (P_{ii}) of the different states of the spin system and differences between diagonal elements are generally referred to as polarisations, while the off-diagonal elements represent phase coherent superpositions of states and are referred to as coherences. The polarisations are related to the classical concept of longitudinal magnetisation and the coherences are related to the transverse magnetisation. The density matrix elements for a system of two coupled spins, for example an electron spin $S = \frac{1}{2}$ and a nuclear spin $I = \frac{1}{2}$, and the possible transitions are depicted in figure 1.9. The coherences can be divided into allowed single-quantum (SQ) coherences and forbidden zero- (ZQ) and double-quantum (DQ) coherences.

The evolution of the density matrix during pulse EPR experiments is described by the Liouville-von Neumann equation

$$\frac{d\hat{\rho}}{dt} = -\frac{i}{\hbar} [\hat{H}, \hat{\rho}] \quad (1.27)$$

where the term in square brackets indicates the commutator between the density matrix and the Hamiltonian acting on the spin system. The Liouville-von Neumann equation can easily be solved for a time-independent Hamiltonian and the solution is

$$\hat{\rho}(t_{\text{final}}) = \hat{R}_t \hat{\rho}(t_{\text{initial}}) \hat{R}_t^{-1} \quad \text{with} \quad \hat{R}_t = \exp[-i(t_{\text{final}} - t_{\text{initial}})\hat{H}] \quad (1.28)$$

The pulse EPR experiments are typically divided into periods of nutation during the pulses and periods of free evolution during the inter-pulse delays governed by different time-independent Hamiltonians.

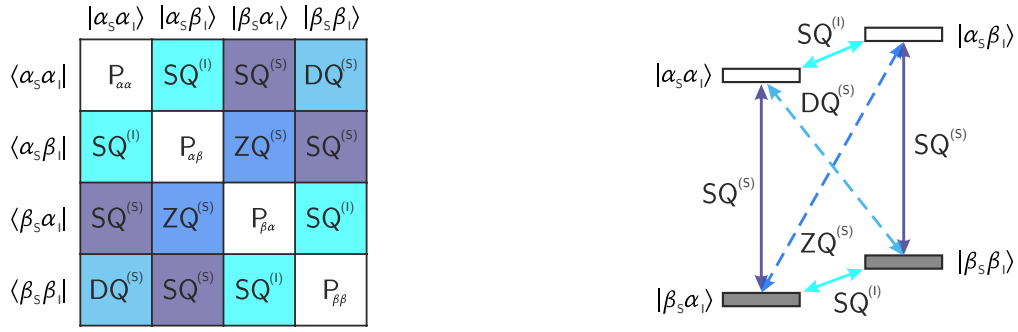


FIGURE 1.9: Density matrix elements and the corresponding transitions for an $S = \frac{1}{2}$, $I = \frac{1}{2}$ spin system. The allowed electron (S) and nuclear (l) transitions are shown in solid lines, the forbidden electron transitions are shown as dashed lines.

The evolution of the density matrix during the pulse sequence is then calculated by consecutively applying the corresponding propagators $\hat{R}_t$. The state of the spin system at the end of a pulse sequence is determined by calculating the trace of the product of $\hat{S}_{x/y}$, or of $(\hat{S}_x + i\hat{S}_y)$ for quadrature detection, and the density matrix at the time of the echo.

At equilibrium only the diagonal elements of the density matrix are non-zero. The microwave pulses in pulse EPR experiments then excite different transitions, generating coherences. Depending on the type of experiment, transfer between coherences and polarisations or between different coherences occurs, and at the end of the experiment the in-phase electron coherence is observed. Pulse EPR experiments can be divided into polarisation-transfer experiments, such as the ENDOR experiments, electron coherence-transfer experiments, such as two-pulse ESEEM, and nuclear coherence-transfer experiments such as three-pulse ESEEM and HYSCORE.

Polarisation-transfer experiments are based on the generation of non-equilibrium polarisation and its detection. Non-equilibrium polarisation can be generated by a single π pulse or by the sequence $\frac{\pi}{2} - \tau - \frac{\pi}{2}$, for detection the polarisation has to be transferred to electron coherence. The Davies and Mims ENDOR experiments fall into this category and are explained in detail in the next chapter.

Electron coherence-transfer experiments are based on the generation of electron coherence and its transfer between different transitions of the spin system. An example of this type of experiment is two-pulse ESEEM, described in the next chapter.

Nuclear coherence-transfer experiments are based on the generation of nuclear coherence and its evolution on nuclear transitions or its transfer between them. Nuclear coherences can be generated by sequences of microwave pulses, if both allowed and forbidden transitions can be excited. At the end of the experiment the nuclear coherence is transferred back to electron coherence for detection. The nuclear coherence-transfer experiments three-pulse ESEEM and HYSCORE are described in the next chapter.

The microwave pulses usually excite not only the desired coherences but also additional unwanted ones, which can interfere with the detection of the wanted signal. These additional coherences can be

suppressed by phase cycling, i.e. repeating the experiment with different phases of specific microwave pulses in the sequence and adding or subtracting the resulting signals. Phase cycling allows the selection of the specific coherence transfer pathways leading to the signal of interest. The phase cycles required for the different types of pulse EPR experiments can generally be found in the literature.

1.3.6 ORIENTATION SELECTION

The microwave pulses typically used in pulse EPR do not have sufficient excitation bandwidth (see section 1.3.4) to excite the whole EPR spectrum in the presence of large anisotropic contributions to the electron spin Hamiltonian, such as g -anisotropy, zero-field splitting or large anisotropic hyperfine couplings. This leads to selection of a subset of orientations of the molecules with respect to the magnetic field. The orientations selected by pulses acting at different positions of the EPR spectrum in the case of an axial g -tensor and for a triplet state with an orthorhombic zero-field splitting tensor are depicted in figure 1.10.

Orientation selection is often an advantage for both hyperfine techniques and dipolar techniques such as DEER, as more information on the spin system can be obtained. On the other hand, orientation selection means that only a subset of the spins in the sample is excited, leading to decreased signal intensities. Orientation selection in hyperfine experiments leads to the detection of hyperfine and nuclear quadrupole couplings in different directions of the molecular frame, providing information on the relative orientation of the hyperfine and nuclear quadrupole tensor with respect to the tensor determining the orientation selection ($\mathbf{g}$ - or ZFS tensor). In DEER experiments, orientation selection allows extraction of information on the relative orientation of the two paramagnetic systems in addition to information on the distance between them, as will be explained in Chapter 5.

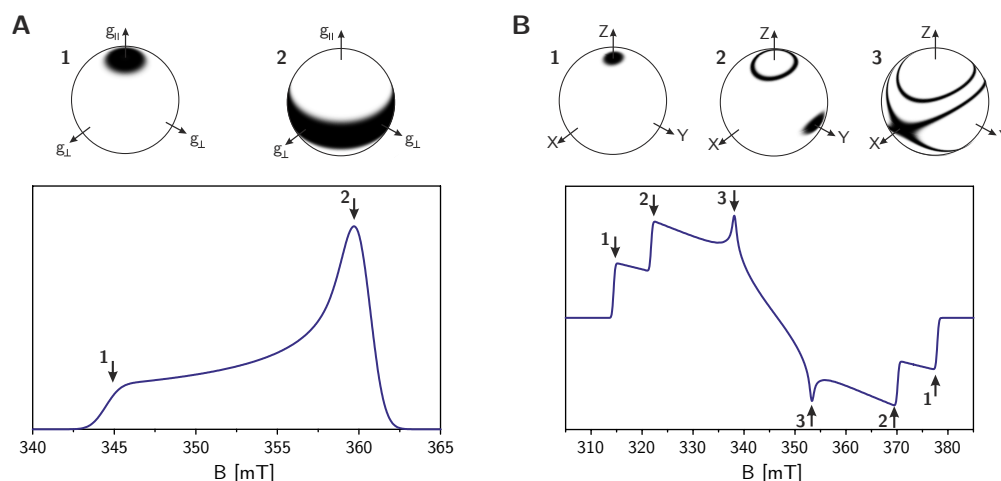


FIGURE 1.10: Calculated powder spectra for an axial g -tensor (A) and for a triplet state with a zero-field splitting with $D, E > 0$ (B). The weights of the orientations excited by pulses with an excitation bandwidth of 60 MHz at the magnetic field positions indicated by arrows are plotted on a unit sphere, darker shades correspond to higher weights, the white areas indicate the orientations not contributing at that field position.

1.3.7 TIME-RESOLVED EPR

The EPR spectra of photoexcited species, such as for example radical pairs or triplet states, and their time evolution after laser excitation can be recorded using time-resolved or transient EPR.

In photoexcited time-resolved EPR, irradiation of the sample with a laser pulse prior to the actual EPR experiment produces the paramagnetic species. The time evolution of the EPR signal after the laser pulse is measured at a fixed magnetic field and under continuous microwave irradiation. Collection of transients at different external magnetic fields yields a two-dimensional spectrum with EPR signal intensity as a function of both the external magnetic field B_0 and the time after the laser pulse. The signal is acquired using a transient recorder and is detected in absorption mode.

Pulse EPR measurements of photoexcited species are performed by preceding the EPR pulse sequence with a laser pulse. The signal of unwanted stable radicals can be suppressed by saturation with a strong microwave pulse before the laser pulse.

1.4 RELAXATION

Relaxation plays an important role in both continuous wave and pulse EPR. In continuous wave EPR, relaxation determines the linewidth of the spectra and has to be considered in order to record spectra not affected by power saturation effects. In pulse EPR, relaxation times determine the maximum length of any pulse sequence and the repetition time of a pulse experiment. Measurement and analysis of relaxation times, while often not straightforward, have the potential of providing useful information on the spin system, its interaction with the surroundings and its dynamic behaviour.

During an EPR experiment, the spin system is perturbed by a continuously oscillating microwave field or nanosecond microwave pulses and then returns to equilibrium by a series of different processes, with different relative contributions depending on the spin system under investigation. Typically two main relaxation mechanisms are distinguished: the longitudinal or spin-lattice relaxation, characterised by the relaxation time T_1 and the transverse or spin-spin relaxation, characterised by the relaxation time T_2 . Both relaxation mechanisms are induced by local fluctuations in the magnetic field experienced by the spins. If these occur at frequencies matching the resonance condition for the spin system, they induce transitions between the energy levels, returning the system to equilibrium. In general, processes leading to a change of the net populations of the electron spin states are classified as spin-lattice relaxation processes and processes leading to a loss of phase coherence or taking spins off-resonance are classified as spin-spin relaxation processes. The two main relaxation mechanisms are briefly discussed in the following sections; much more detailed information can be found, for example, in references [3, 10].

1.4.1 SPIN-LATTICE RELAXATION

The longitudinal or spin-lattice relaxation returns the magnetisation vector to the equilibrium position along the z-axis ($z \parallel B_0$) through electron spin flips induced by dynamic interactions with the surrounding matrix (the solution, powder or crystal lattice). These relaxation processes are characterised by an exchange of energy of the spin system with the surroundings and cause a decrease in enthalpy of the spin system.

Spin-lattice relaxation processes are induced by different mechanisms in solids and liquids. In liquids, the main cause is molecular motion, which modulates the anisotropic interactions of the spin system, such as for example the electron Zeeman, the hyperfine, the exchange or the electron dipole-dipole interaction, and thus generates fluctuating local fields inducing electron spin flip transitions. Another relaxation mechanism in liquid solution is caused by collisions with other paramagnetic species, such as for example oxygen or transition metal ions. In solids, spin-lattice relaxation is induced by thermal motion, i.e. molecular vibrations. The spin system exchanges energy with the lattice by absorption or stimulated emission of phonons, causing a modulation of the spin-orbit coupling or other interactions present in the spin system. Three main processes for this type of relaxation in solids can be distinguished and are characterised by different temperature and magnetic field dependences. The direct process involves phonon absorption or emission, the Raman process involves a virtual excitation and de-excitation to high energy phonon states and the Orbach process is a resonant two-phonon process [3]. The modulation of spin-orbit coupling is often the principal contribution to spin-lattice relaxation in solid samples and hence the relaxation time T_1 decreases significantly from organic molecules to systems containing transition metal ions. Further, relaxation increases for larger deviations of the principal g -values from g_e and for systems with reduced symmetry.

1.4.2 SPIN-SPIN RELAXATION

The transverse or spin-spin relaxation causes loss of phase coherence in the xy -plane through spin flip-flops without exchange of energy with the surroundings. In addition to spin flip-flops, phase coherence is also lost as a consequence of spin flips occurring due to the mechanisms described in the previous section. Hence the overall relaxation time T_2 is determined by two contributions:

$$\frac{1}{T_2} = \frac{1}{T_2'} + \frac{1}{2T_1} \quad (1.29)$$

where T_2' is the relaxation time associated with a flip-flop process and T_1 is the spin-lattice relaxation time associated with spin flip processes.

In most cases in EPR, the empirical parameter phase memory time T_m is introduced, which is the relaxation time related to any process causing loss of electron spin phase coherence and includes T_2 , as defined above. Typical processes determining T_m can be summarised as follows [3]: The local magnetic field experienced by the A spins, the spins excited in a specific experiment, is influenced by

dipolar coupling to other spins not excited in the experiment, called B spins. These B spins can either be other electron spins or nuclear spins. Spin-lattice or spin-spin relaxation of the B spins causes fluctuations of the local magnetic field experienced by the A spins. This leads to a contribution to the phase memory time of the A spins that is inversely proportional to the concentration of B spins. When the coupled B spin is a nuclear spin, this process is called nuclear spin diffusion. In the presence of protons, either on the molecule itself or on the solvent (matrix protons), nuclear spin diffusion usually constitutes the main contribution to T_m . An increase of T_m can usually be achieved by exchange of protons with deuterons, which reduce the importance of this contribution due to the smaller magnetic moment and the smaller dipolar hyperfine couplings to the electron spin.

Additional contributions to T_m arise from processes that change the resonance frequency of the electron spin and thus move spin magnetisation between different regions of the EPR spectrum. In pulse EPR, this movement of the excited spin resonance frequency out of the detection window constitutes an apparent spin relaxation process. Spectral diffusion is one of these processes and occurs if only part of the EPR spectrum is excited due to limited excitation bandwidth. In analogy to the processes described above, the relaxation of B spins, not excited by the microwave pulses, changes the local magnetic field at the position of the excited A spins. The resonance frequency of the A spins thus changes, leading to a transfer of their spin magnetisation to other parts of the spectrum, outside the detection window. Similarly the B spins also change resonance frequency and thus transfer equilibrium spin magnetisation to the region of the spectrum within the detection window. This substitution of A spin magnetisation by B spin equilibrium magnetisation has the effect of an apparent relaxation process. Molecular motion also has a similar effect and causes spectral diffusion.

Instantaneous diffusion can occur when the electron spins interact; in this case, after the microwave pulse the local magnetic field around each spin changes depending on the relative position and orientation of the other spins and causes a shift in resonance frequency. A similar process can also occur between spins excited at different microwave frequencies. Instantaneous diffusion depends on the pulse flip angle and the pulse excitation bandwidth.

2 HYPERFINE SPECTROSCOPY

The hyperfine interaction, discussed in section 1.2.3, provides important information on the electron distribution in a spin system. The isotropic and anisotropic hyperfine couplings can be used to determine the spin density distribution in the paramagnetic system and in addition to that, the dipolar hyperfine tensor can provide information on the position of the coupled nuclei with respect to the spin density distribution, due to the dependence of the dipolar hyperfine interaction on the electron-nuclear separation. Orientation-selective measurements also allow determination of the orientation of the hyperfine tensor with respect to the principal tensor of the system, which can be the g - or the ZFS tensor. In summary, hyperfine spectroscopy has the potential of providing access to an accurate picture of the paramagnetic system and its surroundings.

Several EPR techniques have been developed that allow direct measurement of the hyperfine couplings, which are not well resolved in the EPR spectrum in most cases. The available methods can be divided into two principal categories: Electron Nuclear DOuble Resonance (ENDOR) and Electron Spin Echo Envelope Modulation (ESEEM) techniques. The ENDOR techniques are based on the excitation of both electron and nuclear transitions, while ESEEM techniques only directly excite electron transitions. While ESEEM techniques are restricted to pulse EPR, a continuous wave ENDOR technique is also available, but will not be discussed here. Pulse ENDOR and ESEEM are complementary experiments: ESEEM is often the technique of choice for hyperfine spectroscopy at low nuclear frequencies, for example for ^{14}N and ^2H nuclei, while ENDOR generally performs better at higher radiofrequencies, for example for ^1H nuclei.

Magnetic nuclei typically studied using hyperfine spectroscopy include ^1H , ^2H , ^{13}C , ^{14}N and ^{15}N as well as different isotopes of transition metals. Each type of nucleus is characterised by its Larmor frequency ($g_n\beta_n B_0\hbar^{-1}$, see section 1.2.2), which allows assignment of signals observed by ENDOR or ESEEM to different types of nuclei present in the system. The Larmor frequencies of different isotopes may lie close together at X-band frequencies, which can complicate the analysis of spectra for systems containing more than one type of magnetic nucleus. Higher fields allow better separation of different nuclear Larmor frequencies, as shown in figure 2.1. Therefore hyperfine spectroscopy is often also performed at Q- or W-band. As will be discussed below, in ESEEM experiments the choice of microwave frequency is also of fundamental importance for other reasons.

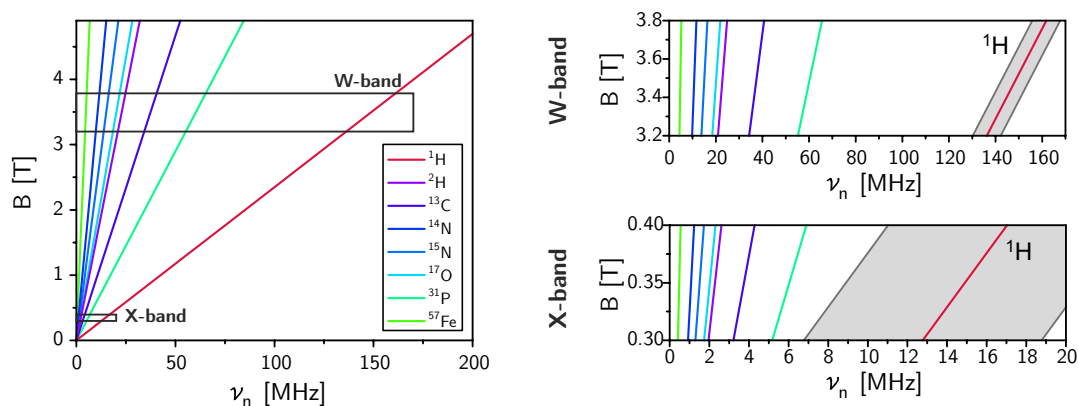


FIGURE 2.1: Larmor frequencies of a series of magnetic nuclei for different static magnetic fields. The Larmor frequencies at X-band and W-band frequencies are highlighted and the region of proton nuclear frequencies up to a hyperfine coupling of 12 MHz is indicated by grey shading. Since in the case of strong coupling, often occurring for nuclei with low gyromagnetic ratios such as ^{14}N , the signals are centred at $\frac{1}{2}A$ (see section 2.1.3), overlap of their signals with the signals arising from proton hyperfine couplings can occur at X-band.

The ENDOR and ESEEM techniques used for the work presented in this thesis are described below. Information on different, more sophisticated extensions of these techniques can be found in reference [3] and in the literature, but they are based on the same principles explained below.

2.1 ENDOR - ELECTRON NUCLEAR DOUBLE RESONANCE

Electron Nuclear DOuble Resonance (ENDOR) techniques are based on the transfer of polarisation between electron and nuclear transitions and the nuclear frequencies are detected through changes of the intensity of electronic transitions. ENDOR thus exploits the greater sensitivity of the electron as compared to the nuclear spin. ENDOR is a double frequency technique and requires excitation of both electron transitions with microwaves, as well as nuclear transitions with radiofrequencies. For this reason, it is technically more demanding than ESEEM, as it requires a radiofrequency source and amplifier, as well as a probehead designed for delivery of both frequencies to the sample.

Two different types of pulse ENDOR experiments have been developed: Mims [11] and Davies [12] ENDOR. The corresponding pulse sequences are shown in figures 2.2 and 2.5. Both experiments have advantages and disadvantages, but typically Davies ENDOR is used for systems with large hyperfine couplings, while Mims ENDOR is preferred for systems with small hyperfine couplings.

2.1.1 DAVIES ENDOR

The principle behind Davies ENDOR can be explained by considering a simple four-level system of an electron spin coupled to a single nuclear spin with $I = \frac{1}{2}$, such as for example a proton. There are two electron or EPR transitions connecting levels with different electron spin quantum numbers and two nuclear or NMR transitions connecting levels with different nuclear spin quantum numbers, as shown

Davies ENDOR

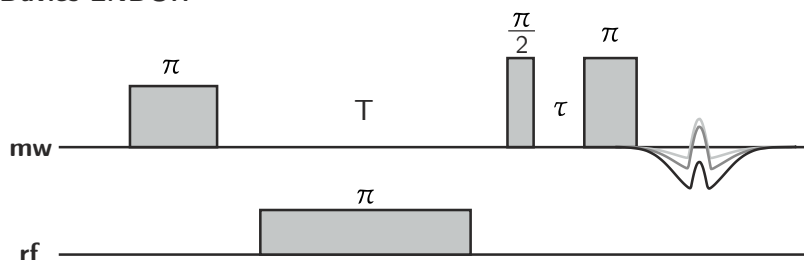


FIGURE 2.2: Pulse sequences for Davies ENDOR. The radiofrequency is swept and the echo intensity is recorded as a function of radiofrequency.

in figure 2.3. At thermal equilibrium, the electronic β states are characterised by a larger population. The first microwave π pulse in the Davies ENDOR sequence is selective for one of the two electron transitions and inverts the corresponding populations. The microwave π pulse is followed by a selective radiofrequency π pulse at varying radiofrequencies. If the radiofrequency is resonant with one of the nuclear transitions, the polarisation of the corresponding transition is inverted and the population difference for the EPR transition is reduced. If the radiofrequency pulse is off resonance, the sublevel populations and EPR intensity do not change. The radiofrequency pulse is followed by a non-selective detection sequence, a two-pulse sequence for echo-detection or a single pulse for FID-detection. The signal amplitude depends on the population difference between the energy levels connected by electron transitions and is thus determined by whether or not the radiofrequency pulse was on resonance with a nuclear transition. Integration over the inverted echo as a function of radiofrequency yields the nuclear frequency spectrum.

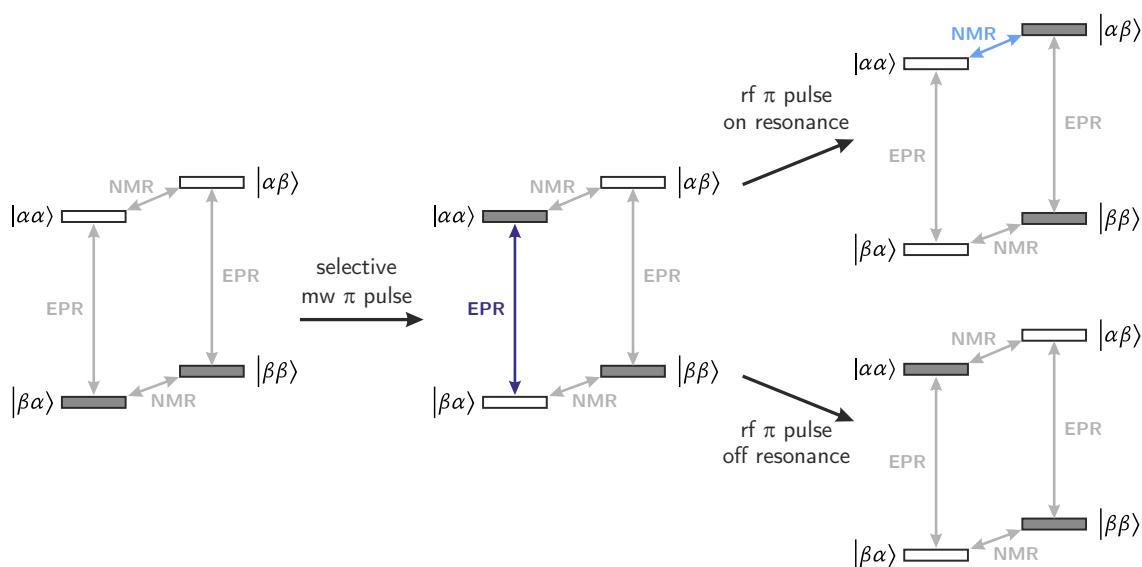


FIGURE 2.3: Effect of the microwave inversion and radiofrequency pulses in a Davies ENDOR experiment on the sublevel populations of a simple four-level system of an electron coupled to a single nuclear spin with $I = \frac{1}{2}$. The different states in the energy level diagrams are denoted by the electron spin state followed by the nuclear spin state, for example $|\beta\alpha\rangle$ corresponds to the $|\beta_e\alpha_n\rangle$ state.

The radiofrequency pulse is required to invert the nuclear populations and thus has to be tuned as a π pulse. The pulse length t_p is given by

$$t_p = \frac{\pi}{\gamma_N B_2} \quad (2.1)$$

where $\gamma_N = g_N \beta_N \hbar^{-1}$ is the nuclear gyromagnetic ratio and B_2 is the apparent strength of the radiofrequency magnetic field. The radiofrequency pulse can be optimised in a nutation experiment by monitoring the echo for increasing pulse lengths at a fixed radiofrequency (on resonance, for example on the matrix line) and choosing the pulse length corresponding to the first minimum observed in the oscillating nutation curve. Typically for protons the radiofrequency pulse has a length of about 15 μ s at X-band, with generally longer pulses for nuclei with smaller gyromagnetic ratios in the absence of the ENDOR enhancement effect.

The linewidth of ENDOR lines is determined by the nuclear phase memory time as $T_m^{(n)-1}$. If the radiofrequency pulse length is shorter than $T_m^{(n)}$, a power broadening of the ENDOR line occurs as the overall linewidth is determined by the convolution of the intrinsic linewidth with the excitation profile of the radiofrequency pulse [13]. The power broadening of ENDOR lines can typically be avoided by carefully choosing the radiofrequency pulse length and the temperature for recording the experiment. Experimentally, the presence of power broadening can be verified by repeating the ENDOR measurement with a longer radiofrequency pulse and by comparing the results.

In Davies ENDOR, the observation of an ENDOR effect depends on the selectivity of the first microwave pulse. If the π pulse inverts the populations for both EPR transitions, the radiofrequency pulse has no effect on the populations and hence no ENDOR effect is observed. In order to avoid this, the pulse length has to be carefully chosen so as to have an excitation bandwidth that does not exceed the hyperfine splitting. If the hyperfine coupling A^n of nucleus n is approximately known, the optimum inversion pulse length can be calculated as [14]

$$t_p = \frac{\eta_n}{A^n} \approx \frac{0.7}{A^n} \quad (2.2)$$

where η_n is called the selectivity parameter. The absolute ENDOR intensity was found to depend on the selectivity parameter as [14]

$$I(\eta) = I_0 \frac{1.4\eta}{\eta^2 + (0.7)^2} \quad (2.3)$$

where I_0 is the maximum ENDOR intensity obtained at $\eta_{max} = 0.7$. The ENDOR intensity function for different lengths of the inversion pulse is depicted in figure 2.4 A. These results predict the absence of an ENDOR signal at the Larmor frequency. However, experimentally a strong matrix line is usually observed for protons. This has been attributed to the fact that the radiofrequency pulse in the matrix region close to the Larmor frequency simultaneously inverts a large number of nuclei and that the induced change of the local magnetic fields affects the echo intensity [15].

ENDOR signals corresponding to weak hyperfine couplings are suppressed by hard inversion pulses and require softer inversion pulses to be observed. This suppression can in some cases be exploited

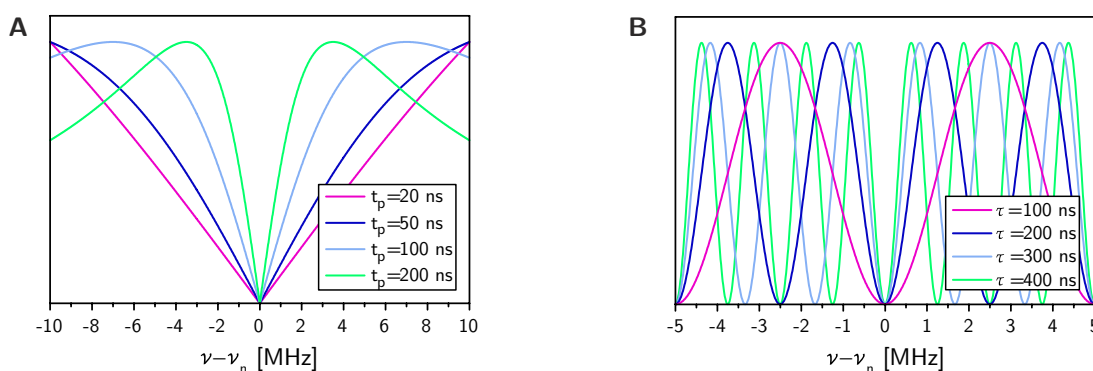


FIGURE 2.4: (A) ENDOR intensity as a function of the length of the microwave inversion pulse in Davies ENDOR calculated based on equation 2.3. (B) ENDOR intensity as a function of the interpulse delay τ in Mims ENDOR calculated based on equation 2.4 ($\nu = \nu_n \pm \frac{1}{2}A$).

for the interpretation of heteronuclear ENDOR spectra with significant overlap of the signals of protons and other nuclei. Hard inversion pulses suppress the ENDOR signals of nuclei with small hyperfine couplings, such as protons, and allow measurement of nearly unperturbed ENDOR spectra of nuclei with stronger hyperfine couplings, such as for example nitrogen nuclei [16].

2.1.2 Mims ENDOR

In the Mims ENDOR experiment, the ENDOR effect is determined by interference effects in the stimulated echo generated by three microwave $\frac{\pi}{2}$ pulses. In contrast to the Davies ENDOR experiment, non-selective microwave pulses are used for Mims ENDOR.

The first two non-selective $\frac{\pi}{2}$ microwave pulses generate a polarisation grating across the EPR line, i.e. the amount of longitudinal magnetisation M_z varies along the EPR line [11] (see figure 2.6). In terms of the vector model, the first $\frac{\pi}{2}$ pulse creates transverse magnetisation which evolves during the interpulse delay as discussed in section 1.3.4; the second $\frac{\pi}{2}$ pulse transfers the magnetisation back to longitudinal magnetisation. The amount of longitudinal magnetisation at each position of the EPR line depends on the interpulse delay τ , the hyperfine coupling and the resonance offset, which determine the position of the magnetisation vector corresponding to a spin packet at the time of

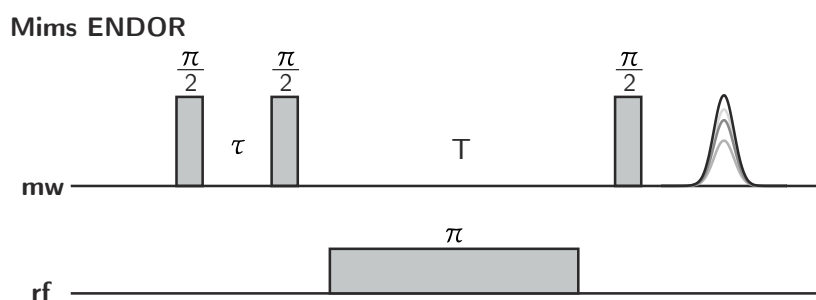


FIGURE 2.5: Pulse sequences for Mims ENDOR. The radiofrequency is swept and the echo intensity is recorded as a function of radiofrequency.

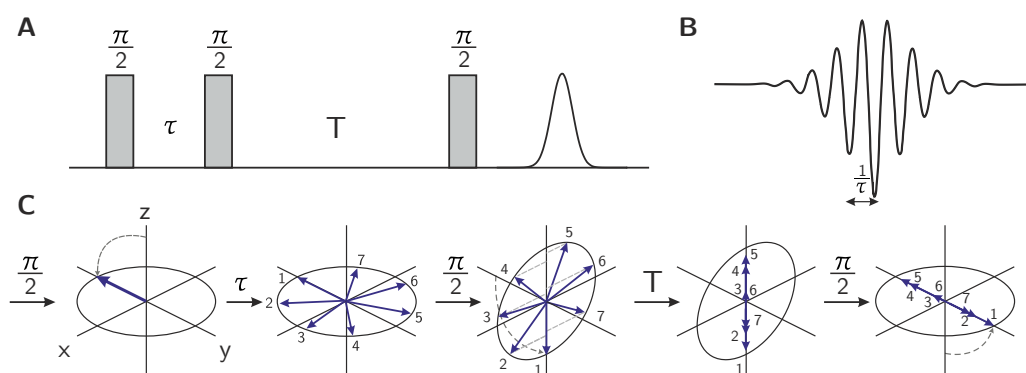


FIGURE 2.6: (A) Stimulated echo sequence. (B) Polarisation pattern across the EPR line after the first two $\frac{\pi}{2}$ microwave pulses for complete excitation. (C) Vector model diagrams showing the evolution of the magnetisation vector in the Mims ENDOR sequence in the absence of relaxation, as explained in the text.

the second $\frac{\pi}{2}$ pulse. For spin packets with a frequency corresponding to that of the rotating frame, the first two microwave pulses have the same effect as a single π pulse and their magnetisation becomes aligned with the $-z$ axis. For spin packets that during the interpulse delay τ have rotated by odd multiples of π , the magnetisation is rotated back to the original position by the second $\frac{\pi}{2}$ pulse. Considering all spin packets, the longitudinal magnetisation varies sinusoidally with a periodicity of $\frac{2\pi}{\tau}$ after the two preparation pulses and this pattern slowly decays through spin-lattice relaxation. In Mims ENDOR the pulses need to be non-selective to ideally generate the polarisation grating over the whole EPR line and the optimum sensitivity is typically obtained for the shortest possible $\frac{\pi}{2}$ pulses [8].

In the absence of the radiofrequency pulse, the last $\frac{\pi}{2}$ microwave pulse transforms the longitudinal magnetisation back into the xy -plane, where it focuses and defocuses, creating a stimulated echo. If the radiofrequency pulse is applied on resonance with a nuclear transition, it causes a frequency shift determined by the hyperfine coupling, which prevents the corresponding spin packet from being focused at the time of the stimulated echo, thereby reducing the echo intensity. Again the radiofrequency pulse is required to invert the nuclear polarisation and the same considerations as in Davies ENDOR apply. Integration over the stimulated echo as a function of radiofrequency yields the nuclear frequency spectrum.

Due to the periodic nature of the polarisation grating, the radiofrequency pulse has little effect on the echo amplitude for certain hyperfine couplings. Hence the ENDOR intensity varies periodically as a function of the hyperfine coupling and the interpulse delay τ :

$$I \propto [1 - \cos(A\tau)] \quad (2.4)$$

The ENDOR effect thus reaches a maximum for $\tau = \left(n + \frac{1}{2}\right) A^{-1}$ and is zero for $\tau = n A^{-1}$ with $n = 0, 1, 2, \dots$. The Mims ENDOR intensity as a function of frequency is shown in figure 2.4 B for different τ values. The blind-spots thus occurring in the Mims ENDOR spectrum can lead to misinterpretation and it is generally recommended to use a τ value sufficiently short to avoid distortions ($A_{max} \approx (2\tau)^{-1}$) or, if this is not possible, to record spectra for a series of different τ values.

Similarly to Davies ENDOR, the absence of an ENDOR effect is expected for very small hyperfine couplings, but a strong matrix line is typically observed. The origin of this effect is the same as explained above for Davies ENDOR; additionally it has been shown that for Mims ENDOR the intensity of the matrix line decreases for increasing τ values [15].

The blind-spot behaviour described above was determined based on the analysis of an $S = \frac{1}{2}$, $I = \frac{1}{2}$ system. ENDOR intensities and τ suppression effects for other spin systems can be determined using the product operator or density matrix formalism [13, 17]. Neglecting the nuclear quadrupole coupling interaction, for a nuclear spin $I = 1$, the ENDOR intensity is found to be proportional to $[1 - \cos(2A\tau)]$. For a $S = 1$, $I = \frac{1}{2}$ system, an analogous behaviour to the simpler $S = \frac{1}{2}$ case is obtained.

The different ENDOR intensity functions for Davies and Mims ENDOR (see figure 2.4) typically lead to a different appearance of the ENDOR spectra recorded with the two techniques and should be taken into account in simulations.

Mims ENDOR is intrinsically more sensitive than Davies ENDOR, as the non-selective pulses in Mims ENDOR excite a larger fraction of spins than the selective inversion pulse of the Davies ENDOR experiment. However, the τ suppression effects in Mims ENDOR reduce the sensitivity for larger hyperfine couplings and in this case the Davies ENDOR experiment is more convenient. The relative sensitivity of Mims and Davies ENDOR for different hyperfine couplings and measurement conditions is discussed in detail in reference [8]. The main conclusion is that Mims ENDOR with a fixed τ value is the best experiment for hyperfine couplings smaller than about 2 MHz, Mims ENDOR averaged over different τ values becomes better for hyperfine couplings between 2 and about 6 MHz and Davies ENDOR is the best choice for couplings greater than 6 MHz.

Since the ENDOR experiments are based on the polarisation inversion and transfer, the ENDOR efficiency increases significantly at very low temperatures, as the thermal equilibrium population difference is larger and the relaxation times are longer.

2.1.3 ANALYSIS OF ENDOR SPECTRA

ENDOR spectra contain peaks at the frequencies of the allowed nuclear transitions of the investigated spin system. ENDOR spectra of $S = \frac{1}{2}$ systems are typically symmetric and centred at the nuclear Larmor frequency in the weak coupling regime ($A < 2\nu_I$, see figure 2.7 A) and centred at half the hyperfine coupling in the strong coupling regime ($A > 2\nu_I$, see figure 2.7 D).

The proton hyperfine couplings are generally small compared to the Larmor frequency and the corresponding ENDOR spectra are thus centred at the proton Larmor frequency. In the case of an isotropic hyperfine interaction an ENDOR spectrum with two peaks separated by a_{iso} is obtained (figure 2.7 A). In general, ENDOR is recorded in frozen solution, where powder patterns are obtained, as shown in figure 2.7 B and C for an axial and an orthorhombic hyperfine tensor, respectively. The ENDOR powder lineshapes are characterised by turning points at the canonical orientations of the hyperfine tensor,

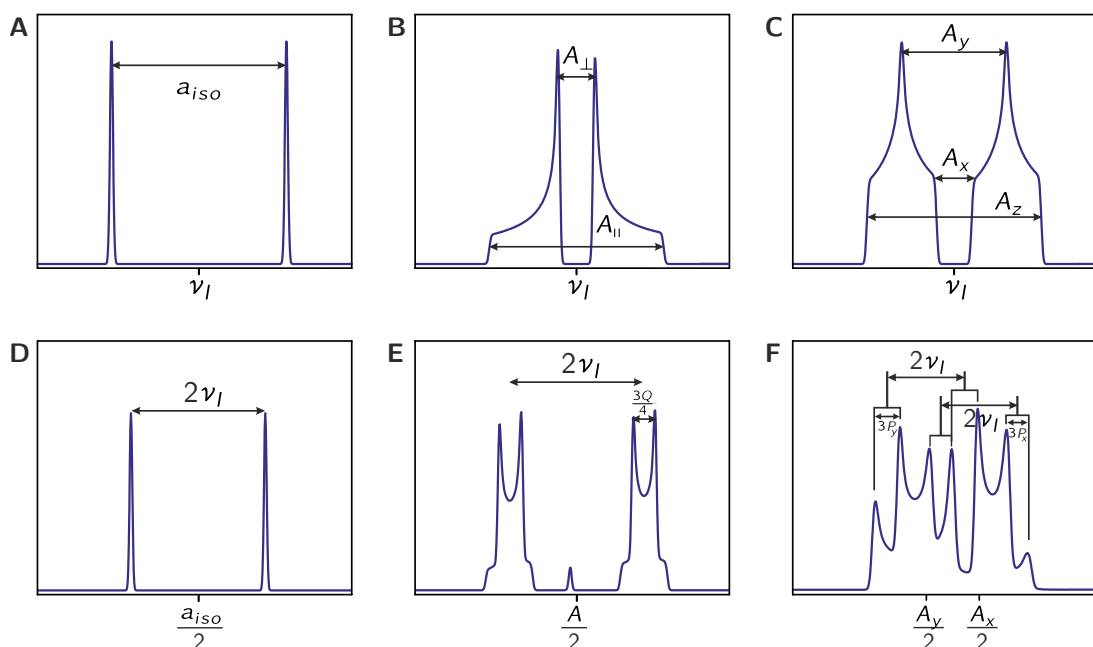


FIGURE 2.7: Simulated ENDOR spectra for a single $I = \frac{1}{2}$ nucleus in the weak coupling regime (A-C, isotropic, axial and orthorhombic hyperfine tensors, respectively) and an $I = 1$ nucleus in the strong coupling regime (D-F, isotropic hyperfine coupling, isotropic hyperfine coupling and a nuclear quadrupole interaction, orthorhombic hyperfine tensor and nuclear quadrupole interaction, respectively).

sometimes allowing the direct determination of the principal values from the spectrum. The analysis of ENDOR spectra is complicated in the presence of multiple nuclei with similar hyperfine interaction and in most cases simulations of the spectra are required for determination of the hyperfine couplings. In the presence of orientation selection, the analysis of ENDOR spectra recorded at different field positions can allow determination of the relative orientations of the g - and hyperfine tensors.

In addition to the peaks corresponding to the hyperfine couplings of the protons in the spin system, weak hyperfine interactions with distant protons or solvent protons (matrix protons) give rise to a central peak at the nuclear Larmor frequency, typically called the matrix peak.

Nitrogen ENDOR spectra at X- and Q-band frequencies can be in the strong coupling regime and the peaks are thus centred at $\frac{1}{2}A$ and separated by $2\nu_I$ as shown in figure 2.7 D for an isotropic hyperfine coupling. Since ^{14}N is an $I = 1$ nucleus, in frozen solution the nuclear quadrupole coupling causes additional splitting depending on the nuclear quadrupole coupling constant Q and the asymmetry parameter η . In the presence of axial or orthorhombic hyperfine couplings as well as nuclear quadrupole interaction the nitrogen ENDOR spectra can become quite complicated to interpret without simulations.

In high-spin electronic systems, ENDOR spectra can become asymmetric with respect to ν_I (or A in case of strong coupling) due to transition selection and yield information on the sign of the hyperfine coupling as well as its magnitude. The sign of the hyperfine coupling for $S = \frac{1}{2}$ systems can only be determined using variants of the ENDOR technique, such as variable mixing time ENDOR [18–20].

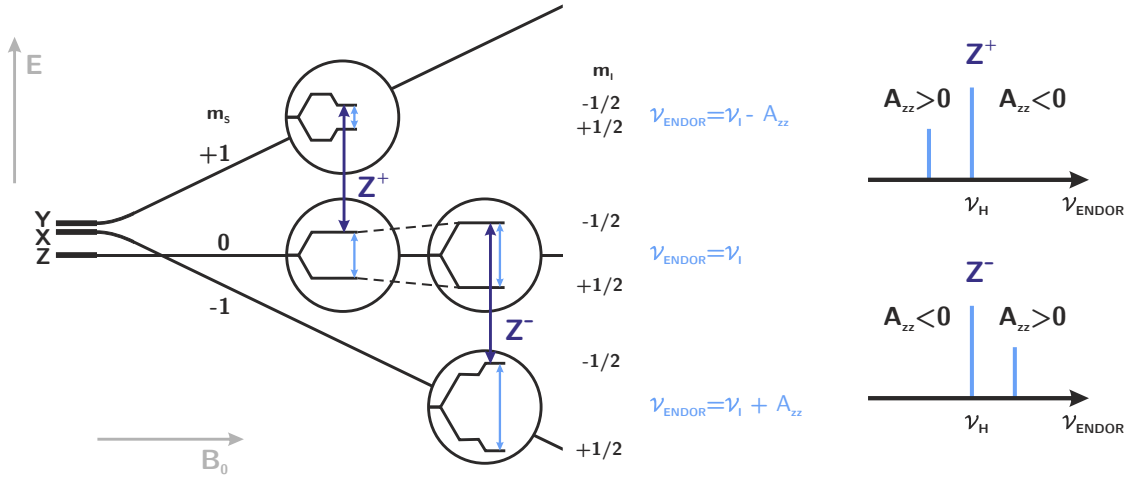


FIGURE 2.8: Energy level diagram for an $S = 1, I = \frac{1}{2}$ spin system with collinear ZFS and hyperfine tensors, $D > 0$, $A > 0$ and with the magnetic field parallel to the Z orientation of the zero-field splitting tensor. Schematic representations of the ENDOR spectra for each of the transitions of the Z canonical orientation illustrate the possibility of determining the sign of the hyperfine coupling for a known sign of D .

In a triplet state, the $m_S = 0 \rightarrow m_S = +1$ and $m_S = -1 \rightarrow m_S = 0$ transitions for each of the canonical orientations can be excited individually. The corresponding ENDOR spectra are characterised by a strong and narrow peak at the nuclear Larmor frequency, arising from the nuclear transition frequencies of the $m_S = 0$ manifold. In the case of a single coupled $I = \frac{1}{2}$ nucleus, an additional peak on one side of the Larmor frequency would appear, as illustrated in figure 2.8. If the sign of the D -value is known, and if thus the canonical field positions in the EPR spectrum can be assigned to the $m_S = 0 \rightarrow m_S = +1$ or $m_S = -1 \rightarrow m_S = 0$ transitions, the sign of the hyperfine coupling can be determined from the position of the hyperfine peak with respect to the nuclear Larmor frequency. For a positive D -value, the hyperfine peak in the spectrum recorded for a $m_S = 0 \rightarrow m_S = +1$ transition will occur at a frequency lower than the nuclear Larmor frequency for a positive hyperfine coupling and higher for a negative hyperfine coupling [21, 22]. The ENDOR spectrum corresponding to the other triplet transition will correspond to the mirror image with respect to the Larmor frequency peak.

In addition to transition selection, triplet state systems are also characterised by orientation selection, hence the hyperfine couplings along the three axes of the zero-field splitting tensor could theoretically be determined by ENDOR measurements at the different canonical field positions. In case of an axial zero-field splitting tensor ($E = 0$), pure orientation selection is only obtained for the Z canonical transition, while the orientations on the whole plane perpendicular to Z are excited for the other canonical transition. If the hyperfine and ZFS tensors are collinear, this analysis would yield the principal values of the hyperfine tensor directly. In practice, such an analysis can often be complicated by overlap of different canonical transitions, the presence of several coupled nuclei and the unknown relative orientation of the different interaction tensors.

The interpretation of ENDOR spectra strongly relies on the simulation of the experimental data to extract the hyperfine and nuclear quadrupole parameters as well as information on relative tensor orientations. In this thesis, the Davies and Mims ENDOR simulations were performed using the EasySpin

[23, 24] routines *salt* and *saffron*, respectively. The *salt* routine for solid state ENDOR simulations is based on the computation of the line positions by diagonalisation of the spin Hamiltonian for each orientation of the magnetic field with respect to the molecular frame and on the determination of the corresponding intensities based on the ENDOR transition rates [23]. The calculation of Mims ENDOR spectra in *saffron* is based on a density matrix approach (see section 1.3.5) as first proposed by Mims [24, 25].

2.2 ESEEM - ELECTRON SPIN ECHO ENVELOPE MODULATION

The Electron Spin Echo Envelope Modulation (ESEEM) experiments are based on the modulation of the electron spin echo by nuclear frequencies of coupled nuclear spins. A nuclear frequency spectrum, similar to the ENDOR spectra discussed above, is obtained by Fourier transform of the modulated spin echo envelope.

The origin of the nuclear modulation of the spin echo envelope can be explained qualitatively by considering an electron and a nuclear spin separated by a distance r in an external magnetic field B_0 [26–28]. Both the electron and the nuclear spin generate a dipolar magnetic field in the position of the other spin, with a magnitude depending on their distance and on the electron and nuclear Bohr magneton, respectively ($B_{e/n} \propto \beta_{e/n} r^{-3}$, $\beta_{e/n} = e\hbar/(2m_{e/n})^{-1}$). The field produced by the nuclear spin at the position of the electron spin is typically so small with respect to B_0 that it can be neglected and the electron spin can be considered aligned with the external magnetic field. The field B_{hf} generated by the electron spin at the position of the nuclear spin, on the other hand, is of comparable magnitude to B_0 , so that the nuclear spin experiences an effective magnetic field given by their vector sum. At equilibrium, the nuclear spin is aligned with this effective magnetic field as shown schematically in figure 2.9. If the electron spin is reoriented by a microwave pulse that is short compared to the Larmor period of the nuclear spin, the effective magnetic field at the nucleus changes too fast for the nuclear spin to follow it and it thus begins to precess around the new effective field ($B_0 + B_{hf}$). This nuclear precession induces time-dependent local fields at the position of the electron spin (figure 2.9)

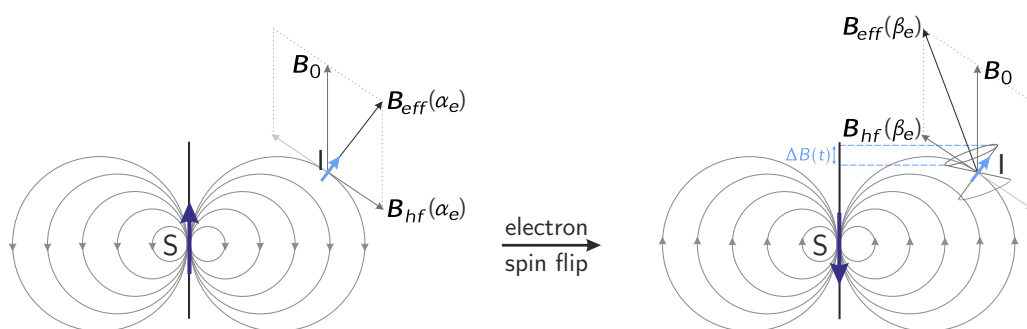


FIGURE 2.9: Schematic diagram showing the origin of the spin echo modulation. The local magnetic fields acting on the nuclear spin are shown as well as the effect of an inversion of the quantisation direction of the electron spin by a microwave pulse (based on reference [26]).

and as a result the electron precession frequency becomes modulated at the precession frequency of the coupled nucleus.

The fundamental requirement for the observation of nuclear modulation effects is that the electron and nuclear spins have different quantisation axes in the external magnetic field. This condition is fulfilled in the intermediate coupling regime, where the nuclear Zeeman interaction and the hyperfine interaction are of comparable magnitude. In this regime, the selection rule $m_S = \pm 1$, $m_I = 0$ breaks down and similar transition probabilities for the "allowed" and "forbidden" transitions, depicted in figure 2.10 A, are obtained. The angle between the quantisation axes for the nuclear spin corresponding to the two opposite orientations of the electron spin is often indicated as 2η and determines the relative intensities of the allowed and forbidden transitions as

$$I_a = \cos^2 \eta \quad I_f = \sin^2 \eta \quad (2.5)$$

The value of η depends on the relative magnitude of the hyperfine and nuclear Zeeman interactions: for a spin $S = \frac{1}{2}$ in the limits of weak coupling ($|A| \ll 2|\omega_I|$) and of strong coupling ($|A| \gg 2|\omega_I|$), η approaches 0, in the case of intermediate coupling ($|A| = 2|\omega_I|$), the value of η is 45° . If $\eta = 0$, $I_f = 0$ and no ESEEM effect is observed. In addition to the limits of weak and strong coupling, this also occurs if only isotropic hyperfine couplings are present or if the external field B_0 is aligned with a principal axis of the hyperfine tensor [3]. ESEEM techniques are thus limited to solid samples.

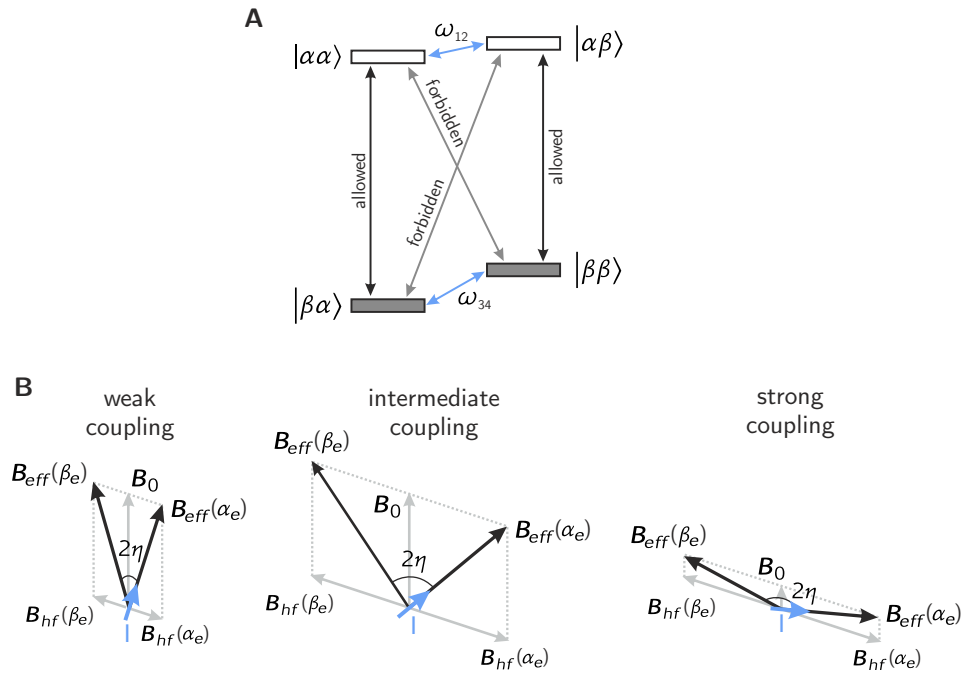


FIGURE 2.10: (A) Energy level diagram for a $S = \frac{1}{2}$, $I = \frac{1}{2}$ spin system indicating the allowed and forbidden electron transitions. (B) Quantisation axes for the nuclear spin in the weak, intermediate and strong coupling regimes. The angle 2η determines the intensities of the allowed and forbidden transitions (see equation 2.5) and the modulation depth in an ESEEM experiment according to equation 2.6.

The depth of the modulations observed in ESEEM experiments is determined by the modulation depth parameter k , which is defined as the product of allowed and forbidden transition probabilities:

$$k = 4I_a I_f = \sin^2 2\eta \quad (2.6)$$

Therefore the maximum modulation depth is obtained in the intermediate coupling regime for the exact cancellation condition ($|A| = 2|\omega_I|$). Interpretation of ESEEM spectra recorded close to the exact cancellation regime is much simplified, especially for $I = 1$ nuclei such as ^{14}N , where this leads to the observation of the pure nuclear quadrupole frequencies in one electron spin manifold. Whenever possible, it is therefore advantageous to perform ESEEM experiments at microwave frequencies approaching this regime.

Nuclear modulation effects can be observed using a number of different pulse sequences; the most important experiments are the one-dimensional two- and three-pulse ESEEM and the two-dimensional HYSCORE (HYperfine Sublevel CORrElation) experiments. The corresponding pulse sequences are shown in figure 2.11.

2.2.1 TWO-PULSE ESEEM

Two-pulse ESEEM is performed by varying the inter-pulse delay τ of the standard two-pulse echo sequence, as depicted in figure 2.11 A, and by recording the echo intensity.

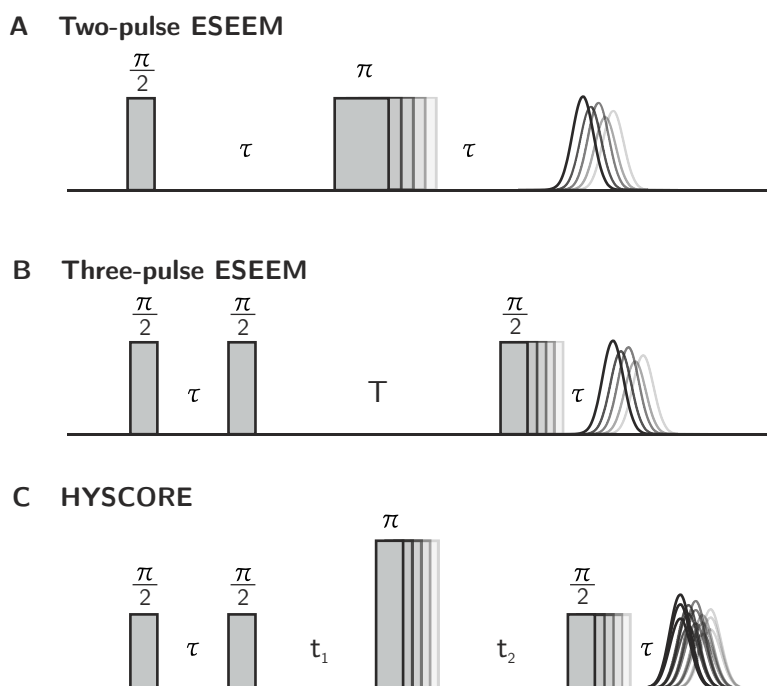


FIGURE 2.11: Pulse sequences for two-pulse (A) and three-pulse (B) ESEEM and HYSCORE (C). The inter-pulse delays τ , T or t_1 and t_2 are incremented for two-pulse ESEEM, three-pulse ESEEM and HYSCORE, respectively, and the echo intensity is recorded in one or two dimensions.

The non-selective $\frac{\pi}{2}$ pulse generates electron coherence on both the allowed and the forbidden electron transitions (see figure 2.12). The evolution of the electron coherence on one of the transitions can again be visualised as magnetisation vector in the xy-plane, precessing during the delay τ with a frequency determined by the frequency offset and the hyperfine couplings. The π pulse flips the magnetisation vectors around the x-axis as explained in section 1.3.4, but also redistributes the coherences among the allowed and forbidden electron spin transitions. The magnetisation vectors corresponding to the four electron transitions are aligned after the second pulse and then start to precess at different frequencies. The transverse magnetisation along the y axis is detected at a time τ after the π pulse. Due to the coherence transfer between different transitions, the precession frequency of a specific magnetisation vector before and after the π pulse may differ and hence the magnetisation vectors are refocused at different positions in the xy-plane. The refocusing of the coherence originally excited by the $\frac{\pi}{2}$ pulse gives rise to the primary echo, while the refocusing of coherence on a different transition to that on which it has defocused leads to coherence transfer echoes [29]. The frequency differences between evolution before and after the π pulse correspond to the nuclear frequencies ω_α and ω_β and to their sum and difference. Incrementing the inter-pulse delay τ leads to a modulation of the electron spin echo at these frequencies, due to oscillating contributions of the coherence transfer echoes.

The echo amplitude modulation formula for the two-pulse ESEEM experiment can be derived using the density matrix formalism [25, 26, 30]. For an $S = \frac{1}{2}$, $I = \frac{1}{2}$ spin system, the modulation formula is:

$$E_{mod}(\tau) = 1 - \frac{k}{4} \left[2 - 2 \cos \omega_\alpha \tau - 2 \cos \omega_\beta \tau + \cos(\omega_\alpha - \omega_\beta) \tau + \cos(\omega_\alpha + \omega_\beta) \tau \right] \quad (2.7)$$

where ω_α and ω_β are the nuclear frequencies of the α and β manifolds (in angular frequency units) and the modulation depth parameter k is defined as:

$$k = \left(\frac{B\omega_I}{\omega_\alpha \omega_\beta} \right) \quad (2.8)$$

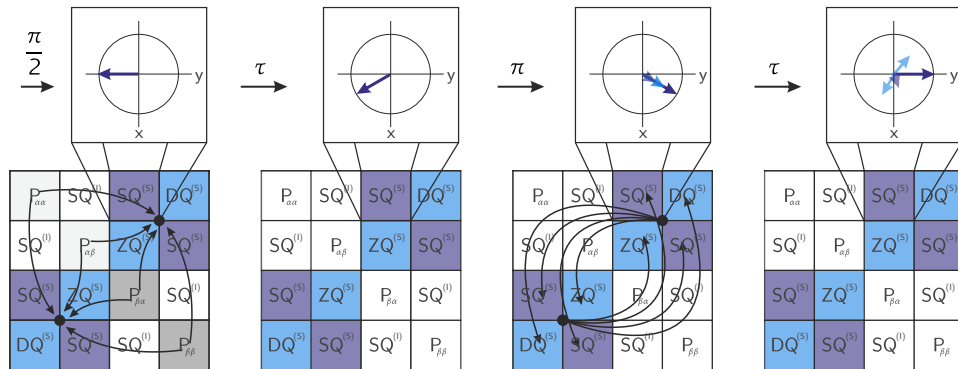


FIGURE 2.12: Schematic representation of the two-pulse ESEEM experiment in terms of evolution of density matrix elements and showing the evolution of the coherence on one of the allowed transitions. The evolution of the coherences corresponding to the other transitions are analogous. The effects of the microwave pulses on the elements of the density matrix are depicted by arrows.

Equation 2.7 shows that the echo amplitude is modulated by the nuclear frequencies and by their sum and difference. The different signs and coefficients lead to a nuclear frequency spectrum with two positive peaks at frequencies corresponding to ω_α and ω_β and two negative peaks with half the intensity at the combination frequencies.

Relaxation during the inter-pulse delay leads to a decay of the signal amplitude with time, and the observed echo intensity in the two-pulse experiment can be written as:

$$I_{echo}(\tau) = E_{mod}(\tau) \exp\left(-\frac{2\tau}{T_m}\right) \quad (2.9)$$

where T_m is the electron phase memory time defined in section 1.4.2. The length of the two-pulse ESEEM trace that can be recorded thus depends on the electron phase memory time. For data analysis, the decay is subtracted before performing the Fourier transform. The phase memory time of the electron also determines the linewidths in two-pulse ESEEM spectra.

The expression in equation 2.7 refers to the coupling of the electron spin to a single nucleus. In the presence of multiple nuclei coupled to the electron, the modulation formula describing the two-pulse ESEEM trace is given by the product of the traces for the individual nuclei:

$$E_{mod}^N(\tau) = \prod_{k=1}^N E_{mod}^k(\tau) \quad (2.10)$$

In the limit of small modulation depths k for all nuclei, the overall ESEEM signal can also be approximated by the sum of the signals of individual nuclei.

For spin systems with $S > \frac{1}{2}$, such as for example a triplet state, similar expressions for the ESEEM signal can be obtained by assuming selective excitation of a single transition and thus treating the spin system as a fictitious $S = \frac{1}{2}$ system [31, 32].

The expressions given above were derived for a spin system in a single defined orientation with respect to the external magnetic field, as would be the case for single crystals. Typically ESEEM is however performed on disordered systems, such as powders or frozen solutions, where the spin system can assume an arbitrary orientation with respect to the external field. The echo envelope modulation pattern for disordered samples is obtained by averaging over all orientations of the spin system with respect to the external field:

$$\langle E_{mod} \rangle_{\theta, \phi} = \frac{1}{4\pi} \int_0^\pi \int_0^{2\pi} E_{mod}(\theta, \phi) \sin \theta \, d\theta \, d\phi \quad (2.11)$$

where the angles θ and ϕ define the orientation of the hyperfine tensor with respect to the external magnetic field. The powder average leads to an attenuation of the modulation frequencies with time. The damping of the modulations depends on the size of the anisotropy of the hyperfine and nuclear quadrupole interactions [27].

2.2.2 THREE-PULSE ESEEM

In a three-pulse ESEEM experiment, the delay T between the second and third pulse of a stimulated echo sequence is varied in order to record the modulation of the electron spin echo as shown in figure 2.11 B.

The first $\frac{\pi}{2}$ pulse generates electron coherence on the allowed and forbidden transitions as in the two-pulse ESEEM experiment. The second $\frac{\pi}{2}$ pulse converts the electron coherence to nuclear coherence. This can be achieved since a pulse acting on an allowed transition generates electron coherence and a pulse acting on a forbidden transition generates both electron and nuclear coherence, hence the combination of two pulses can lead to the creation of just nuclear coherence. The nuclear coherence evolves during the inter-pulse delay T and is transformed back to electron coherence by the third $\frac{\pi}{2}$ pulse. The electron spin echo is formed after a time τ , equal to the delay between the first two pulses, and its intensity is recorded as a function of T .

The modulation formula for the three-pulse ESEEM experiment can also be derived with the density matrix formalism and is given by:

$$\begin{aligned} E_{mod}(\tau, T) &= \frac{1}{2} \left[E_{mod}^{\alpha}(\tau, T) + E_{mod}^{\beta}(\tau, T) \right] \\ &= 1 - \frac{k}{4} \left[(1 - \cos \omega_{\alpha} \tau) (1 - \cos \omega_{\beta} (\tau + T)) + (1 - \cos \omega_{\beta} \tau) (1 - \cos \omega_{\alpha} (\tau + T)) \right] \end{aligned} \quad (2.12)$$

The modulation formula for the three-pulse ESEEM experiment only contains the nuclear frequencies ω_{α} and ω_{β} , the combination frequencies are absent. This reduction of the number of peaks with respect to the two-pulse ESEEM experiment is often an advantage, as it simplifies the spectrum obtained after Fourier transform.

As can be seen from equation 2.12, the three-pulse echo modulation depends on both inter-pulse delays, τ and T . The $(1 - \cos \omega_i \tau)$ terms in equation 2.12 describe the dependence of the amplitude of the modulation at the nuclear frequency of one electron spin manifold on the inter-pulse delay τ and on the nuclear frequency of the other manifold. The modulation at the nuclear frequency $\omega_{\alpha/\beta}$ thus vanishes if $\omega_{\beta/\alpha} = 2\pi n \tau^{-1}$ with $n = 0, 1, \dots$. The resulting blind-spots in the three-pulse ESEEM spectrum can lead to misinterpretation and it is thus common practice to record ESEEM traces for different τ values. In some cases, the blind-spot behaviour of this experiment can be exploited to suppress strong matrix lines, e.g. for ^1H nuclei, that often prevent the measurement of small hyperfine couplings.

The electron coherences decay with the electron phase memory time T_m during the τ intervals and the nuclear coherences decay with the nuclear phase memory time $T_m^{(n)}$ during the time T . The final expression for the echo intensity including relaxation effects under the assumption of $T_{1e} > T_m$ is thus:

$$I_{echo}(\tau, T) = E_{mod}(\tau, T) \exp\left(-\frac{2\tau}{T_m}\right) \exp\left(-\frac{T}{T_m^{(n)}}\right) \quad (2.13)$$

Since three-pulse ESEEM is based on the evolution of nuclear coherences, the linewidth is determined by the nuclear phase memory time, which is generally longer than the electron phase memory time, leading to smaller linewidths and increased resolution in three-pulse ESEEM as compared to two-pulse ESEEM.

In the case of more than one nucleus coupled to the electron spin, the overall modulation formula can again be obtained using the product rule. However, since the electron coherences are in a defined state during the evolution time T , only combinations of nuclear frequencies within the same electron spin manifold are possible. This leads to the following expression:

$$E_{mod}^N(\tau, T) = \frac{1}{2} \left[\prod_{k=1}^N E_{mod}^{k,\alpha}(\tau, T) + \prod_{k=1}^N E_{mod}^{k,\beta}(\tau, T) \right] \quad (2.14)$$

Similarly to the two-pulse experiment, the combination peaks are weak for small modulation depth parameters and the ESEEM spectra can then be interpreted as sums of the spectra corresponding to the different nuclei.

2.2.3 HYSORE

HYSORE (HYperfine Sublevel CORrElation) is a two-dimensional variant of the ESEEM experiment [33] and is performed by incrementing the inter-pulse delays t_1 and t_2 shown in figure 2.11 C in two dimensions. A mixing pulse leads to correlation of the nuclear frequencies of different electron manifolds, improving the resolution and providing additional information on the spin system [33, 34].

The first part of the HYSORE pulse sequence is analogous to the three-pulse ESEEM sequence; two $\frac{\pi}{2}$ pulses separated by τ generate nuclear coherence, which then evolves during the time t_1 . The mixing π pulse then interchanges nuclear coherence between the two electron spin manifolds. The transfer of nuclear coherence between the two electron spin manifolds occurs as the π pulse inverts the electron spin and thus inverts the hyperfine field B_{hf} experienced by the nuclei. The nuclear coherence then continues to evolve at a different frequency during the time t_2 and is then transformed back to electron coherence by the last $\frac{\pi}{2}$ pulse and refocuses to give an echo after a time τ . Nuclear coherence evolving with the frequency of one electron spin manifold during t_1 , evolves with the frequency of the other electron spin manifold during t_2 and gives rise to cross-peaks in the HYSORE spectrum. A non-ideal mixing pulse leads to incomplete transfer of nuclear coherence between the two manifolds and therefore gives rise to diagonal peaks. The transfer of coherences in the HYSORE experiments is shown pictorially in figure 2.13.

The modulation formula for the HYSORE experiment on a $S = \frac{1}{2}$, $I = \frac{1}{2}$ system is given in equation 2.15 [35]. The expression is usually divided into two contributions to emphasise the different evolution pathways of the nuclear spins: $E_{mod}^{\alpha\beta}/E_{mod}^{\beta\alpha}$ describe the nuclear spins precessing with the

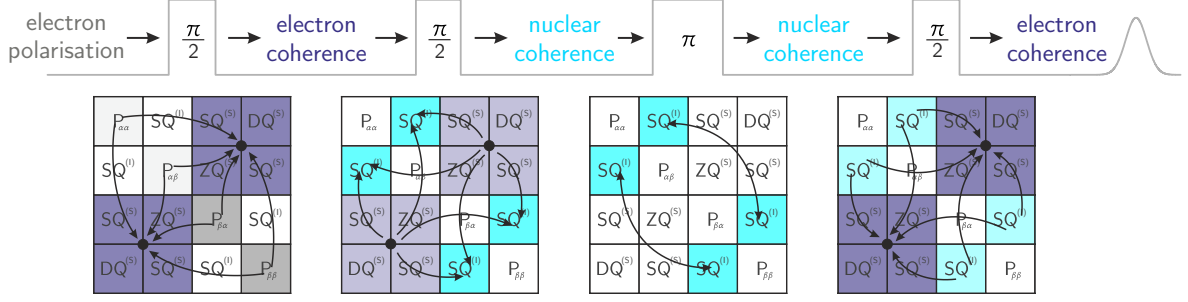


FIGURE 2.13: Schematic representation for the generation and transfer of coherences in a HYSCORE experiment applied to a $S = \frac{1}{2}$, $I = \frac{1}{2}$ system. The effects of the microwave pulses on the elements of the density matrix are depicted and the arrows indicate transfers between polarisation and coherences and between different types of coherences.

frequencies of the α/β manifold during t_1 and of the β/α manifold during t_2 .

$$\begin{aligned}
 E_{mod}(\tau, t_1, t_2) &= \frac{1}{2} [E_{mod}^{\alpha\beta}(\tau, t_1, t_2) + E_{mod}^{\beta\alpha}(\tau, t_1, t_2)] \\
 E_{mod}^{\alpha\beta}(\tau, t_1, t_2) &= 1 - k \left[\frac{C_0}{4} + \frac{C_\alpha}{2} \cos\left(\omega_\alpha t_1 + \frac{\omega_\alpha \tau}{2}\right) + \frac{C_\beta}{2} \cos\left(\omega_\beta t_2 + \frac{\omega_\beta \tau}{2}\right) \right. \\
 &\quad \left. + \frac{C_c}{2} \left\{ c^2 \cos\left(\omega_\alpha t_1 + \omega_\beta t_2 + \frac{\omega_+ \tau}{2}\right) - s^2 \cos\left(\omega_\alpha t_1 - \omega_\beta t_2 + \frac{\omega_- \tau}{2}\right) \right\} \right] \\
 E_{mod}^{\beta\alpha}(\tau, t_1, t_2) &= 1 - k \left[\frac{C_0}{4} + \frac{C_\alpha}{2} \cos\left(\omega_\alpha t_2 + \frac{\omega_\alpha \tau}{2}\right) + \frac{C_\beta}{2} \cos\left(\omega_\beta t_1 + \frac{\omega_\beta \tau}{2}\right) \right. \\
 &\quad \left. + \frac{C_c}{2} \left\{ c^2 \cos\left(\omega_\alpha t_2 + \omega_\beta t_1 + \frac{\omega_+ \tau}{2}\right) - s^2 \cos\left(\omega_\alpha t_2 - \omega_\beta t_1 + \frac{\omega_- \tau}{2}\right) \right\} \right]
 \end{aligned} \tag{2.15}$$

$$\begin{aligned}
 C_0 &= 3 - \cos \omega_\alpha \tau - \cos \omega_\beta \tau - s^2 \cos \omega_+ \tau - c^2 \cos \omega_- \tau \\
 C_\alpha &= c^2 \cos\left(\omega_\beta \tau - \frac{\omega_\alpha \tau}{2}\right) + s^2 \cos\left(\omega_\beta \tau + \frac{\omega_\alpha \tau}{2}\right) - \cos\left(\frac{\omega_\alpha \tau}{2}\right) \\
 C_\beta &= c^2 \cos\left(\omega_\alpha \tau - \frac{\omega_\beta \tau}{2}\right) + s^2 \cos\left(\omega_\alpha \tau + \frac{\omega_\beta \tau}{2}\right) - \cos\left(\frac{\omega_\beta \tau}{2}\right) \\
 C_c &= -2 \sin\left(\frac{\omega_\alpha \tau}{2}\right) \sin\left(\frac{\omega_\beta \tau}{2}\right)
 \end{aligned}$$

$$c^2 = \cos^2 \eta = \frac{1}{2} (1 + \sqrt{1 - k}) \quad s^2 = \sin^2 \eta = \frac{1}{2} (1 - \sqrt{1 - k})$$

where ω_α and ω_β are the nuclear frequencies of the α and β manifolds and the modulation depth parameter k is defined in equation 2.8.

The different terms in equation 2.15 give rise to different types of peaks in the HYSCORE spectrum obtained after 2D Fourier transform along the time dimensions t_1 and t_2 (see also figure 2.14):

- C_0 is an unmodulated term as it does not depend on t_1 or t_2 ;
- the terms $\cos(\omega_i t_{1/2} + \frac{1}{2} \omega_i \tau)$ depend on either t_1 or t_2 and give rise to axial peaks $(\omega_i, 0)$ or $(0, \omega_i)$, which are, however, usually eliminated by the baseline correction;

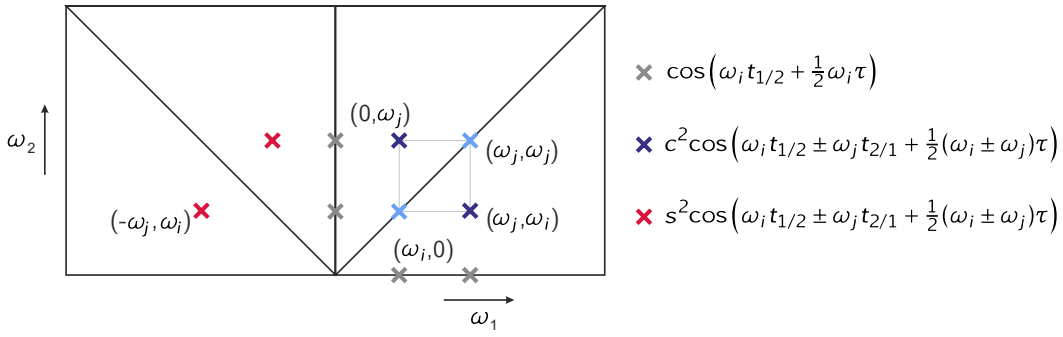


FIGURE 2.14: Schematic drawing of the upper quadrants of a HYSCORE spectrum showing the possible types of peaks and the corresponding expressions in the modulation formula given in equation 2.15: cross-peaks in different quadrants for weak and strong coupling (blue and red, respectively), diagonal peaks (light blue) and axial peaks (grey).

- the terms $\cos(\omega_i t_{1/2} \pm \omega_j t_{2/1} + \frac{1}{2}(\omega_i \pm \omega_j)\tau)$ depend on both t_1 and t_2 and thus give rise to cross-peaks at $(\pm\omega_i, \omega_j)$.

The weighting factors c^2 and s^2 determine whether the cross-peaks appear in the $(+, +)$ or $(-, +)$ quadrant of the HYSCORE spectrum and depend on the strength of the hyperfine coupling compared to the nuclear Zeeman interaction. In the limit of weak coupling $c^2 \approx 1$ and $s^2 \approx 0$, leading to cross-peaks in the $(+, +)$ quadrant, while in the limit of strong coupling $c^2 \approx 0$ and $s^2 \approx 1$ and the cross-peaks appear in the $(-, +)$ quadrant. A first indication of the strength of the hyperfine coupling is thus provided by the position of the cross-peaks in the weak or strong coupling quadrant. In addition to this, the distribution of peaks over both quadrants reduces peak overlap and facilitates interpretation.

Equation 2.15 also shows that a similar blind-spot behaviour to the three-pulse ESEEM experiment is also expected for HYSCORE. It is thus advantageous to perform HYSCORE experiments for different values of the inter-pulse delay τ .

In the presence of several nuclei, a product rule analogous to the one reported for the three-pulse ESEEM experiment also applies for HYSCORE.

In the presence of a single nucleus of spin $I = \frac{1}{2}$, as can be seen from equation 2.15, only two cross-peaks are expected in each quadrant. A similar expression can also be derived for an electron coupled to a nuclear spin with $I = 1$ [36] and shows that 16 cross-peaks arising from $\Delta m_I = \pm 1$ nuclear transitions and 20 cross-peaks also involving $\Delta m_I = 2$ nuclear transitions are predicted. However, the intensity of most of these cross-peaks is too low to be detected experimentally.

2.2.4 MATCHED ESEEM AND HYSCORE

The signal intensity in ESEEM and HYSCORE experiments can be greatly increased with the use of matched microwave pulses [37]. Matched pulses are high turning angle (HTA) pulses with a microwave strength optimised for the generation and transfer of coherences.

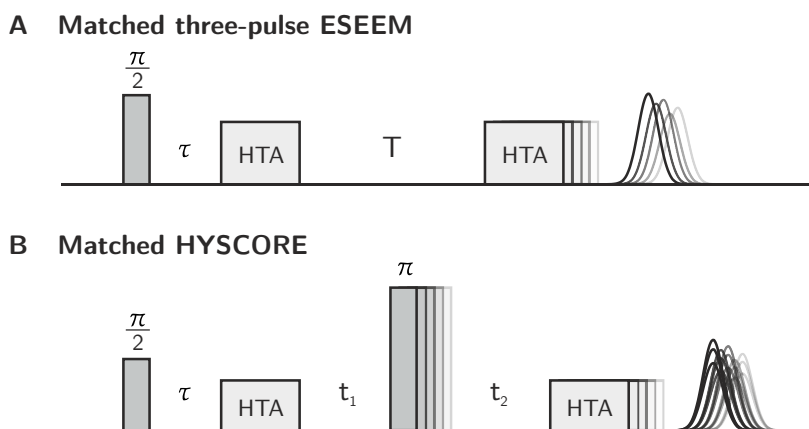


FIGURE 2.15: Pulse sequences for matched three-pulse ESEEM (A) and matched HYSCORE (B).

The three-pulse ESEEM and HYSCORE experiments are based on the generation of nuclear coherence, which then evolves during the mixing time, eventually leading to the desired signals. Nuclear coherence can be generated from electron polarisation with semi-selective microwave pulses, which simultaneously excite two transitions with a common level, for example an allowed and a forbidden electron transition involving the $|\beta\alpha\rangle$ level in the two-level system in figure 2.10. In an experiment using matched pulses, the microwave strength and the pulse length are optimised for the creation of a specific nuclear coherence. These optimum parameters depend on the spin system and hence have to be determined experimentally, as the hyperfine couplings are usually not known in advance.

In general, the microwave field strength is matched to the nuclear Zeeman frequency for weakly coupled nuclei, while the maximum available microwave strength is used for strongly coupled nuclei [37, 38]. In three-pulse ESEEM and HYSCORE typically the second and last microwave pulses are replaced by matched pulses (see figure 2.15). The pulse lengths are optimised by performing the three-pulse ESEEM experiment and gradually incrementing the pulse lengths of the last two microwave pulses. The optimum pulse length gives rise to the largest modulation depth and strongest ESEEM signal intensity.

The use of matched pulses can lead to enhancements in signal intensity often exceeding one order of magnitude [37]. In the matched HYSCORE experiment, the enhancement factor is particularly large for multiple quantum peaks, hence the use of matched pulses is particularly useful for nitrogen HYSCORE, where it can often lead to the appearance of double and multiple quantum peaks that could not be detected in a standard HYSCORE experiment [37].

2.2.5 ANALYSIS OF ESEEM SPECTRA

ESEEM and ENDOR both indirectly measure the nuclear transition frequencies, therefore the peak positions in ESEEM spectra obtained after Fourier transform of the time domain data are the same as in the corresponding ENDOR spectrum for $I = \frac{1}{2}$ nuclei. In the presence of $I = 1$ nuclei, the ENDOR

techniques only give peaks at the single-quantum (sq, $\Delta m_I = \pm 1$) frequencies, while double-quantum (dq, $\Delta m_I = \pm 2$) peaks are also observed and often dominate the ESEEM spectrum. In addition to this, sum and difference frequencies are also observed in two-pulse ESEEM spectra, as discussed in section 2.2.1.

ESEEM spectra are characterised by considerably different powder lineshapes as compared to the corresponding ENDOR spectra. The powder lineshapes in ENDOR spectra are determined by the statistical weights of the different orientations, leading to turning points at the nuclear frequencies corresponding to the hyperfine couplings along a principal direction of the hyperfine tensor. In ESEEM, the powder lineshapes are determined by the angular dependence of the modulation depth parameter k and by the absence of modulations for orientations along the principal axes of the hyperfine tensor, often leading to structureless peaks [39, 40]. The lineshapes of ENDOR and ESEEM spectra for an $I = \frac{1}{2}$ nucleus in different coupling regimes are compared in figure 2.16. In three-pulse ESEEM the intensities of peaks at the nuclear frequency of one manifold also depend on the nuclear frequency of the other manifold through the terms $\frac{1}{4}k(1 - \cos \omega_{\alpha/\beta}\tau)$ in equation 2.12, leading to different intensities of the low and high frequency peaks as opposed to ENDOR, where they usually have approximately equal intensities (see figure 2.16). Matching of the nuclear Zeeman and hyperfine interactions ($A \approx 2\nu_I$), often also referred to as exact cancellation, leads to line narrowing and an increase in line intensity; for $I = \frac{1}{2}$, a strong narrow line is then observed at low frequencies and a broader weak line at approximately $2\nu_I$ [41].

The analysis of $I = 1$ ESEEM spectra is greatly simplified under exact cancellation conditions, i.e. when the nuclear Zeeman and hyperfine interactions cancel in one of the manifolds (see figure 2.18 A). The

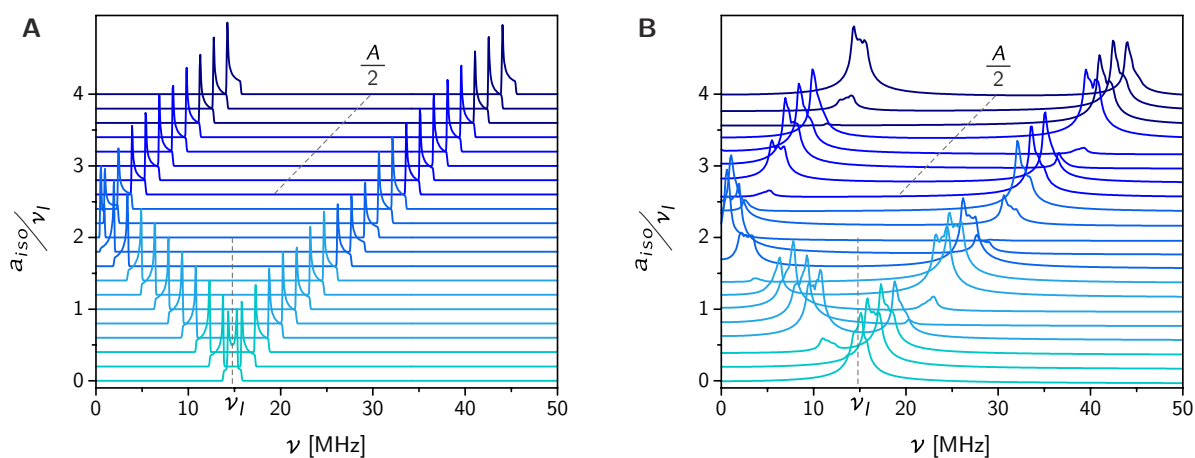


FIGURE 2.16: Simulations of ENDOR spectra (A) and three-pulse ESEEM spectra with $\tau = 120$ ns (B) for a single ^1H nucleus with fixed anisotropic hyperfine coupling and varying isotropic hyperfine coupling. The spectra change from the weak coupling regime ($a_{iso} < 2\nu_I$) on the bottom through the matching regime ($a_{iso} \approx 2\nu_I$) to the strong coupling regime ($a_{iso} > 2\nu_I$) on top. The intensities are normalised for better comparison of the lineshapes.

nuclear frequencies in that manifold are then determined solely by the nuclear quadrupole interaction, yielding peaks at the pure nuclear quadrupole frequencies:

$$\nu_0 = \frac{Q}{2}\eta \quad \nu_- = \frac{Q}{4}(3-\eta) \quad \nu_+ = \frac{Q}{4}(3+\eta) \quad (2.16)$$

The corresponding ESEEM spectra are characterised by three narrow lines at low frequencies (ν_0 , ν_- and ν_+ , with $\nu_0 + \nu_- = \nu_+$) and a weaker double-quantum (dq) peak corresponding to the other manifold at the frequency:

$$\nu_{dq} = 2\sqrt{\left(\nu_+ + \frac{A}{2}\right)^2 + \frac{Q^2}{16}(3+\eta^2)} \quad (2.17)$$

The single-quantum peaks of this manifold are typically too broad to be observed [42]. The intensities of the lines at the pure nuclear quadrupole frequencies are determined by the relative orientations of the nuclear quadrupole and hyperfine tensors and by τ [42]. The ESEEM time trace and spectrum in the exact cancellation regime are compared to the corresponding data in the weak and strong coupling regimes in figure 2.17.

For ESEEM spectra of high-spin systems, similar considerations as already described for ENDOR in section 2.1.3 apply. In the case of a triplet state coupled to an $I = 1$ nucleus in the cancellation regime, which for a triplet state corresponds to $A \approx \nu_I$, the nuclear frequencies in two of the manifolds do not depend on the hyperfine coupling. The nuclear frequencies of the $m_S = 0$ manifold always depend only on the nuclear Zeeman and the nuclear quadrupole interaction (see figure 2.18 B). In the cancellation regime, the nuclear frequencies of another manifold also depend on only the nuclear quadrupole interaction, which means that for one of the two triplet transitions no information on the hyperfine interaction is contained in the corresponding ESEEM spectrum. The information on the hyperfine coupling is contained in the double quantum peak of the ESEEM spectrum corresponding to the other triplet transition. In general, triplet state ESEEM powder spectra of $I = 1$ nuclei are dominated by the nuclear frequencies of the $m_S = 0$ manifold, as the hyperfine couplings show a more

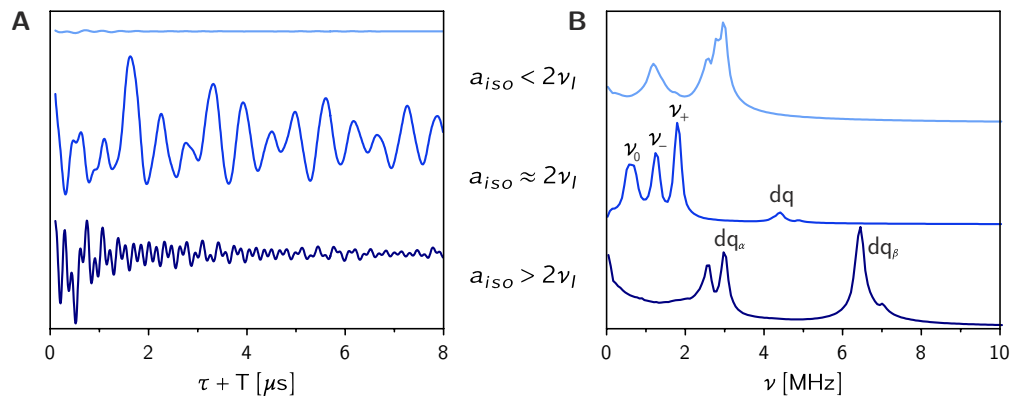


FIGURE 2.17: Simulations showing the main characteristics of the time (A) and the frequency (B) domain of three-pulse ESEEM ($\tau = 120$ ns) data for a ^{14}N nucleus in the weak coupling ($a_{iso} < 2\nu_I$), matching ($a_{iso} \approx 2\nu_I$) and strong coupling regimes ($a_{iso} > 2\nu_I$).

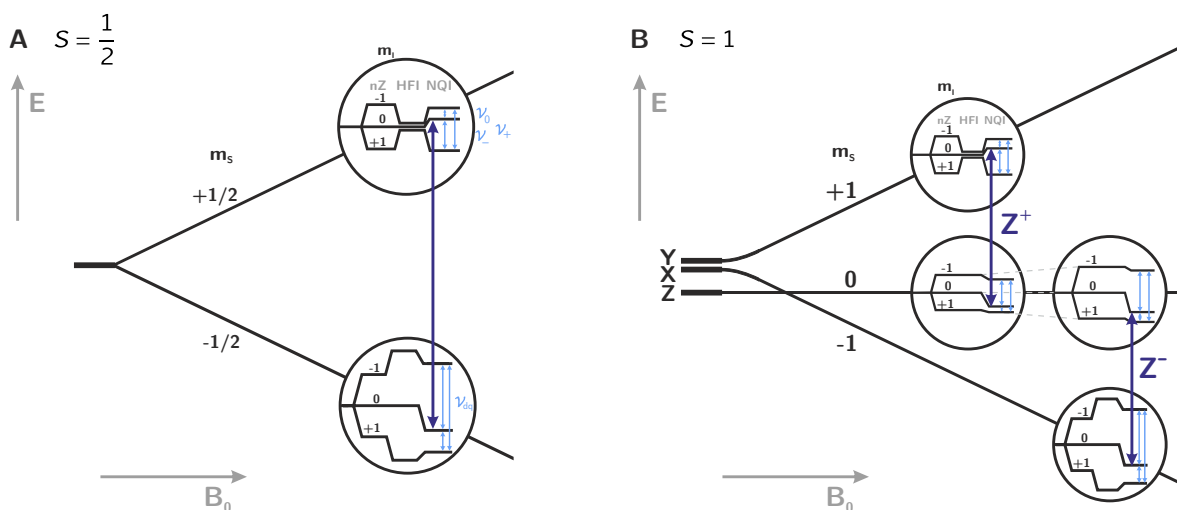


FIGURE 2.18: Energy level diagram for an $S = \frac{1}{2}, I = 1$ (A) and $S = 1, I = 1$ (B) spin system close to the cancellation regime for a positive hyperfine coupling. For the $S = 1$ system D is positive and the magnetic field is aligned with the Z orientation of the zero-field splitting tensor.

significant orientation dependence than the nuclear quadrupole interaction and hence the nuclear frequencies of the manifolds with significant contributions of the hyperfine coupling are often too broad to be clearly identified.

In addition to the frequency domain, the time domain of ESEEM experiments can also provide information on the hyperfine interactions. The modulation depth and the modulation damping depend on the number of coupled nuclei and on the strength and anisotropy of their hyperfine coupling [27]. A simultaneous fitting of the time and frequency domain of ESEEM data, ideally recorded at different microwave frequencies, generally leads to the most accurate determination of the hyperfine coupling parameters.

ESEEM simulations are generally performed using a density matrix approach introduced by Mims [25, 30], implemented, for example, in EasySpin's *saffron* routine [24]. The computation is based on the determination of the ESEEM frequencies and amplitudes, which are then used to construct the frequency domain spectrum [24]. The time domain data are obtained by inverse Fourier transform, achieving increased computational efficiency with respect to direct computation of the ESEEM trace.

2.2.6 ANALYSIS OF HYSCORE SPECTRA

HYSCORE spectra of disordered samples are characterised by so-called correlation ridges, arising from a series of cross-peaks between the nuclear frequencies of different manifolds for contributing relative orientations of the magnetic field with respect to the spin system. The typical HYSCORE powder patterns for an $S = \frac{1}{2}, I = \frac{1}{2}$ system with an axial hyperfine coupling tensor in the weak and strong coupling case are shown in figure 2.19 [3, 38].

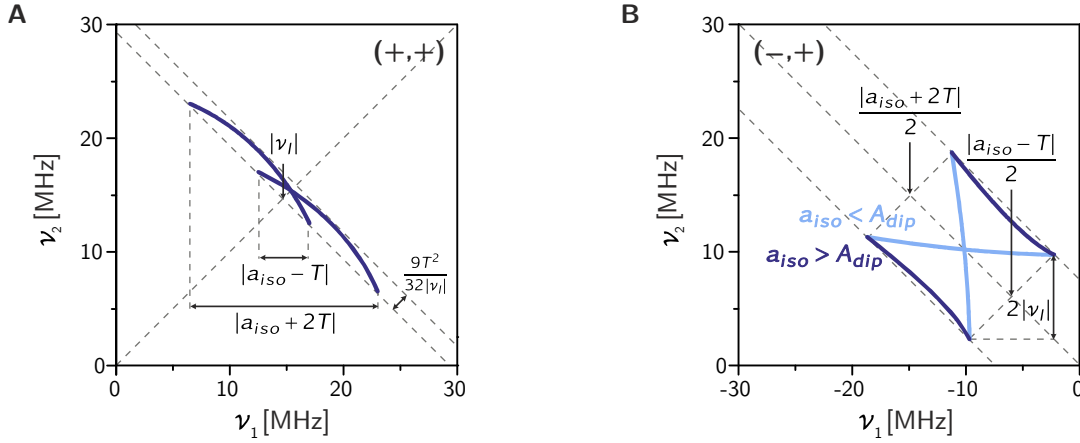


FIGURE 2.19: Hyscore cross-peak positions for an $S = \frac{1}{2}$, $I = \frac{1}{2}$ spin system with an axial hyperfine tensor ($A = A_{dip} + a_{iso}$, $A_{dip} = [-T, -T, 2T]$) in the weak (A) and strong (B) coupling regime [38]. The weak coupling simulations (A) are for ^1H at X-band with $T = 7$ MHz and $a_{iso} = 2.5$ MHz; the strong coupling simulations (B) for ^{13}C at X-band with $T = 6$ MHz and $a_{iso} = 18$ MHz (dark blue) or $T = 14$ MHz and $a_{iso} = 2$ MHz (light blue).

In the case of weak coupling ($A < 2\nu_I$), the strongest correlation ridges occur in the $(+, +)$ quadrant of the Hyscore spectrum and lie along the antidiagonal. Information on the isotropic and dipolar part of the hyperfine tensor can be inferred from the extrapolated distances between the end points of the ridges [38, 43]. The curvature of the ridges perpendicular to the diagonal is also related to the dipolar hyperfine coupling as shown in figure 2.19.

In the case of strong coupling ($A > 2\nu_I$), correlation ridges parallel to the diagonal or crossing the diagonal are observed in the $(-, +)$ quadrant of the Hyscore spectrum as shown in figure 2.19 B. The edges of the ridges again correspond to the principal values of the hyperfine tensor and are thus separated by $2\nu_I$. The isotropic and dipolar hyperfine coupling constants for an axial hyperfine interaction can be determined from the position of the crossing point of a line connecting the two extrapolated endpoints of the ridges and the diagonal. The shape of the correlation ridges depends on the relative sign and magnitude of the isotropic and dipolar hyperfine coupling, determining the signs of the three eigenvalues of the hyperfine tensor. If the hyperfine coupling changes sign, the ridges cross, otherwise they remain approximately parallel to the diagonal [38, 43].

As discussed in section 2.2.3, the number of cross-peaks increases significantly for coupling to an $I = 1$ nucleus, however not all of them are generally observed in the Hyscore spectrum. In the presence of small nuclear quadrupole couplings, such as for example for ^2H , the Hyscore spectrum is dominated by the single-quantum features, while in the presence of strong nuclear quadrupole interactions, such as for ^{14}N , the double-quantum features are typically characterised by the highest intensity [38]. The double-quantum transition frequencies do not depend on the nuclear quadrupole coupling to first order (see equation 2.17) and thus the double-quantum features of $I = 1$ nuclei resemble the correlation ridges in Hyscore spectra of $I = \frac{1}{2}$ nuclei, but with a splitting of $2\nu_I$. The

single-quantum features, however, depend on the nuclear quadrupole coupling and are thus broadened, which makes them more difficult to identify. As discussed above, the hyperfine interaction leads to splittings along the antidiagonal of the HSCORE spectrum, whilst the nuclear quadrupole interaction leads to splittings along the diagonal.

Examples for powder patterns of HSCORE spectra of $I = 1$ nuclei are shown in figure 2.20. Initial information on the hyperfine and nuclear quadrupole interaction can often be obtained directly from the distances between specific types of peaks as shown in the figure. This analysis is based on the assumption of collinear $\mathbf{g}$ -, hyperfine and nuclear quadrupole tensors and can in most cases only be used for the determination of initial parameters for simulation or fitting. The assignment of different peaks in the HSCORE spectrum can often be complicated, however in ^{14}N HSCORE spectra the features with the highest intensity can be identified as double-quantum peaks and used to estimate the hyperfine coupling, either from the distance between the peaks (weak coupling regime) or from the crossing point of the diagonal and the line connecting the double-quantum peaks (strong coupling).

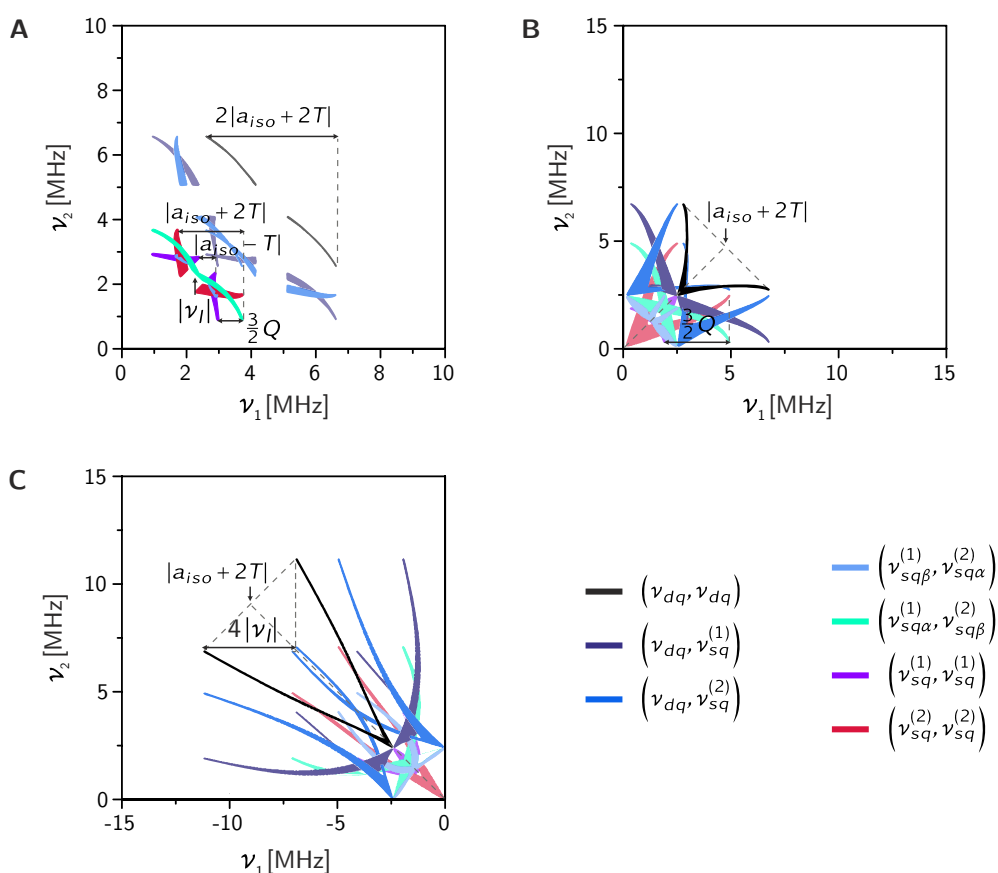


FIGURE 2.20: Calculated HSCORE cross-peak positions for $S = \frac{1}{2}$, $I = 1$ spin systems with an axial hyperfine tensor ($A = A_{dip} + a_{iso}$, $A_{dip} = [-T, -T, 2T]$). (A) ^2H HSCORE spectrum for $T = 0.5$ MHz, $a_{iso} = 1$ MHz, $Q = 0.5$ MHz and $\eta = 0.5$. (B) ^{14}N HSCORE spectrum for $T = 1.5$ MHz, $a_{iso} = 1.5$ MHz, $Q = 2$ MHz and $\eta = 0.5$. (C) ^{14}N HSCORE spectrum for $T = 3$ MHz, $a_{iso} = 3$ MHz, $Q = 2$ MHz and $\eta = 0.5$. The features corresponding to different types of cross-peaks are indicated in different colours and the features with higher intensities in experimental HSCORE spectra are highlighted.

In the presence of orientation selection, only parts of the correlation ridges are excited at each field position and simulations are typically required to determine the hyperfine couplings, especially if signals of different coupled nuclei overlap. An alternative approach is based on the analysis of slopes and intercepts in a plot of ν_α^2 against ν_β^2 [44]. HYSCORE spectra recorded at field positions with strong orientation selection, for example at a $g_{||}$ field position, resemble single-crystal spectra with narrow peaks instead of ridges. The hyperfine values determined from those spectra correspond to the hyperfine coupling along the corresponding axis of the g -tensor.

In high-spin systems, both orientation and transition selection influence the appearance of HYSCORE spectra. For a triplet state this means that HYSCORE spectra only contain nuclear frequencies of two of the three manifolds ($m_S = +1$ and $m_S = 0$, or $m_S = -1$ and $m_S = 0$). Both contain the nuclear frequencies of the $m_S = 0$ manifold, determined exclusively by the nuclear Zeeman and nuclear quadrupole interaction (for $I = 1$) (see figure 2.18). As in triplet state ENDOR, the sign of the hyperfine coupling can be determined from the HYSCORE spectra if the sign of the ZFS parameter D is known.

The correlation ridges for triplet state HYSCORE powder spectra are expected to be parallel to the frequency axes, since the orientation-dependence of the hyperfine couplings would lead to a distribution of nuclear frequencies in the $m_S = +1$ and $m_S = -1$ manifolds, but not in the $m_S = 0$ manifold. The sign of the hyperfine coupling can be determined based on the position of the cross-peaks with respect to the nuclear Larmor frequency for each of the two triplet transitions in the weak coupling regime. The correlation ridges for $I = 1$ nuclei are also approximately parallel to the axes, since the orientation-dependent changes in hyperfine coupling are usually more significant with respect to those of the nuclear quadrupole interaction. As already discussed for ESEEM, for $I = 1$ nuclei in the cancellation regime, in addition to the nuclear frequencies of the $m_S = 0$ manifold, the nuclear frequencies of one of the other manifolds also do not depend on the hyperfine coupling and reduce to the pure nuclear quadrupole frequencies of equation 2.16 ($m_S = -1$ for $A < 0$ and $m_S = +1$ for $A > 0$). The nuclear frequencies of the two manifolds will be very similar in most cases, one depending only on the nuclear quadrupole interaction and the other on the nuclear quadrupole and nuclear Zeeman interaction, and peaks on or close to the diagonal will be observed in the HYSCORE spectrum for that transition. All the information on the hyperfine coupling is in this case contained in the HYSCORE spectrum of the triplet state transition involving the other m_S manifold. The sign of the hyperfine coupling can be determined from the relative positions of the double-quantum cross-peaks in the two triplet state transitions corresponding to each orientation. If the hyperfine coupling is positive, the larger nuclear frequencies are observed for the $m_S = -1 \rightarrow m_S = 0$ transition (see figure 2.18 B), if it is negative, the larger nuclear frequencies are observed for the $m_S = 0 \rightarrow m_S = +1$ transition. Examples of triplet state HYSCORE spectra are shown and discussed in section 4.3.4 of Chapter 4.

The rich information contained in HYSCORE spectra can only be completely extracted through simulation of the experimental results. The efficient and accurate simulation of HYSCORE is thus of fundamental importance, but due to its being a two-dimensional technique, computation of the spectra can easily become computationally very intensive. Numerical simulations of HYSCORE spectra have been implemented in GAMMA [45, 46] and also in Matlab with the *saffron* function in EasySpin [24],

already previously mentioned for ESEEM simulations. Since simulations including both peak positions and intensities are very time consuming and an accurate simulation of the intensities requires the consideration of not only the spin Hamiltonian parameters, but also of the relaxation times, the properties of the pulses used in the experiment and cross-suppression effects [34, 47], the initial analysis of the experimental data often involves just the simulation of the cross-peak positions. In this thesis, the cross-peak positions were computed with a Matlab routine written by Dr. Jeffrey Harmer and based on the exact diagonalisation of the spin Hamiltonian. In some cases, the full HYSCORE spectra, including the cross-peak intensities, were also simulated using EasySpin [24].

3 ELECTRON DELOCALISATION IN RADICAL CATIONS OF PORPHYRIN ARRAYS

The electron delocalisation in radical cations and triplet states of linear and cyclic supramolecular porphyrin structures was studied using a variety of EPR techniques. All of the investigated structures were designed and synthesised in the group of Prof. Harry L. Anderson, who is the main collaborator on this project.

The electron delocalisation in the radical cations generated by chemical oxidation was investigated both at room temperature and at low temperatures using continuous wave EPR as well as different hyperfine techniques. The extent of delocalisation in the corresponding photoexcited triplet states was determined by time-resolved EPR experiments and will be the topic of Chapter 4.

3.1 SUPRAMOLECULAR PORPHYRIN STRUCTURES

Supramolecular assemblies of porphyrins gained considerable interest due to their role in natural photosynthetic processes, where they are responsible for efficient energy and electron transfer [48]. In natural photosynthetic antenna complexes and reaction centres, assemblies of chlorophyll or bacteriochlorophyll molecules are responsible for the absorption of light as well as energy and electron transfer. The geometries of these chromophore arrays are determined by the surrounding protein matrix and were found to be crucial for efficient electronic communication between the single monomeric units. Examples of such assemblies include large cyclic bacteriochlorophyll arrays found in the light harvesting complexes of purple photosynthetic bacteria [49], self-assembled stacks of chlorophylls in chlorosomes [50] and face-to-face dimers of chlorophylls or bacteriochlorophylls (typically referred to as the special pair) responsible for charge separation in plant and bacterial photosynthetic reaction centres.

A lot of effort has been focused on the design of artificial linear chromophore arrays and more sophisticated branched and cyclic structures with porphyrins as building blocks. Due to the versatile optoelectronic properties of porphyrins, these supramolecular assemblies represent potential candidates for a wide range of applications, including artificial photosynthesis [51–55], novel optical materials [56, 57] and molecular-scale electronics [58–60]. One of the fundamental properties required for the

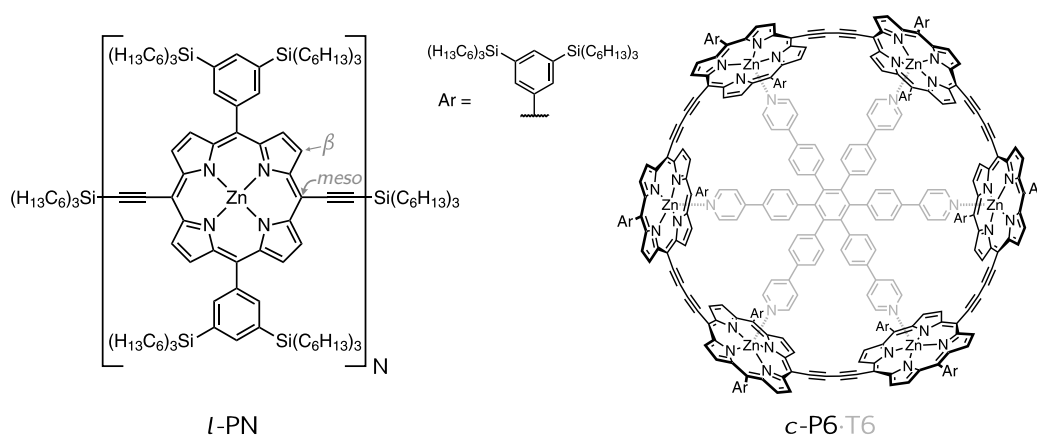


FIGURE 3.1: Molecular structures of the linear porphyrin oligomers *l*-PN (*N* = 1-6) and porphyrin nanorings *c*-P6 (black) and *c*-P6-T6 (T6 template shown in grey). The *meso* and *β* positions on the porphyrin are indicated.

use of porphyrin arrays in molecular engineering is an efficient electronic communication between the single porphyrin units. A profound understanding of the factors determining electronic communication between the single units constituting the supramolecular architecture is of paramount importance for the rational design of optoelectronic devices based on this type of material. With this in mind, conjugated porphyrin oligomers with a wide variety of linkers and linking geometries have been designed and investigated using a series of different techniques [56, 61–70].

The linear and cyclic porphyrin arrays investigated here were designed and synthesised in the research group of Prof. Harry L. Anderson, for the projects described in this thesis specifically by Dr. Patrik Neuhaus and Dr. Georg Fischer. The molecular structures of the investigated porphyrin systems are shown in figure 3.1. The porphyrins are linked at the *meso* positions by butadiyne bonds, which promote conjugation between the different units and allow a wide range of conformations due to the free rotation around this type of linker. The end groups and side groups on the phenyl rings are trihexylsilyl (THS) groups, expected to reduce aggregation by steric hindrance. The synthesis of the linear oligomers is described in reference [60]. The six-membered ring is synthesised using Vernier templating [69, 71] and was investigated both with and without the rigid template used for its synthesis. In addition to this, a double porphyrin ring or nanotube (*t*-P12-T6₂) [72], with butadiyne units linking both the porphyrins within each six-membered ring as well as the porphyrins on the two rings at the *meso* positions, was also investigated.

3.2 EPR OF RADICAL CATIONS OF PORPHYRIN SYSTEMS

EPR spectroscopy is a well established technique for probing the ground state electronic communication between porphyrins and has been applied to radical cations of several natural [73–78] and artificial [64, 66, 68, 79–84] multiporphyrin arrays. Most of these studies have focused on the EPR linewidth as a measure of the extent of electron delocalisation, as first proposed by Norris et al. for the identification of the primary donor in photosynthetic organisms [73].

A narrowing of the EPR line with respect to the single unit is observed in systems with hole/electron hopping on a timescale faster than the hyperfine interactions of the systems under investigation (usually of the order of 10^7 s^{-1} for porphyrins). This is based on the decrease of the hyperfine interaction between the electron spin and magnetic nuclei of the system, as the number of units over which the electron is hopping/delocalised increases. At room temperature, delocalisation over up to seven units in linear *meso-to-meso* ethyne-bridged zinc porphyrin arrays, corresponding to a delocalisation length of ca. 75 Å [66], and over 8-12 porphyrins in a dodecameric ring system with *meta*-phenylene linkers [68], have been demonstrated using continuous wave EPR. While the use of the EPR linewidth as an indicator of electron delocalisation is quite straightforward at room temperature, low temperature EPR linewidths are determined by factors other than just the isotropic hyperfine interactions. These factors need to be taken into account as well in order to reliably determine the electron delocalisation length at low temperatures and especially in frozen solutions.

ENDOR, which allows a direct measurement of the hyperfine couplings of the electron spin to the nuclei of the molecule, can also be used to obtain information on the distribution of the unpaired electron spin. The first application of ENDOR for the determination of electron delocalisation lengths was in the identification of a bacteriochlorophyll dimer as primary donor in the photosynthetic apparatus of purple bacteria by comparison of ENDOR spectra recorded of the pigment *in vitro* and *in vivo* [85]. Similar further studies on both natural photosynthetic systems [76] and porphyrin dimer and trimer model compounds [68, 79, 82, 86] were performed, again mainly at or close to room temperature using continuous wave ENDOR.

3.3 EXPERIMENTAL METHODS

3.3.1 SAMPLE PREPARATION

The EPR measurements were performed on 200 μM solutions of linear and cyclic porphyrin oligomers in toluene- d_8 :THF- d_8 : CD_2Cl_2 1:1:1. The specific solvent mixture was chosen for its glass-forming properties and for the relative stability of the radical cations in this mixture compared to, for example, pure THF or toluene. Pyridine, which is usually added in order to reduce aggregation, could not be used in this study as it preferentially reacts with the oxidant and also with the porphyrin radical cations. The investigated porphyrins do, however, have large side groups, which should limit aggregation. No effects of concentration on the experimental spectra could be identified in the range from 100 to 600 μM at room temperature.

The radical cations were generated by addition of 0.75 equivalents of the oxidant thianthrenium hexachloroantimonate (ThSb) to a deaerated solution kept in the EPR tube under argon. The oxidant was added immediately before the room temperature measurements and for the low temperature measurements the samples were shock-frozen in liquid nitrogen after addition of the oxidant. Measurements using a different type of oxidant, tris(4-bromophenyl)aminium hexachloroantimonate (BAHA), were also performed and yielded similar results.

3.3.2 CW-EPR MEASUREMENTS

Continuous wave (cw) X-band EPR measurements were performed at room temperature and 100 K on a Bruker Biospin EMX spectrometer equipped with a high sensitivity Bruker probe head and an Oxford Instruments helium-flow cryostat. EPR spectra were recorded at a microwave frequency of ca. 9.40 GHz with modulation amplitudes and frequencies of 0.01 mT and 100 kHz, respectively. The microwave power was adjusted in order to avoid spectral distortions. EPR experiments on samples with different radical cation concentrations were performed and no concentration-dependence of the linewidth or lineshape was detected.

3.3.3 PULSE EPR

The pulse EPR measurements were performed on a W-/X-band Bruker Elexsys 680 spectrometer equipped with helium gas-flow cryostats from Oxford instruments. The echo-detected field sweep W-band EPR spectra were recorded by integrating the echo of a two-pulse sequence, $\frac{\pi}{2}-\tau-\pi-\tau$ -echo, with pulse lengths $t_{\pi/2} = 32$ ns, $t_{\pi} = 64$ ns and $\tau = 180$ ns and using a two-step phase cycle.

^1H Mims ENDOR spectra were recorded with the pulse sequence $\frac{\pi}{2}-\tau-\frac{\pi}{2}-T-\frac{\pi}{2}-\tau$ -echo with mw pulse lengths of $t_{\pi/2} = 44$ ns, $\tau = 200$ ns and a radiofrequency pulse with a length of 18 μs applied during the time T ; the rf power was adjusted in order to obtain a π pulse. The ENDOR spectra were recorded at the magnetic field corresponding to the echo maximum and ca. +1 mT from the echo maximum for the linear oligomers, -1 mT for c-P6 and -0.8 mT for c-P6-T6 to investigate orientational selectivity. Mims ENDOR spectra recorded with a 100 μs radiofrequency π pulse only showed negligible differences in the central region of the ENDOR spectrum. Mims ENDOR spectra recorded with different τ values and ENDOR spectra recorded with the Davies ENDOR pulse sequence, $\pi-T-\frac{\pi}{2}-\tau-\pi-\tau$ -echo, with mw pulse lengths of $t_{\pi/2} = 100$ ns, $t_{\pi} = 200$ ns and $T = 200$ μs , $\tau = 200$ ns and a radiofrequency pulse with a length of 18 μs applied during the delay T , were compared in order to rule out the presence of any distortions due to blind-spots in the recorded spectra. No temperature dependence of the appearance of the ENDOR spectra was detected in the range from 6 to 100 K.

X-band HYSCORE experiments were performed at 100 K and/or 80 K with the pulse sequence $\frac{\pi}{2}-\tau-\frac{\pi}{2}-t_1-\pi-t_2-\frac{\pi}{2}-\tau$ -echo with mw pulses of lengths of $t_{\pi/2} = 16$ ns, $t_{\pi} = 16$ ns and $\tau = 120$ ns, and starting times $t_1 = t_2 = 64$ ns with increments $dt = 16$ or 24 ns (data matrix 256×256 or 170×170). A four-step phase cycle was used to remove unwanted echoes. The experimental data were processed in Matlab; the time traces were baseline corrected by a 3rd order polynomial, apodised with a Hamming window and zero-filled to 1024 points in each dimension. A 2D Fourier transform was applied and the absolute-value spectrum was calculated.

X-band three-pulse ESEEM experiments were performed at 80 K with the pulse sequence $\frac{\pi}{2}-\tau-\frac{\pi}{2}-T-\frac{\pi}{2}-\tau$ -echo with mw pulses of length $t_{\pi/2} = 16$ ns and an initial T value of 64 ns with increments $dt = 8$ ns (1024 data points were collected). A four-step phase cycle was used to remove unwanted echoes. The ESEEM traces were recorded for three different τ values (100, 140 and 200 ns) and the FT spectra

were summed to avoid distortions due to blind-spots. The experimental data were processed with a home-written Matlab routine, the time traces were baseline corrected with a stretched exponential, apodised with a Hamming window and zero-filled to 2048 data points. The frequency spectrum was calculated using the cross-term averaging procedure [87] as implemented in EasySpin [23].

W-band ^{14}N Mims ENDOR spectra were recorded at 60 K with the pulse sequence given above, with mw pulse lengths of $t_{\pi/2} = 44$ ns, $\tau = 120 - 160$ ns and a radiofrequency pulse with a length of 55 μs .

3.3.4 COMPUTATIONAL METHODS

DFT geometry optimizations were performed using Turbomole [88] with the B3LYP functional and the SV(P) (split-valence, polarised) basis set [89] using the RI-J (resolution of identity for the Coulomb energy) approximation [90]. The trihexylsilyl groups were replaced by hydrogen atoms and the resulting structures were optimized without symmetry constraints. The hyperfine and nuclear quadrupole interaction parameters were calculated on the optimised structures of *l*-P1-6 with ORCA [91] using the same hybrid functional, B3LYP, and the EPRII basis set, purposely built for the calculation of EPR hyperfine interaction values, for the C, N and H nuclei [92–94] and the 6-31G(d) basis set for Zn. The spin densities were obtained from a Mulliken population analysis. The principal values and orientations of the *g*-tensors of *l*-P1-5 were also calculated; calculations on the larger systems were unfortunately not successful.

3.3.5 SIMULATION METHODS

The room temperature cw-EPR spectra were simulated with the EasySpin function *garlic* and simulations for the low temperature (frozen solution) cw-EPR and W-band spectra were performed with the *pepper* function [23, 95].

The dipolar hyperfine coupling tensors for the larger systems and for different distributions of spin density were calculated with a Matlab routine based on the distributed dipole approximation of equation 1.11. The spin density distribution was modelled by rescaling either the *l*-P1 $^{\cdot+}$ or the *l*-P2 $^{\cdot+}$ DFT spin density distribution and projecting it onto the optimised geometries for the longer oligomers. Calculations were performed both for spin densities evenly distributed across the whole porphyrin chain and for different ratios of spin densities on the porphyrin units. The spin density distributions thus obtained were used for the calculation of the dipolar hyperfine couplings. The validity of this method was tested by comparing the dipolar hyperfine couplings obtained for the smaller systems (*l*-P1 $^{\cdot+}$ to *l*-P3 $^{\cdot+}$) to the corresponding values predicted by DFT. In order to obtain a good agreement it was found to be necessary to model the spin density as localised in *p*-type orbitals above and below the molecular plane for the porphyrin unit. The distance to the porphyrin plane was optimised for best agreement with the DFT results obtained for *l*-P1 $^{\cdot+}$ to *l*-P3 $^{\cdot+}$ and was found to correspond to ± 52 pm.

The proton isotropic hyperfine coupling constants were estimated by extrapolation from the values available from DFT by using the relationship

$$a_{iso}(N) = \frac{a_{iso}(N=1)}{N} \quad (3.1)$$

where $a_{iso}(N=1)$ is the isotropic hyperfine coupling constant of l -P1^{•+} for each type of proton. In case of uneven spin density distributions, the values were scaled depending on the amount of spin density on each of the porphyrin units.

The nitrogen isotropic hyperfine coupling constants could be estimated relatively accurately by using the McConnell relationship

$$a_{iso}^{(i)} = C\rho^{(i)} \quad (3.2)$$

with a coefficient C determined as an average from the available DFT a_{iso} values ($C=36.2$ MHz) and the spin density $\rho^{(i)}$ on the considered nitrogen nucleus.

The nuclear quadrupole interaction parameters Q and η did not vary considerably with oligomer size in the DFT calculations and hence an average was used in the simulations ($Q=2.23$ MHz, $\eta=0.70$).

ENDOR and ESEEM simulations were performed using the EasySpin routine *saffron* for Mims ENDOR and three-pulse ESEEM [23, 24]. The ENDOR spectra were computed for each nucleus separately and then summed with intensities adjusted based on the number of equivalent nuclei. The ESEEM spectra were simulated for groups of four nitrogen nuclei at a time and summed together, the simulations were performed for the three experimental τ values and the frequency domain data were obtained from the simulated time domain data using the same procedure applied to the experimental data. HYSCORE simulations of just the cross-peak positions were obtained with a Matlab program written by Dr. Jeffrey Harmer, based on the exact diagonalisation of the spin Hamiltonian to determine the nuclear frequencies.

3.4 RESULTS AND DISCUSSION

3.4.1 ROOM TEMPERATURE CW-EPR

The room temperature X-band cw-EPR spectra of the radical cations of the one to six-membered linear oligomers (l -P1 to l -P6), of the six-membered ring without (c -P6) and with the six-membered template (c -P6-T6) and of the double ring (t -P12-T6₂), obtained through chemical oxidation with thi-anthrenium hexachloroantimonate (ThSb), in deuterated toluene:THF:CD₂Cl₂ 1:1:1 are shown in figure 3.2 A and B.

The spectrum of l -P1^{•+} shows nine hyperfine lines due to coupling to the four nitrogen nuclei and a superimposed partially resolved hyperfine structure due to protons. The spectrum can be simulated by assuming four equivalent nitrogen nuclei with an isotropic hyperfine coupling constant of 4.05 MHz

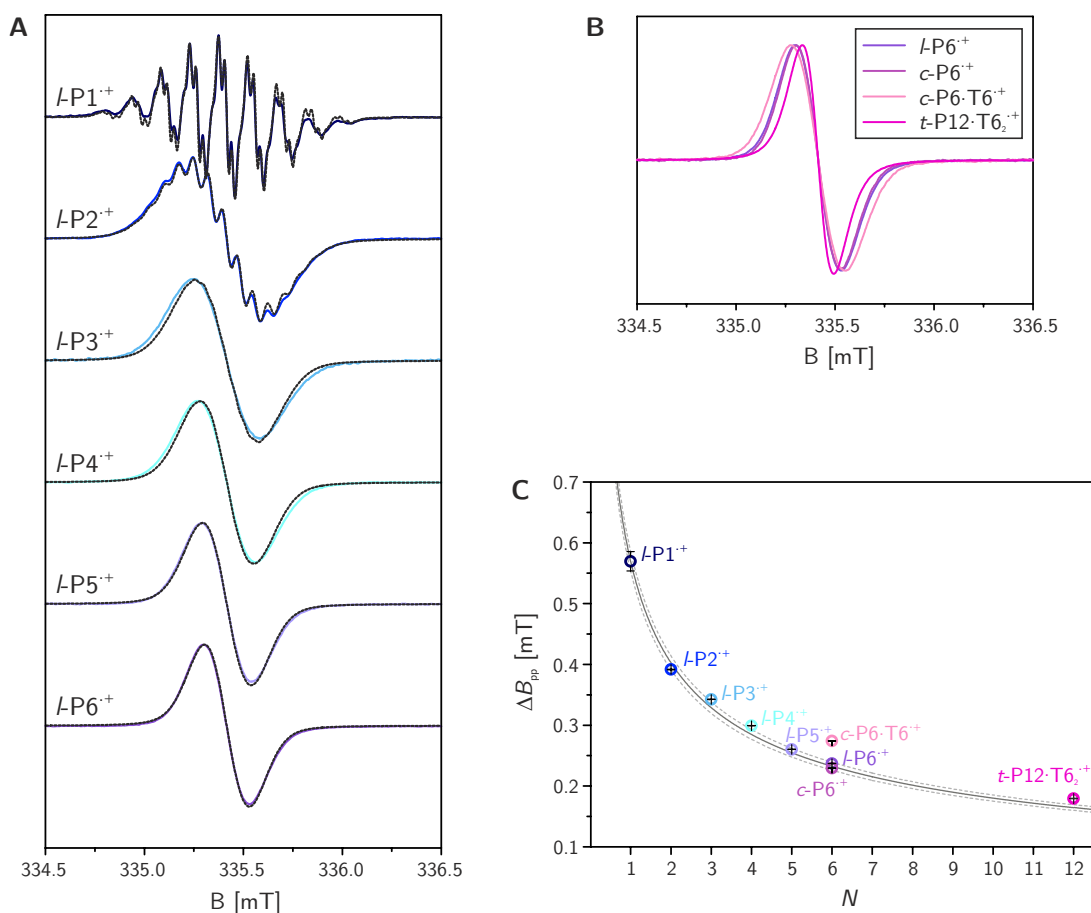


FIGURE 3.2: (A) Experimental room temperature X-band cw-EPR spectra (solid lines) of the radical cations of $l\text{-P1}$ to $l\text{-P6}$ obtained through oxidation with ThSb. The experimental data are compared to simulations (dashed lines) performed using EasySpin [23] as explained in the text. (B) Experimental room temperature X-band cw-EPR spectra of the radical cations of $l\text{-P6}$, $c\text{-P6}$, $c\text{-P6}\cdot\text{T6}$ and $t\text{-P12}\cdot\text{T6}_2$. (C) Derivative peak-to-peak linewidths of the room temperature X-band EPR spectra of the radical cations of $l\text{-P1}$ to $l\text{-P6}$, $c\text{-P6}$ and $c\text{-P6}\cdot\text{T6}$ determined through least-square fitting and plotted as a function of the number of porphyrin units. The grey solid line is the theoretical curve from equation 3.7 calculated for the experimental $l\text{-P1}^+$ peak-to-peak linewidth, the two dashed lines indicate the region within the standard deviation of the $l\text{-P1}^+$ peak-to-peak linewidth.

and four equivalent protons with a hyperfine coupling of 0.90 MHz. The simulation is shown as a dashed line in figure 3.2 A. In previous deuteration studies on Zn tetraphenylporphyrin, similar proton couplings have been assigned to protons on the phenyl rings [96, 97].

The spectrum of $l\text{-P2}^+$ also shows partially resolved hyperfine structure and can be simulated satisfactorily by assuming eight equivalent nitrogen nuclei with an isotropic hyperfine coupling constant corresponding to half that of $l\text{-P1}^+$; the proton hyperfine coupling was halved and included as well, but is not resolved. For the longer oligomers, the hyperfine couplings become too small to be resolved and lead to inhomogeneous broadening of the EPR line. The spectra can be simulated satisfactorily by increasing the number of coupled nuclei and reducing the isotropic hyperfine coupling constants accordingly (see equation 3.1), while keeping the same linewidth used for the simulation of the $l\text{-P2}^+$

spectrum. This indicates complete delocalisation or fast hopping over the whole porphyrin array for all of the linear porphyrin oligomers.

The EPR spectrum of $c\text{-P6}^{\cdot+}$ is almost identical to the spectrum of $l\text{-P6}^{\cdot+}$, but the EPR spectrum of $c\text{-P6}\cdot\text{T6}^{\cdot+}$ is slightly broader (see figure 3.2 B). The increased linewidth observed for $c\text{-P6}\cdot\text{T6}^{\cdot+}$ is most likely due to the presence of additional nitrogen hyperfine couplings on the template and not to different extents of delocalisation, since the ^1H ENDOR results indicate an increased delocalisation for $c\text{-P6}\cdot\text{T6}^{\cdot+}$ with respect to $c\text{-P6}^{\cdot+}$ (see section 3.4.4). A clear narrowing of the linewidth is observed in the spectrum of $t\text{-P12}\cdot\text{T6}_2^{\cdot+}$.

The EPR linewidth of radical cation spectra of porphyrin-like systems has been related to the delocalisation length of the unpaired electron [73], and has been used as a measure of electron delocalisation length in several instances [66, 68, 78, 80].

The second moment of an EPR spectrum can be used as a measure of the line broadening of the spectrum and is defined as:

$$M_2 = \frac{\int (x - x_0)^2 y dx}{\int y dx} \quad (3.3)$$

If the line broadening can be attributed to the hyperfine interactions, the second moment can be expressed in terms of the hyperfine coupling constants. The second moment for a monomeric unit ($N=1$) can be defined as:

$$M_2(N=1) = \sum_i^n l_i x_i^2 \quad (3.4)$$

with

$$\sum_i^n l_i x_i = 0 \quad \text{and} \quad \sum_i^n l_i = 1 \quad (3.5)$$

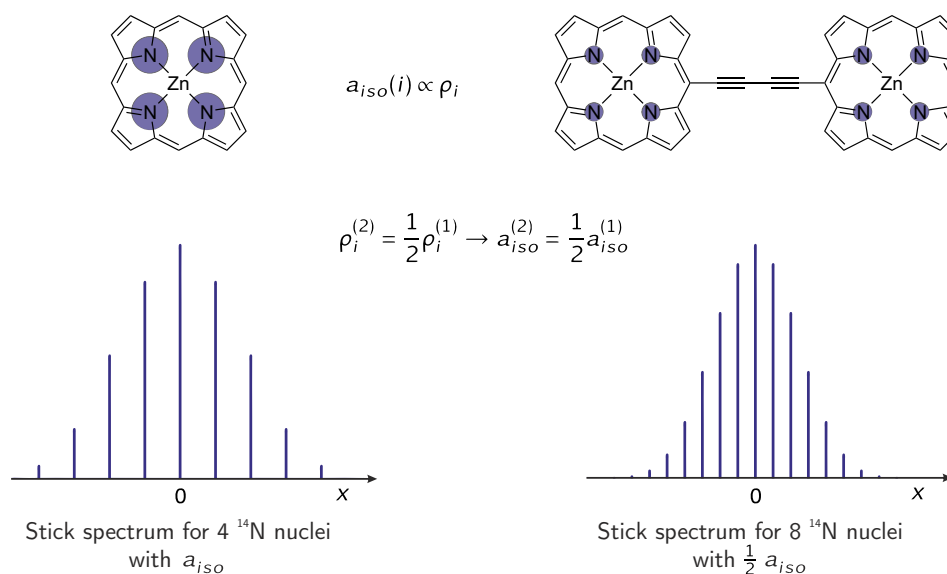


FIGURE 3.3: Schematic representation exemplifying the relationship between the second moment and the number of equal monomeric units carrying spin density. Only the nitrogen isotropic hyperfine couplings are considered, giving 9 lines for the monomeric unit and 17 lines for the dimer.

where x_i represent the field positions of the lines in a stick spectrum and I_i are the corresponding intensities (see figure 3.3).

If the spin density is delocalised over two identical units, based on the McConnell relationship, the hyperfine couplings will be reduced by a factor of two. Since there are now twice as many nuclei, the number of lines will increase and their positions will be given by $\frac{1}{2}(x_i + x_j)$. The second moment in this case is defined as shown in equation 3.6 and can be expressed in terms of the hyperfine couplings in the monomer.

$$M_2(N=2) = \sum_{i,j} I_i I_j \left(\frac{x_i}{2} + \frac{x_j}{2} \right)^2 = \frac{1}{2} \sum_i I_i x_i^2 = \frac{1}{2} M_2(N=1) \quad (3.6)$$

The cross terms in the first expression vanish because of symmetry.

Since for a Gaussian line the second moment M_2 is proportional to the square of the peak-to-peak linewidth, a general theoretical relationship relating the peak-to-peak linewidth ΔB_{pp} to the number of equivalent units N over which the electron spin is delocalised or hopping can be defined:

$$\Delta B_{pp} = \frac{1}{\sqrt{N}} \Delta B_{pp}(N=1) \quad (3.7)$$

The experimental room-temperature spectra all exhibit Gaussian lineshapes (see figure 3.6 A), indicating that the main contribution to line broadening is due to the unresolved hyperfine couplings. Therefore the peak-to-peak linewidth can be related to the extent of delocalisation according to the theory proposed by Norris et al. [73]. The derivative peak-to-peak linewidth, determined through derivative Gaussian fitting of the experimental spectra using a least-square fitting routine, decreases with increasing oligomer size; the values and their standard deviations are reported in table 3.1 and the experimental linewidth is compared to the theoretical relationship of equation 3.7 in figure 3.2 C. The number of porphyrin units N the electron is delocalised over in each oligomer was calculated according to equation 3.7 and is reported in the table as well. The experimental linewidths approximately follow the theoretical prediction for all of the linear oligomers under investigation. The linewidth of the ring without template is slightly smaller than that of the linear hexamer, while that of the ring with

TABLE 3.1: Peak-to-peak linewidth determined through derivative Gaussian fitting of the experimental room temperature EPR spectra and number N of molecular sites the electron spin is delocalised over as calculated using equation 3.7.

	ΔB_{pp} [mT]	N
<i>l</i> -P1 ^{•+}	0.5697 ± 0.0161	1.0 ± 0.1
<i>l</i> -P2 ^{•+}	0.3917 ± 0.0006	2.1 ± 0.1
<i>l</i> -P3 ^{•+}	0.3424 ± 0.0002	2.8 ± 0.2
<i>l</i> -P4 ^{•+}	0.2992 ± 0.0001	3.6 ± 0.2
<i>l</i> -P5 ^{•+}	0.2604 ± 0.0001	4.8 ± 0.3
<i>l</i> -P6 ^{•+}	0.2370 ± 0.0001	5.8 ± 0.3
<i>c</i> -P6 ^{•+}	0.2294 ± 0.0001	6.2 ± 0.3
<i>c</i> -P6-T6 ^{•+}	0.2742 ± 0.0001	4.3 ± 0.2
<i>t</i> -P12-T6 ₂ ^{•+}	0.1795 ± 0.0003	10.1 ± 0.6

template is larger, most probably due to the additional nitrogen hyperfine couplings to the template. In the presence of the template, the $l\text{-P1}^{\cdot+}$ linewidth is not an accurate reference for estimating N , leading to an underestimate of the number of porphyrin units the electron is delocalised over in the six-membered ring and the double ring with template. If the linewidth of $c\text{-P6}\cdot\text{T6}^+$ is taken as a reference and delocalisation over six units is assumed, a delocalisation over twelve units is determined for $t\text{-P12}\cdot\text{T6}_2^+$. The good agreement suggests that, at room temperature, the electron is either delocalised over the whole π -system or hopping between the porphyrin units on a faster timescale than that associated with the ^{14}N hyperfine interactions ($>10^7 \text{ s}^{-1}$) in all of the investigated systems. In the present case, continuous wave EPR alone cannot distinguish a fast hopping mechanism from a delocalisation of the electron spin in a molecular orbital extended over the whole multiporphyrin π system. In the presence of resolved hyperfine couplings for the larger oligomers, the hopping rates could be extracted by simulation and a temperature dependence could potentially help distinguish the two mechanisms. An analysis of this type is described in reference [98] for thallium-substituted porphyrin dimers. Thallium-substitution into the porphyrin systems investigated here was attempted by Dr. Patrik Neuhaus, but was unfortunately not successful.

Small deviations from the curve predicted by Norris, i.e. to smaller linewidths for $l\text{-P2}^{\cdot+}$ and to larger linewidths for the longer oligomers, can be rationalised in terms of the partial breakdown of the assumptions behind its derivation. These are, firstly, that the unpaired electron is evenly distributed over the whole system and, secondly, that the spin density distribution in each single porphyrin is identical to that in $l\text{-P1}^{\cdot+}$. The results of DFT calculations suggest that in the case of coherent delocalisation of the unpaired electron, the spin density would be slightly larger on the central porphyrin units of the oligomers with respect to the external ones (see figure 3.4). Uneven spin density distributions would lead to larger linewidths than expected based on equation 3.7, as observed for oligomers with three to six units. The DFT results also predict a distortion of the spin density along the porphyrin-linking butadiyne bond in $l\text{-P2}^{\cdot+}$ and in the longer oligomers, which leads to additional spin density on the

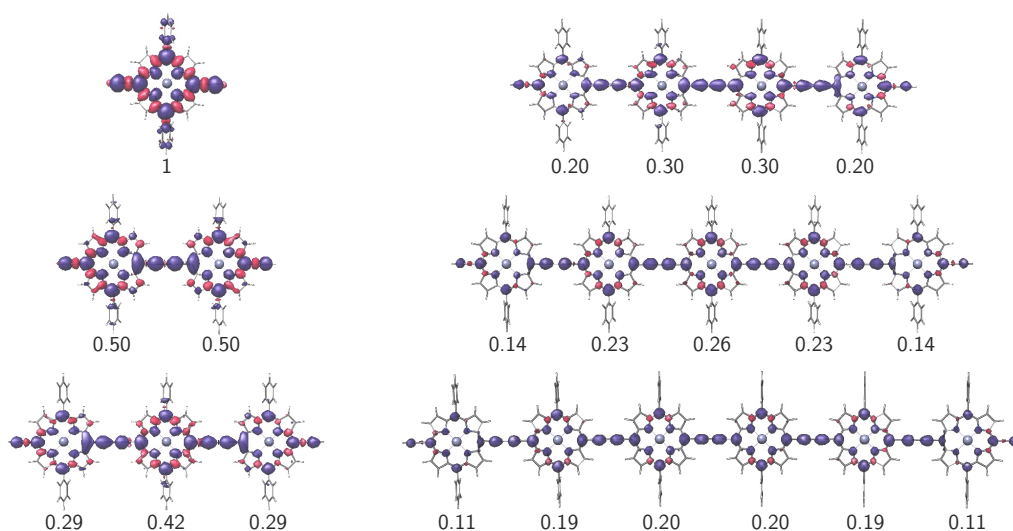


FIGURE 3.4: Mulliken spin densities calculated in ORCA using B3LYP/EPRII for $l\text{-P1}^{\cdot+}$ to $l\text{-P6}^{\cdot+}$ after geometry optimisations performed in Turbomole. The ratios of spin densities on the single porphyrin units are reported.

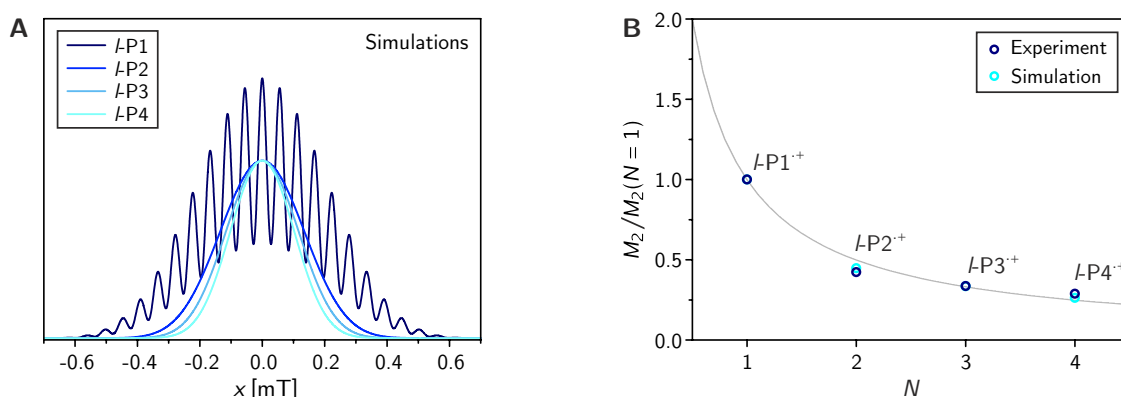


FIGURE 3.5: (A) EPR spectra constructed by computing the stick spectrum according to reference [99], based on both proton and nitrogen hyperfine couplings predicted by DFT, and by using a Gaussian broadening with a FWHM of 0.5 MHz. (B) Second moments of the experimental EPR spectra for $l\text{-P1}^{\cdot+}$ to $l\text{-P4}^{\cdot+}$ and second moments calculated for EPR stick spectra shown in A.

internal β carbon atoms, on which only negligible spin density was found for $l\text{-P1}^{\cdot+}$. This would be in keeping with a decrease in the nitrogen hyperfine coupling, hence the linewidth is expected to be smaller than predicted by equation 3.7. Model calculations of the EPR spectra taking this into consideration were performed for $l\text{-P1}^{\cdot+}$ to $l\text{-P4}^{\cdot+}$ and qualitatively reproduce the experimentally observed deviations from the theoretical curve (see figure 3.5).

The lineshapes of EPR spectra in systems characterised by electron/hole hopping can also be analysed based on one-dimensional random walk processes as described by Tang et al. [100] for open chains and closed loops. Their theoretical study predicts a Gaussian lineshape in case of fast or slow hole/electron hopping with respect to the timescale of the hyperfine interaction, while intermediate hopping rates give rise to deviations from Gaussian lineshapes. In previous studies on artificial multiporphyrin systems, Lorentzian contributions to the EPR lineshape were observed for larger oligomers (6 to 12 porphyrin units arranged linearly or in rings) [66, 68, 83], which were attributed to slower hopping rates. In the systems investigated here, Gaussian lineshapes are observed for all the linear oligomers with up to six porphyrin units and for the six-membered ring with and without template, seemingly indicating a faster hopping rate with respect to systems previously investigated. Deviations from the Gaussian lineshape are observed for the double ring, potentially indicating a slower hopping rate in this system containing twelve porphyrin units. The calculations of Tang et al. [100] also suggest that for a given hopping rate, open chain systems would have a larger EPR linewidth compared to the corresponding closed-loop systems. Hence the fact that the same linewidth is observed for $l\text{-P6}^{\cdot+}$ and $c\text{-P6}^{\cdot+}$ could be interpreted as a slightly larger hopping rate in the ring system. The differences are however very small in the fast-hopping regime [100].

The expressions for the calculation of the response function for hopping along a linear chain or a closed loop are given in reference [100]. The EPR line is related to the response function by Fourier transform. This analysis is also based on a variation of the second moment as one over N . A fitting of the hopping rate constant to the experimental room temperature spectra was performed. The results of this analysis were that a reduction of the second moment by a factor N occurs for all

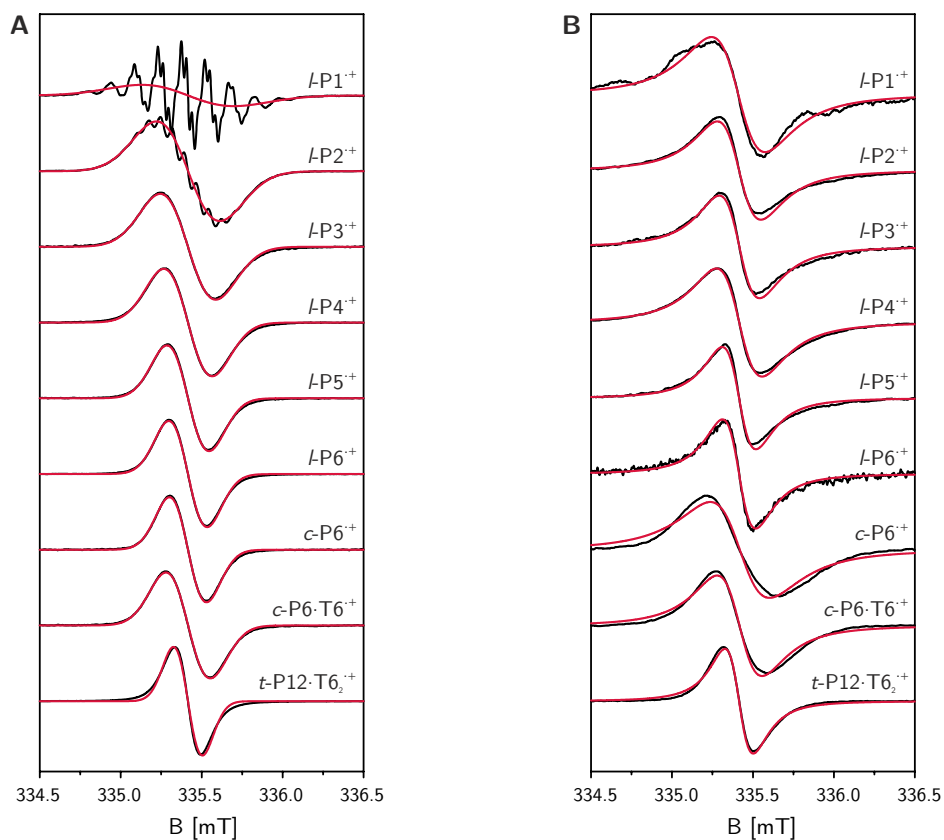


FIGURE 3.6: Experimental room temperature (A) and 100 K (B) X-band cw-EPR spectra (black) of the radical cations of $l\text{-P1}$ to $l\text{-P6}$, $c\text{-P6}$, $c\text{-P6}\cdot\text{T6}$ and $t\text{-P12}\cdot\text{T6}_2^{\cdot+}$ and fitted derivative Gaussian (A) and Lorentzian (B) functions (red).

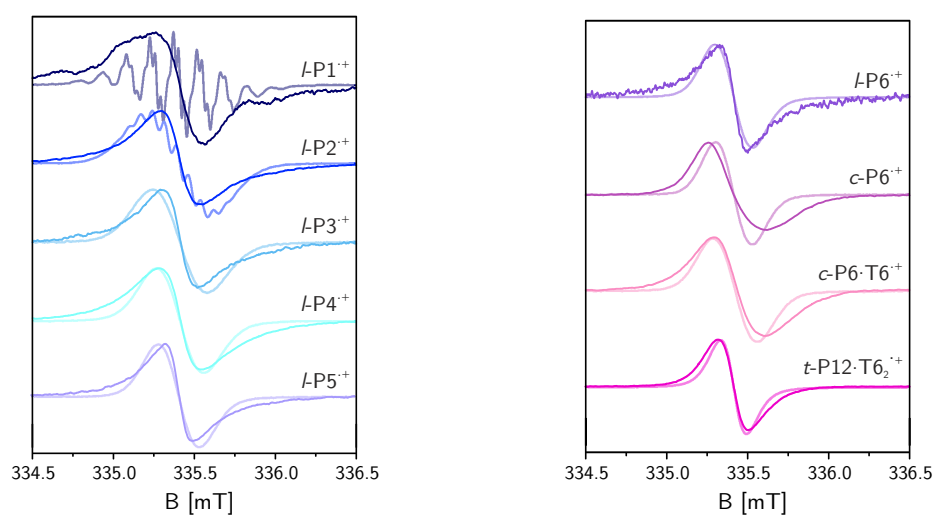


FIGURE 3.7: Experimental X-band cw-EPR spectra of the radical cations of $l\text{-P1}$ to $l\text{-P6}$, $c\text{-P6}$, $c\text{-P6}\cdot\text{T6}$ and $t\text{-P12}\cdot\text{T6}_2^{\cdot+}$ recorded at 100 K compared to the corresponding room temperature EPR spectra shown in the background.

systems except for $c\text{-P6}\cdot^+$ and that the fitted hopping rates have a magnitude of $10^8\text{-}10^9\text{ s}^{-1}$, indicating hopping on a timescale more rapid than that associated with the hyperfine interaction ($10^6\text{-}10^7\text{ s}^{-1}$). The uncertainty on the hopping rates was too large to identify any trends along the series of oligomers with different lengths.

3.4.2 LOW TEMPERATURE CW-EPR

The frozen solution X-band cw-EPR spectra were recorded at 100 K and are shown in figure 3.7. No hyperfine structure is resolved even for $l\text{-P1}^+$ and $l\text{-P2}^+$, and the spectra of the linear oligomers are all characterised by approximately Lorentzian lineshapes with some small asymmetry, as shown in figure 3.6 B. The low temperature EPR spectra of $c\text{-P6}\cdot^+$ and $c\text{-P6}\cdot\text{T6}\cdot^+$ are characterised by more significant asymmetries and the lineshape is a convolution of a Gaussian and a Lorentzian line.

Direct comparison of the room and low temperature cw-EPR spectra shows only small differences in the peak-to-peak linewidth for the longer oligomers (see figure 3.7). Similar results have previously been interpreted in terms of complete delocalisation even at low temperatures while not explicitly considering the clearly different lineshape [66]. The theory for the interpretation of the EPR linewidth in terms of delocalisation length, first derived by Norris [73] and later extended as described in references [78, 100], is only valid when the lineshape is determined by the hyperfine couplings of the nuclei and only for spectra with approximately Gaussian lineshapes. The low temperature lineshapes of the linear oligomers cannot be explained by the theory described by Tang et al. [100], which, even for intermediate and slow hopping rates, predicts a lineshape that doesn't approach a Lorentzian. Hence any interpretation of the linewidth based on that theory is not possible. The peak-to-peak linewidths determined from the low temperature cw-EPR spectra show a high sample-to-sample variability and a dependence on the method of freezing. The experimental linewidths of the low temperature spectra

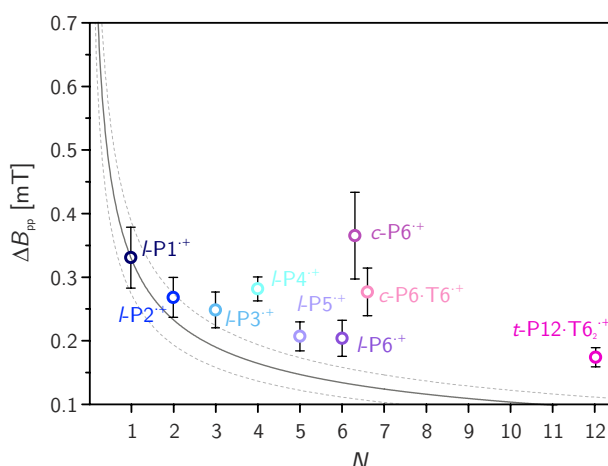


FIGURE 3.8: Experimental derivative peak-to-peak linewidths of the 100 K X-band EPR spectra of $l\text{-P1}^+$ to $l\text{-P6}^+$, $c\text{-P6}^+$, $c\text{-P6-T6}^+$ and $t\text{-P12-T6}^+$, determined through least-square Lorentzian fitting, plotted as a function of oligomer size. The grey solid line is the theoretical curve from equation 3.7 calculated for the experimental $l\text{-P1}^+$ peak-to-peak linewidth, the two dashed lines indicate the region within the standard deviation of the $l\text{-P1}^+$ peak-to-peak linewidth.

with standard deviations estimated based on this variability are plotted as a function of the number of porphyrin units in figure 3.8 and show that the linewidths do not follow any clear trend.

An interpretation of the low temperature cw-EPR spectra was attempted by simulation of the spectra including the anisotropic hyperfine interaction. The 100 K EPR spectrum of $l\text{-P1}^+$ is characterised by an approximately Lorentzian lineshape and by significant asymmetry. A simulation of this spectrum was only possible by taking a distribution of hyperfine couplings and g -values in the out-of-plane orientation into account. The individual spectra and the final simulation are shown in figure 3.9 A and B. The assumption of a distribution of hyperfine couplings and g -values can be explained by a variation of the degree of distortion in the porphyrin plane due to specific porphyrin-solvent interactions. The ^{14}N hyperfine interactions in $l\text{-P1}^+$ are characterised by a large anisotropy in the out-of-plane orientation (see section 3.4.5), thus the hyperfine coupling in that direction is very sensitive to changes in the porphyrin plane. Distortions in the porphyrin plane can occur in the presence of coordinating solvents, such as for example the THF used in our case, as the change from four- to five-coordination of the Zn ion influences the degree of saddle distortion of the porphyrin plane [101], which could cause the observed effect. A similar effect has been previously observed in a Co(II) corrin molecule by ^{14}N HYSCORE [102] and is also present in the triplet state of $l\text{-P1}$ as described in section 4.3.4.

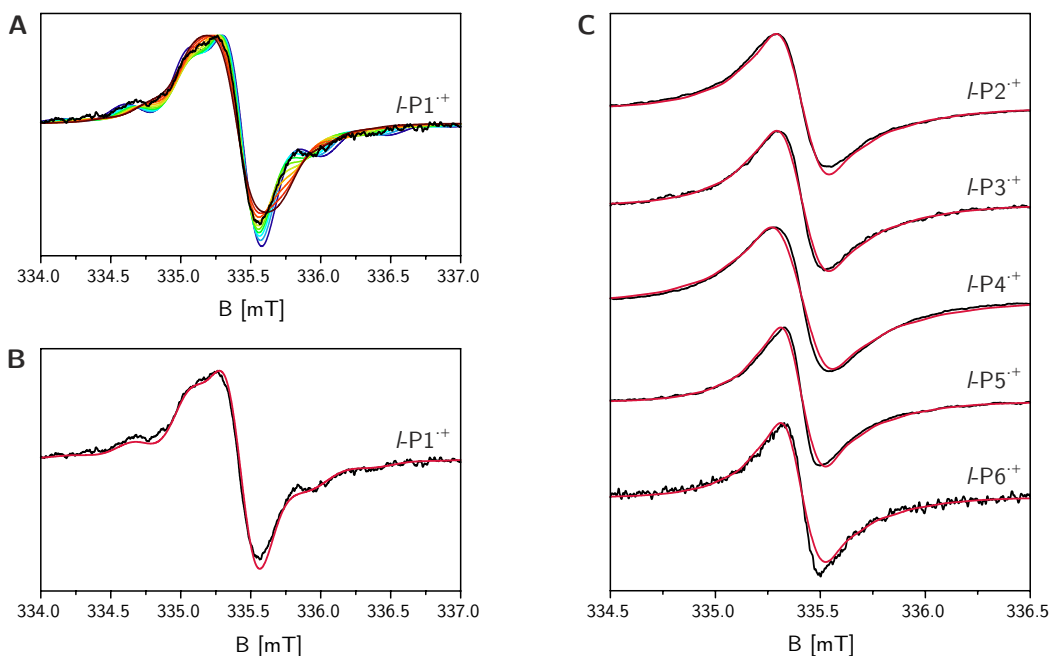


FIGURE 3.9: (A) Individual simulations of the $l\text{-P1}^+$ cw-EPR spectrum at 100 K compared to the experimental result (black). The simulations were performed using a distribution of A_z from 8.5 to 13 MHz and of g_z from 2.0024 to 2.0015 (red to blue) with the same a_{iso} value used for the room temperature simulations and an axial hyperfine tensor with $A_{\perp} = \frac{1}{2}(3a_{iso} - A_z)$, $A_{||} = A_z$ for the four ^{14}N nuclei. (B) Simulation calculated as a sum over the distributions in g_z and A_z shown in A with a Gaussian weighting, compared to the experimental spectrum. (C) Comparison of the experimental 100 K cw-EPR spectra of $l\text{-P2}^+$ to $l\text{-P6}^+$ (black) to simulations (red). The simulations were performed using the EasySpin function *pepper* for solid state EPR spectra with eight ^{14}N nuclei with an axial hyperfine coupling ($A_{\perp} = 0.4$ MHz, $A_{||} = 5.2$ MHz) and a line broadening accounting for the proton hyperfine couplings. The parameters were optimised for $l\text{-P2}^+$ and in order to fit the spectra of the longer oligomers deviations of $\pm 15\%$ from these values were needed (-5% for $l\text{-P3}^+$, $+15\%$ for $l\text{-P4}^+$, -15% for $l\text{-P5}^+$, -15% for $l\text{-P6}^+$).

The optimised geometry of $l\text{-P2}^+$ showed much reduced distortions in the porphyrin plane (the maximum out-of-plane deviation is reduced from 0.22 to 0.10 Å) and the experimental spectrum could be simulated without resorting to a distribution of hyperfine couplings (see figure 3.9 C). Simulations of the spectrum for different degrees of anisotropy in the nitrogen hyperfine coupling and using only a Gaussian broadening to account for the unresolved proton hyperfine couplings have shown that the Lorentzian lineshape can be accounted for by a large anisotropy of the ^{14}N hyperfine couplings. The low temperature EPR spectra of $l\text{-P2}^+$ and of the longer oligomers up to $l\text{-P6}^+$ can all be simulated by eight ^{14}N nuclei with hyperfine couplings close to those used for simulation of the $l\text{-P2}^+$ spectrum. The hyperfine couplings and linewidths of $l\text{-P2}^+$ were scaled by factors of 0.95, 1.15, 0.85 and 0.85 for $l\text{-P3}^+$, $l\text{-P4}^+$, $l\text{-P5}^+$ and $l\text{-P6}^+$, respectively, in order to simulate the experimental spectra (see figure 3.9 C). These results seem to suggest that delocalisation is limited to about two porphyrin units in the longer oligomers.

The low temperature EPR spectra of $c\text{-P6}^+$ and $c\text{-P6}\cdot\text{T6}^+$ are characterised by a different lineshape and by a significant asymmetry which, based on the W-band results described in the next section, cannot be explained by g -anisotropy. Temperature-dependent EPR measurements show that the asymmetry, as well as the deviation from the Gaussian lineshape, occur gradually at first with more pronounced changes close to the freezing point of the solvent mixture, between 120 K and 100 K (see figure 3.10). Due to the appearance of asymmetry, the EPR spectra cannot be simulated by the approach of reference [100]. The behaviour of the porphyrin ring systems in solution is too poorly understood to interpret the low temperature EPR spectra, particularly since aggregation cannot be excluded even in the presence of the THS side groups and its effects on the EPR spectrum are not known. Therefore the frozen solution cw-EPR spectra of the ring systems could not be conclusively interpreted in terms of the extent of delocalisation.

In contrast to the majority of linear systems investigated so far, where frozen solution EPR measurements yielded broader spectra with a linewidth similar to that of the corresponding monomeric unit at temperatures below the freezing point of the solvent [68, 83, 84], the frozen solution spectra obtained here for the linear oligomers revert to the dimeric rather than monomeric linewidth, indicating more extended conjugation in these butadiyne-linked arrays.

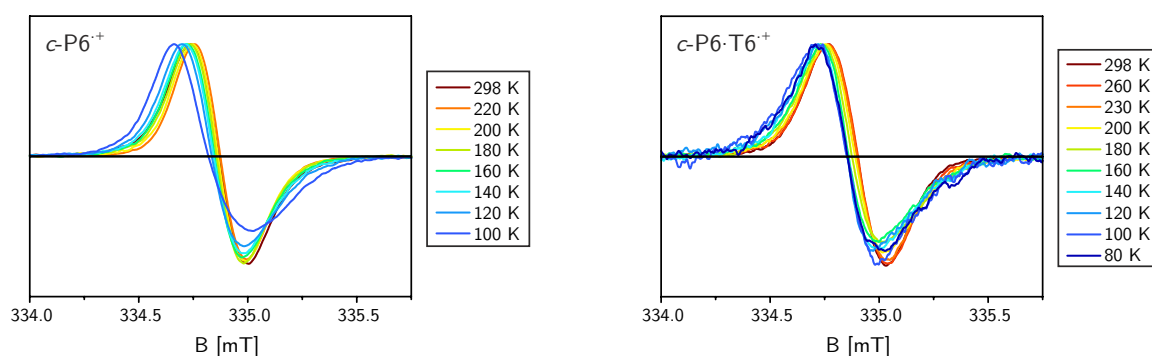


FIGURE 3.10: Experimental X-band cw-EPR spectra for $c\text{-P6}^+$ and $c\text{-P6}\cdot\text{T6}^+$ from room temperature to frozen solution. Measurements at lower temperatures were not possible due to power saturation.

3.4.3 W-BAND EPR

The g -anisotropy of the radical cation spectra was investigated by W-band EPR. Echo-detected field sweeps were recorded in the temperature range from 120 K to 6 K and are shown in figure 3.11. The temperature-dependent data show a significant change from an approximately isotropic to a rhombic or axial EPR spectrum between 120 K and 100 K, due to freezing of the solvent mixture. In most of the cases no pronounced further changes are observed upon lowering the temperature below 100 K down to 20 K. A significant temperature dependence is, however, observed for the six-membered ring without template in the whole of the investigated temperature range. The W-band EPR spectrum of $c\text{-P6}^{\bullet+}$ changes from an almost isotropic spectrum at 120 K to a spectrum with a shoulder of increasing intensity with respect to the maximum at lower magnetic fields.

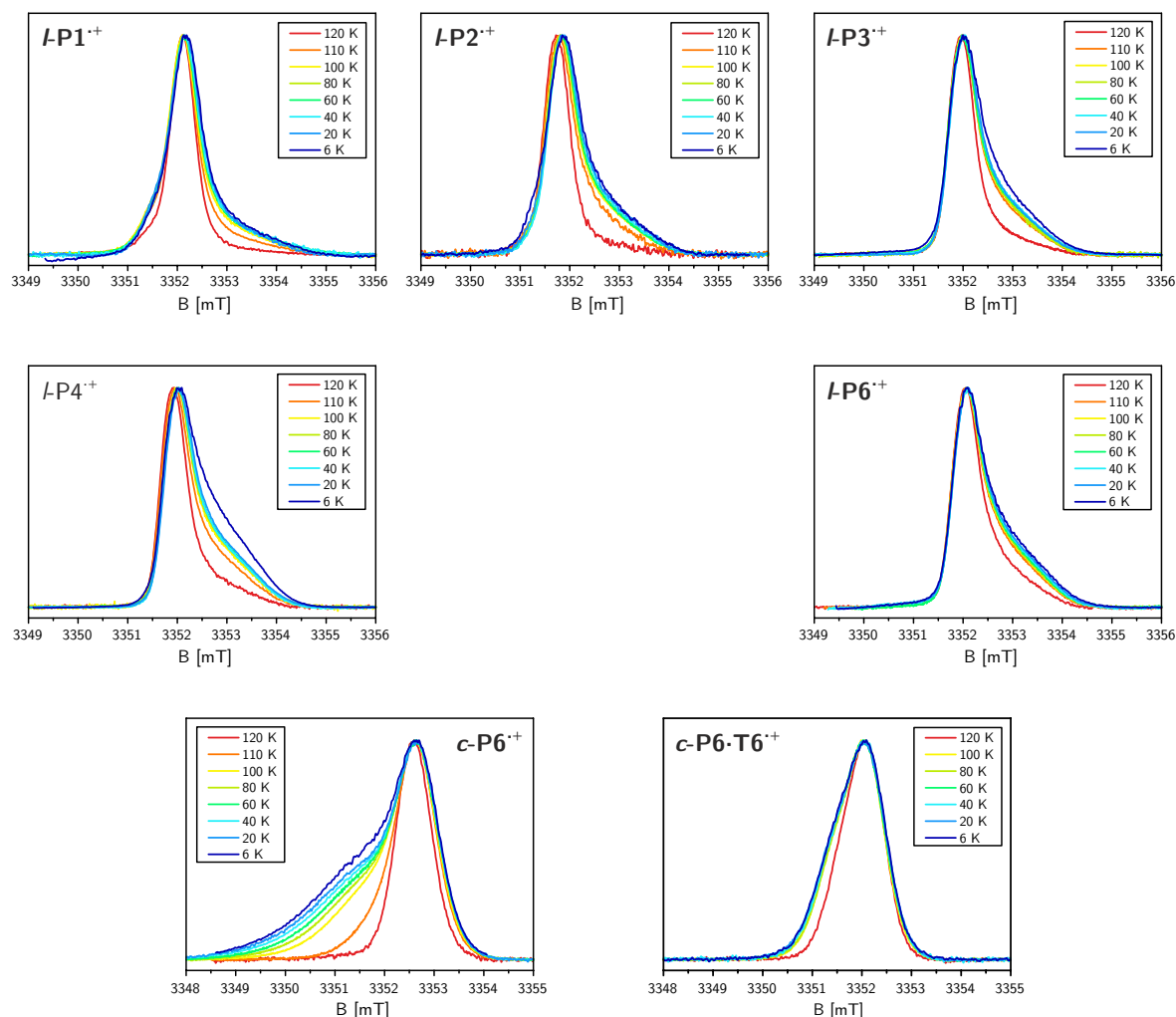


FIGURE 3.11: Experimental W-band echo-detected field sweeps of the radical cations of $l\text{-P1}$ to $l\text{-P6}$, $c\text{-P6}$ and $c\text{-P6-T6}$ in toluene:THF:CH₂Cl₂ 1:1:1 recorded at different temperatures in the range from 120 to 6 K. Experimental details are described in section 3.3.3. No temperature-dependent measurements were performed for $l\text{-P5}^{\bullet+}$.

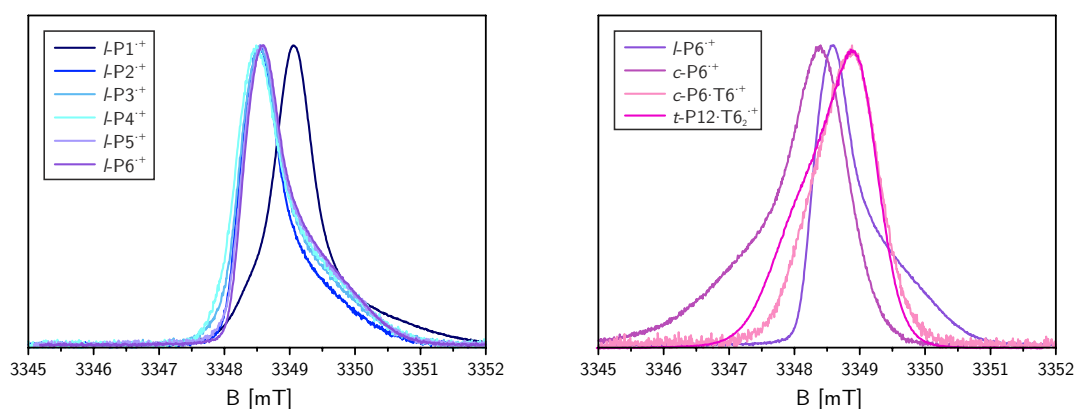


FIGURE 3.12: Experimental W-band echo-detected EPR spectra of the radical cations of *l*-P1 to *l*-P6, *c*-P6, *c*-P6·T6 and *t*-P12·T6₂⁺ recorded at 100 K.

The W-band echo-detected EPR spectra of the different porphyrin oligomers at 100 K are compared in figure 3.12. In order to compare both spectral shape and *g*-values for the different oligomers, the magnetic field axis of the W-band data was calibrated based on simultaneous simulation of the X- and W-band EPR spectra, determining the relative *g*-values from the W-band spectra and the absolute position from the X-band spectra. The W-band EPR spectrum of *l*-P1⁺ is characteristic of a rhombic *g*-tensor, while the spectra of the longer oligomers have the typical shape of systems with approximately axial *g*-tensors. The *g*-anisotropy is nevertheless small in all cases. While there is a distinct difference in lineshape between the spectra of *l*-P1⁺ and *l*-P2⁺, only very small differences are observed for the longer linear oligomers. A comparison of the spectra for *l*-P6⁺, *c*-P6⁺ and *c*-P6·T6⁺ indicates the presence of an inversion of the relative magnitude of the *g*_{||}- and *g*_⊥-values from the linear to the cyclic geometry. The W-band EPR spectrum of *c*-P6⁺ further exhibits a much greater *g*-anisotropy with respect to the more rigid and therefore likely more symmetric *c*-P6·T6⁺, potentially due to the increased flexibility of the template-free ring. The W-band EPR spectrum of *t*-P12·T6₂⁺ is similar to the spectrum obtained for *c*-P6·T6⁺, except for an increase of the *g*-value in the out-of-plane direction of the porphyrin ring (*g*_{||}). This increase in *g*-anisotropy reflects the increase in the dimension of the ring system in the out-of-plane direction.

TABLE 3.2: *g*-values determined from simulation of the W-band EPR spectra of *l*-P1⁺ to *l*-P6⁺, *c*-P6⁺ and *c*-P6·T6⁺ at 100 K with an axial *g*-tensor. The simulations are compared to the experimental data in figure 3.13. The error on the *g*-values given in the table is approximately 0.00030.

	<i>g</i> _⊥	<i>g</i>	<i>g</i> _{iso}
<i>l</i> -P1 ⁺	2.00325	2.00295	2.00315
<i>l</i> -P2 ⁺	2.00360	2.00290	2.00337
<i>l</i> -P3 ⁺	2.00359	2.00285	2.00334
<i>l</i> -P4 ⁺	2.00364	2.00275	2.00334
<i>l</i> -P5 ⁺	2.00359	2.00270	2.00329
<i>l</i> -P6 ⁺	2.00359	2.00270	2.00329
<i>c</i> -P6 ⁺	2.00355	2.00450	2.00387
<i>c</i> -P6·T6 ⁺	2.00325	2.00375	2.00342
<i>t</i> -P12·T6 ₂ ⁺	2.00317	2.00317	2.00404

The high-field EPR measurements reveal a g -anisotropy that is not detectable at X-band and which might provide additional information on the characteristics of the electron distribution in these porphyrin arrays. Similar measurements on photosynthetic reaction centres have prompted a detailed g -tensor analysis for model systems of stacked aromatic molecules to aid interpretation of the experimental data obtained for natural photosynthetic systems [103]. It was found that qualitative trends in g -values could be predicted based on DFT calculations on model systems; a quantitative discussion was however prevented by the fact that the systems soon become too large for accurate DFT predictions in reasonable computation times.

The g -tensors predicted by DFT for l -P1 $^{\cdot+}$ to l -P5 $^{\cdot+}$ qualitatively reproduce the change in g -anisotropy and relative magnitude of the g -values (see figure 3.13 B and C): there is an increase in g_{iso} from l -P1 $^{\cdot+}$ to the longer oligomers as well as an increase in g -anisotropy. The differences predicted for the linear oligomers with more than two porphyrin units are slightly larger than those observed experimentally. Calculation of the g -tensor for the linear and cyclic systems with six porphyrin units were unfortunately not computationally feasible.

The changes in g -values observed experimentally and predicted by DFT can be rationalised by simple considerations based on Stone's theory of g -values [104–106]. The analysis of g -values for aromatic molecules led Stone to the conclusion that the deviations from the free electron g -value are an additive property of the different groups constituting the aromatic molecule [104]. The overall deviation from the free electron g -value can then be estimated from the sum of the deviations arising from different groups of orbitals [104]. A similar approach has been applied to the porphyrin systems investigated here, by considering the deviations from g_e determined experimentally for l -P1 $^{\cdot+}$ and by calculating the overall g -values of the larger systems as linear combinations based on the different geometries. For the linear oligomers such an approach predicts an increase in g_{iso} and an increase in g -anisotropy, as also observed experimentally. The magnitude of the changes is, however, significantly overestimated by this simple approach.

The same approach has also been applied to the calculation of the g -values of the six-membered ring system and it was found that the lineshape of the simulated spectra is very sensitive to the g -values for the single porphyrin unit and to the number of porphyrin units over which the spin density is considered to be delocalised. The theory predicts small g -anisotropies for extensive delocalisation of the unpaired electron over the porphyrin ring system, and spectra with larger g -anisotropies for localisation of the unpaired electron on only part of the ring system. For some of the considered spin density distributions, an inversion of the $g_{||}$ and $g_{\perp}$ values with respect to the linear systems was also predicted. This apparent strong dependence of the g -values on electron delocalisation would allow the changes observed between c -P6 $^{\cdot+}$ and c -P6·T6 $^{\cdot+}$ to be assigned to increased delocalisation for the templated ring. A more quantitative analysis was precluded by the limitations of this approach and by the absence of reliable estimates of the deviation from the g_e value for the single porphyrin unit in a bent geometry.

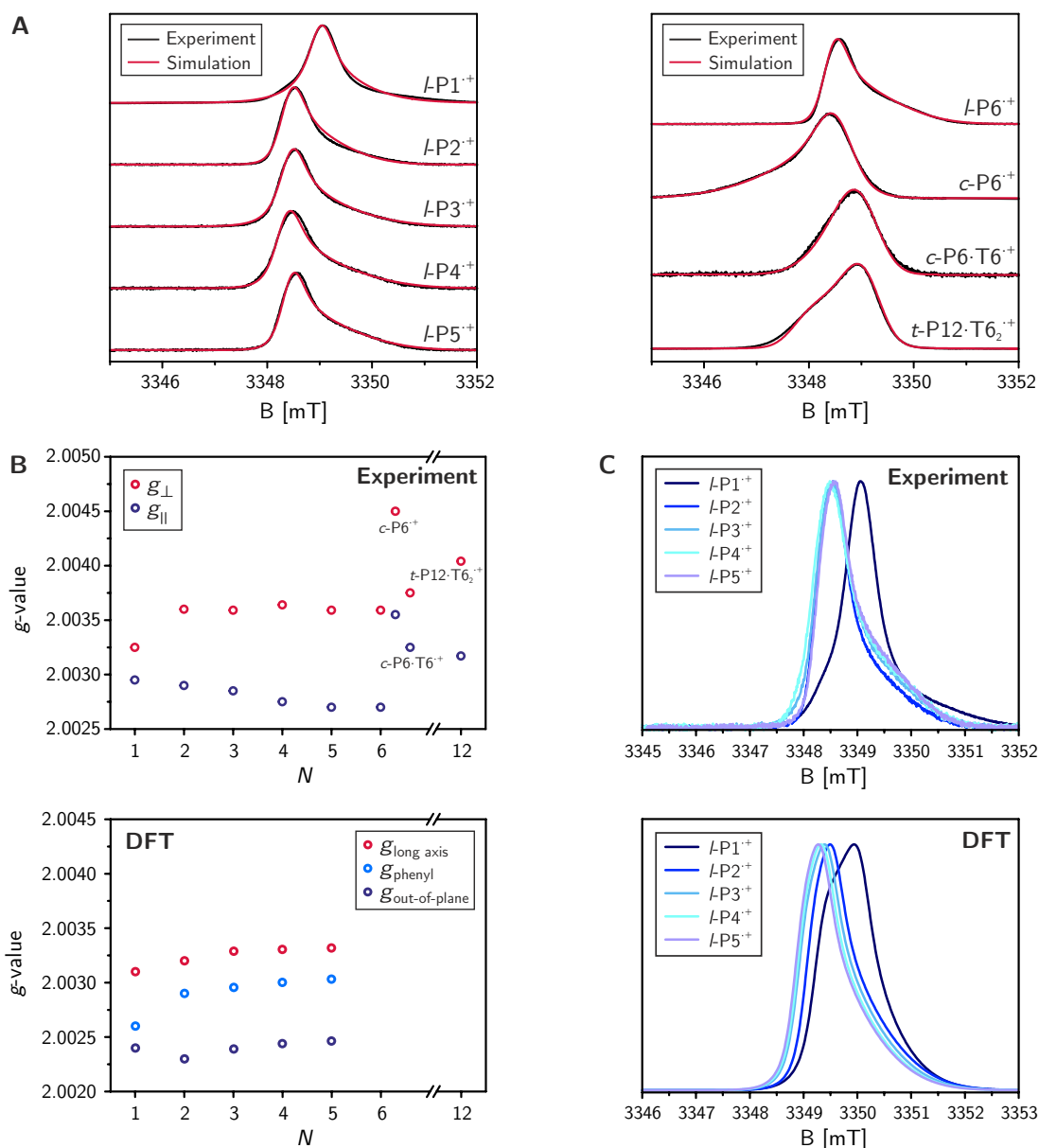


FIGURE 3.13: (A) Experimental W-band echo-detected EPR spectra of the radical cations of $l\text{-P1}$ to $l\text{-P6}$, $c\text{-P6}$, $c\text{-P6}\cdot\text{T6}$ and $t\text{-P12}\cdot\text{T6}_2^{\cdot+}$ recorded at 100 K and simulations for an axial g -tensor with the values given in table 3.2. (B) Top: $g_{\perp}$ - and $g_{\parallel}$ -values for the linear and cyclic oligomers determined from the simulations shown in A. Bottom: g -values obtained from DFT calculations performed with B3LYP/EPRII. The principal axes of the g -tensor are aligned with the out-of-plane axis, the axis pointing along the phenyl substituents and the long axis of the molecules (along the acetylene bonds). (C) Experimental W-band EPR spectra for $l\text{-P1}^{\cdot+}$ to $l\text{-P5}^{\cdot+}$ (top) and simulations using the g -values from DFT calculations (bottom).

The sensitivity of the W-band EPR spectrum of the porphyrin ring systems to the extent of delocalisation might explain the observed temperature dependence. In order to determine whether the temperature dependence of the W-band spectrum observed for $c\text{-P6}^{\cdot+}$ could be explained by changes in delocalisation, further measurements were performed. The first derivatives of the echo-detected field sweeps recorded at different temperatures are compared to the cw-EPR spectrum recorded at 100 K in figure 3.14. The cw-EPR spectrum recorded at 100 K is characterised by a pronounced shoulder

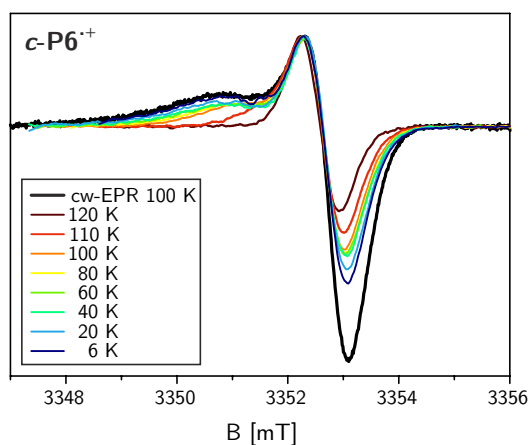


FIGURE 3.14: Comparison of the W-band cw-EPR spectrum of $c\text{-P6}^{\bullet+}$ at 100 K with the first derivatives of the echo-detected field sweeps recorded at different temperatures in the range from 6 to 120 K. The cw-EPR spectra were recorded with a microwave power of $2.530 \mu\text{W}$, a modulation amplitude of 5 G and a time constant of 5.12 ms.

at lower magnetic fields and resembles the echo-detected spectrum recorded at 6 K rather than the echo-detected spectrum recorded at the same temperature of 100 K. Continuous wave EPR measurements at temperatures lower than 100 K were not possible due to saturation. Based on the good agreement between the 100 K cw-EPR and the 6 K echo-detected EPR spectra it seems likely that the observed temperature dependence of the spectral shape is limited to the pulse EPR measurements, which would exclude changes in the extent of delocalisation as a possible cause.

The origin of the temperature dependence in the echo-detected EPR spectra has been investigated further by measurements of the relaxation time T_m using a two-pulse echo sequence with variable delay τ . The measurements were performed at different temperatures and at different field positions for $c\text{-P6}^{\bullet+}$ and as a comparison also for $l\text{-P2}^{\bullet+}$ and $c\text{-P6}\cdot\text{T6}^{\bullet+}$ (see figure 3.15 B). The phase memory time T_m was determined by fitting the time traces with a stretched exponential (stretching parameter varying between 0.7 and 1.7) and the results are depicted in figure 3.15 C. The porphyrin dimer and the six-membered ring show a clear field dependence of T_m , while almost no field-dependent changes are observed for the six-membered ring with template. In all systems the phase memory times increase for decreasing temperatures and the average T_m at each temperature is similar for the two ring systems, but slightly longer for $l\text{-P2}^{\bullet+}$. The two ring systems are also characterised by a slightly larger temperature dependence of T_m with respect to $l\text{-P2}^{\bullet+}$.

For both $l\text{-P2}^{\bullet+}$ and $c\text{-P6}^{\bullet+}$, T_m at a fixed temperature is longest at the field position corresponding to the echo maximum and decreases on both sides. The ratios of T_m values at the echo maximum ($g_{\perp}$) and on the shoulder ($g_{\parallel}$) decrease at lower temperatures, providing an explanation for the differences observed in the echo-detected spectra recorded at different temperatures. The relaxation time at magnetic field positions corresponding to $g_{\parallel}$ is smaller, leading to a faster decay of the corresponding echo signal as compared to the magnetic field positions corresponding to $g_{\perp}$. Since the ratio in relaxation times at the two types of field positions depends on the temperature, changes in

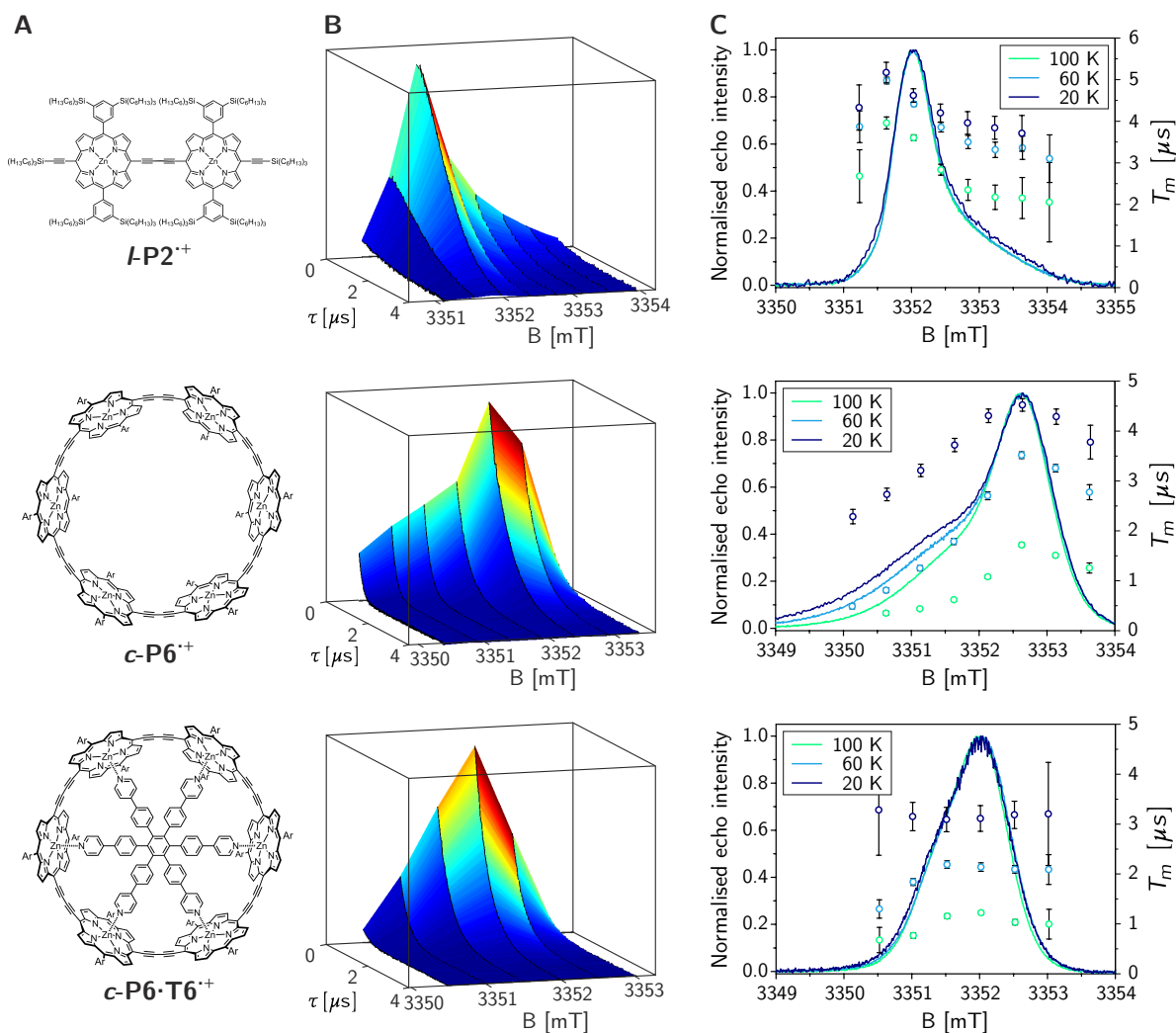


FIGURE 3.15: (A) Molecular structures of the porphyrin dimer, six-membered ring and six-membered ring with template. (B) T_m relaxation measurements performed at different field positions for $l\text{-P2}^{\bullet+}$ (top), $c\text{-P6}^{\bullet+}$ (middle) and $c\text{-P6-T6}^{\bullet+}$ (bottom) at a temperature of 100 K. The T_m measurements were performed with a two-pulse sequence with a $\frac{\pi}{2}$ pulse of 32 ns. (C) Echo-detected field sweeps with $\tau=180$ ns recorded at three different temperatures and the corresponding field-dependent T_m values determined by fitting of the traces shown in B at three different temperatures.

relative intensity of the two parts of the spectrum are observed. The ratios of T_m values at $g_{\perp}$ and $g_{\parallel}$ are significantly larger for $c\text{-P6}^{\bullet+}$ as compared to $l\text{-P2}^{\bullet+}$ (3.7, 2.8 and 1.4 for $c\text{-P6}^{\bullet+}$ and 1.7, 1.3, 1.2 for $l\text{-P2}^{\bullet+}$ at 100, 60 and 20 K, respectively). This explains why such a pronounced temperature dependence is observed for the six-membered ring, while only minor temperature-dependent changes are observed in the echo-detected spectra of $l\text{-P2}^{\bullet+}$. Anisotropic relaxation times with the longest values along the canonical orientations of the g -tensor have previously been observed for quinone radicals [107–109] and have been interpreted in terms of relaxation induced by modulation of the effective g -value through molecular motion. Since oscillations along a particular axis lead to small modulations of the g -value for the orientation with the magnetic field aligned with this axis and to larger modulations of the g -values for all other orientations, the T_m relaxation time will be longest along the axis of oscillation [107].

In the case of $l\text{-P2}^{+\cdot}$, the longest T_m is observed at the highest g -value. Based on the DFT calculations, the corresponding principal axis is predicted to be parallel to the long axis of the molecule, which would thus correspond to the preferential axis of libration of the molecule. The absence of anisotropic relaxation for the six-membered ring with the rigid internal template and the presence of a strong anisotropy of the relaxation time for the six-membered ring without template suggest that in this case the anisotropic relaxation might be caused by some type of breathing motions or distortions of $c\text{-P6}$ that are prevented by the template in $c\text{-P6.T6}$.

3.4.4 CHARACTERISATION OF THE PROTON HYPERFINE INTERACTION

The electron delocalisation in the radical cations at low temperatures was investigated by measuring the proton hyperfine couplings using W-band Mims ENDOR. ^1H Mims ENDOR was measured at different temperatures from 6 K to 100 K in a deuterated solvent mixture and no temperature dependence was observed in this range for any of the systems.

The ENDOR spectrum of the radical cation of the porphyrin monomer does not show any features that can be easily assigned to the hyperfine couplings of different types of protons, since the different types of protons are all coupled to the electron spin with similar weak hyperfine couplings. DFT calculations were performed on the radical cation of $l\text{-P1}$ to compare the experimental results with the theoretical predictions and to attempt a characterisation of the radical state. The optimised geometry of $l\text{-P1}^{+\cdot}$ is characterised by slight saddle-type distortions of the porphyrin plane and the phenyl substituents form an angle of about 61° with the porphyrin plane, in good agreement with literature data on crystal structures of *meso*-aryl Zn porphyrins from the Cambridge Crystallographic Database (CCD) [58] (see figure 3.16 A and B). The structure has approximate D_2 symmetry. In the ground state, the HOMO and HOMO-1 are very close in energy and two different radicals can potentially be formed: the radical arising from the removal of one electron from the HOMO (2B_1 radical) or that arising from removal of one electron from the HOMO-1 (2A radical) (see figure 3.16 C and D). Porphyrins are generally characterised by significant configuration interaction and hence it is quite likely that the real radical state is a linear combination of these states. As can be seen from the spin densities of the two states (shown in figure 3.16 C), the hyperfine couplings of the nitrogen nuclei and of the β -protons will differ significantly for the two states. The proton hyperfine couplings for both types of radicals were calculated and are compared to the experimental results in figure 3.17. Even though the agreement between simulation and experiment is not perfect, this comparison clearly identifies the observed radical as belonging to the 2B_1 radical state. The largest hyperfine coupling is observed for the protons in the *ortho* positions of the phenyl substituents, as also previously determined for Zn tetraphenylporphyrin [96, 97]. The value for this hyperfine coupling is in good agreement with the isotropic hyperfine coupling needed to reproduce the partially resolved proton hyperfine structure in the room temperature cw-EPR spectrum (figure 3.2).

The ENDOR spectrum of $l\text{-P2}^{+\cdot}$ is characterised by a significant reduction in overall width with respect to $l\text{-P1}^{+\cdot}$. ENDOR measurements performed at field positions corresponding to $g_\perp$ and $g_\parallel$ show the

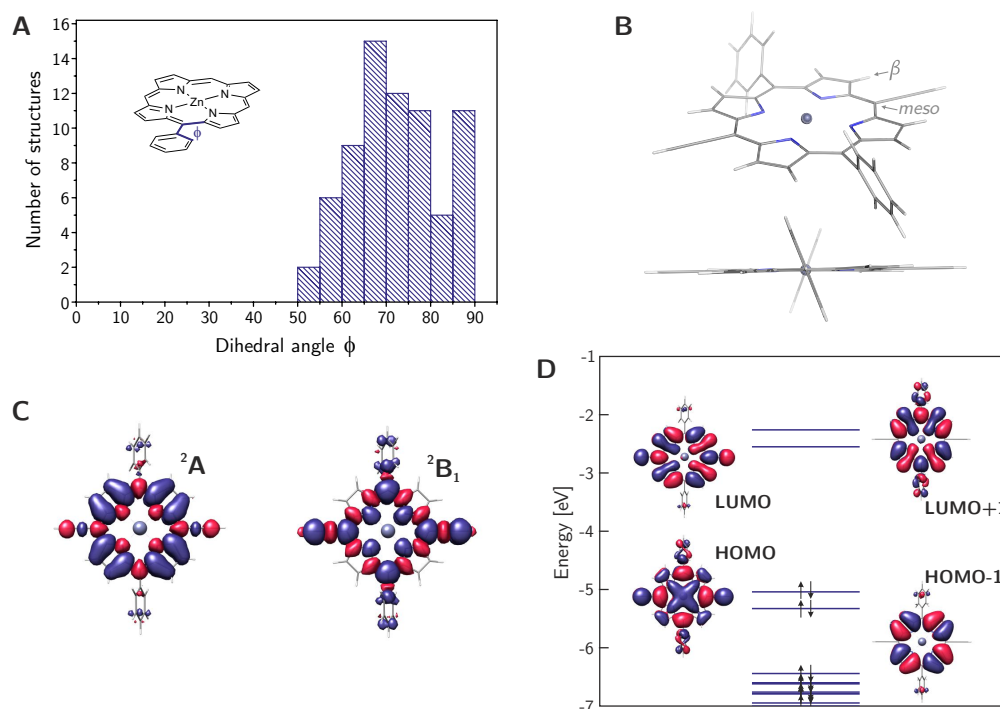


FIGURE 3.16: (A) Distribution of dihedral angles in *meso*-aryl Zn porphyrins from the Cambridge Crystallographic database (CCD), excluding structures with *R* factors greater than 10% or with disorder [58]. (B) Optimised geometry for l -P1 $^{+}$ at B3LYP/6-31G(d) level, the side view shows the presence of slight saddle distortions. (C) Radical cation spin density for the two possible radical states generated by removing an electron from either the HOMO or the HOMO-1. (D) Ground state molecular orbital energy level plot and depiction of the frontier orbitals calculated at B3LYP/6-31G(d) level.

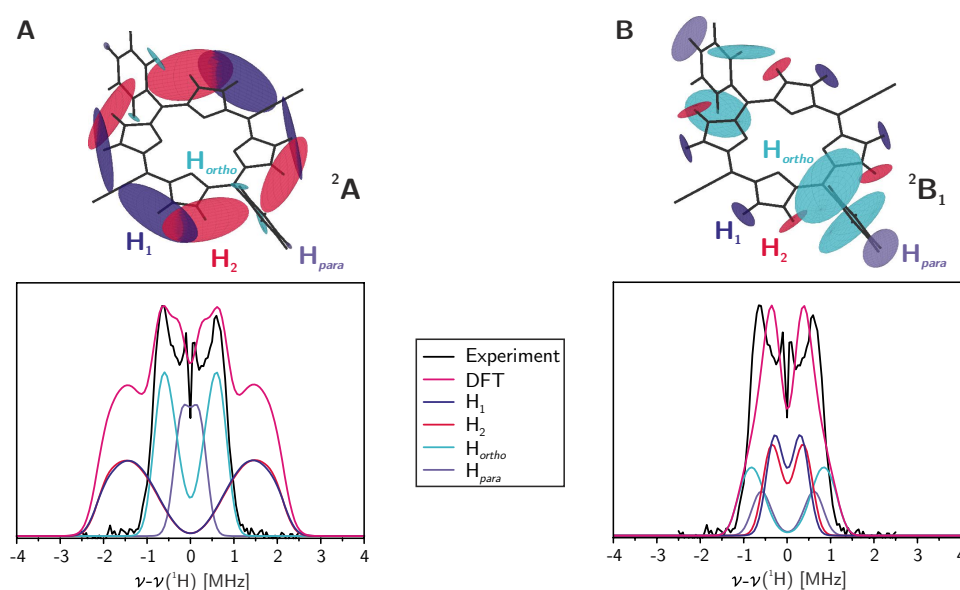


FIGURE 3.17: Proton hyperfine tensors predicted by DFT (B3LYP/EPRII) for the 2A (A) and the 2B_1 (B) radical states of l -P1 and a comparison of the corresponding calculated ENDOR spectra with the experimental Mims ENDOR spectrum recorded for l -P1 $^{+}$ in deuterated solvent at 80 K. The ENDOR spectra were calculated using EasySpin [23] for each type of proton individually and then summed with coefficients taking the number of equivalent protons into account.

presence of some degree of orientation selection. The experimental results were again compared to the results of DFT calculations, which predict delocalisation of the unpaired electron over both porphyrin units (see figure 3.18). The presence of the second porphyrin unit causes changes in the spin density distribution with respect to the porphyrin monomer in the central region of the molecule. The presence of additional spin density on the internal β carbon atoms (indicated by arrows in figure 3.18) leads to increased hyperfine couplings for the protons attached to them (labelled H'_1 and H'_2 in figure 3.18 B), exceeding the hyperfine coupling of the *ortho* protons. The overall agreement between the experimental spectra and the spectra calculated based on DFT hyperfine interaction parameters is good, with the exception of the hyperfine coupling of the internal H'_1 protons which give rise to shoulders with larger hyperfine couplings than observed experimentally in the ENDOR spectrum recorded for the $g_{\perp}$ orientation.

The ENDOR spectra of $l\text{-P1}^{\cdot+}$ and $l\text{-P2}^{\cdot+}$ are compared to the ENDOR spectra of the longer oligomers, recorded at the $g_{\perp}$ and $g_{\parallel}$ field positions at 80 K, in figure 3.19. The comparison shows a distinct change in hyperfine couplings between $l\text{-P1}^{\cdot+}$ and $l\text{-P2}^{\cdot+}$, but the changes observed for the larger oligomers are only very small. Slightly more pronounced changes are observed for the two six-membered ring systems. In order to exclude effects of power broadening on the appearance of the ENDOR spectra, the experiment was repeated with a longer radiofrequency pulse for $c\text{-P6}^{\cdot+}$ (100 μs instead of 18 μs). No changes in the linewidth of the ENDOR spectrum were observed.

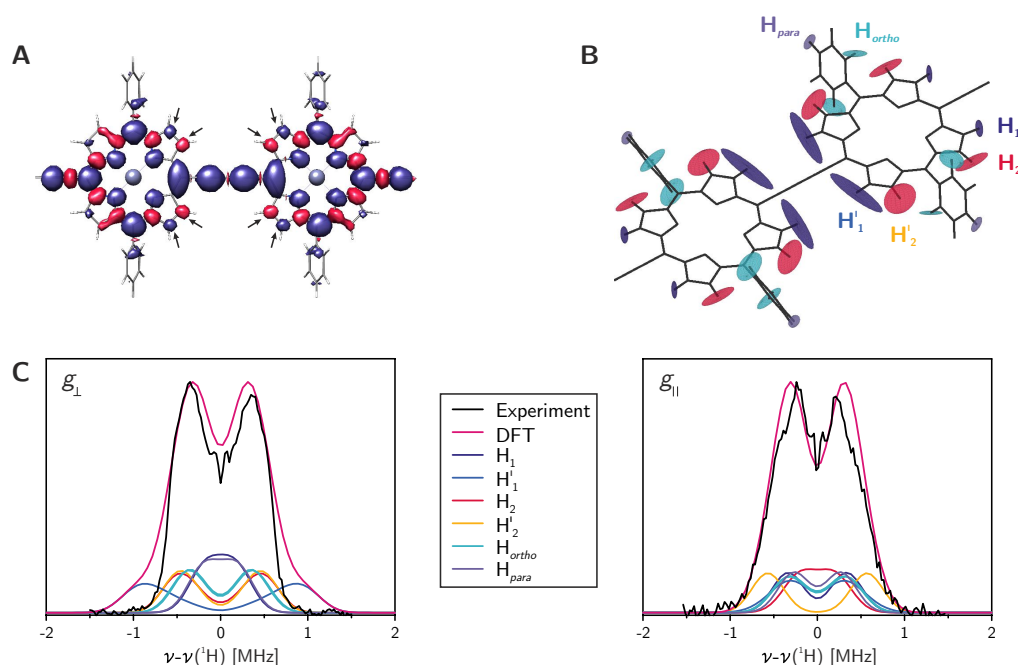


FIGURE 3.18: (A) Spin density distribution in the radical cation of *l*-P2 predicted by DFT calculations at B3LYP/EPRII level. The nuclei in the central region with significantly increased spin density compared to *l*-P1 $^{\cdot+}$ are indicated by arrows. (B) Proton hyperfine tensors predicted by DFT (B3LYP/EPRII) for the spin density distribution shown in A. (C) ENDOR spectra calculated based on the hyperfine couplings predicted by DFT compared to the experimental Mims ENDOR spectrum recorded for *l*-P2 $^{\cdot+}$ in deuterated solvent at two field positions corresponding to $g_{\perp}$ and $g_{\parallel}$ at 80 K. The ENDOR spectra were calculated using EasySpin [23] for each type of proton individually and then summed with coefficients taking the number of equivalent protons into account.

The determination of individual hyperfine couplings from the ENDOR spectra was not possible due to the absence of features in the ENDOR spectra that could be used to identify couplings of different protons. However, the approximate width of the spectrum can still be related to the largest proton hyperfine coupling. The hyperfine couplings are proportional to the spin density and if that decreases as N^{-1} with increasing oligomer size, as assumed by Norris et al. [73], the width of the ENDOR spectrum should change accordingly. The prediction by DFT that different types of protons give rise to the largest hyperfine couplings in l -P1 $^{+\bullet}$ and l -P2 $^{+\bullet}$ and thus determine the overall width of the ENDOR spectrum however complicates this type of analysis. Therefore a different approach for estimating the extent of delocalisation in these radical cation systems was employed.

The proton hyperfine couplings for different geometries and different distributions of spin density over the investigated porphyrin systems were calculated using a distributed point-dipole model. The spin density distributions were constructed by projecting the DFT spin density distribution for the porphyrin monomer onto the optimised geometries of each of the larger porphyrin systems; the spin densities were localised above and below the plane of the molecule to model the π system. The DFT spin density distribution of the porphyrin dimer was also used in some cases to investigate the effect of the distortion of the spin density in the presence of an adjacent porphyrin unit. The hyperfine couplings were then calculated based on these model spin density distributions and used for the ENDOR simulations. A schematic representation of this approach is shown in figure 3.20 and a detailed description can be found in section 3.3.5.

Simulations performed for complete delocalisation of the unpaired electron over the linear porphyrin oligomers are compared to the experimental results in figure 3.21. Apart from some small differences in shape and relative intensities, the spectra calculated for l -P1 $^{+\bullet}$ and l -P2 $^{+\bullet}$ agree quite well with the experimental data, showing coupling of the electron spin with protons on both porphyrin units in l -P2 $^{+\bullet}$. However, from l -P3 $^{+\bullet}$ onwards, the calculated spectra are narrower than the experimental ENDOR data; the differences become more pronounced as the length of the porphyrin arrays

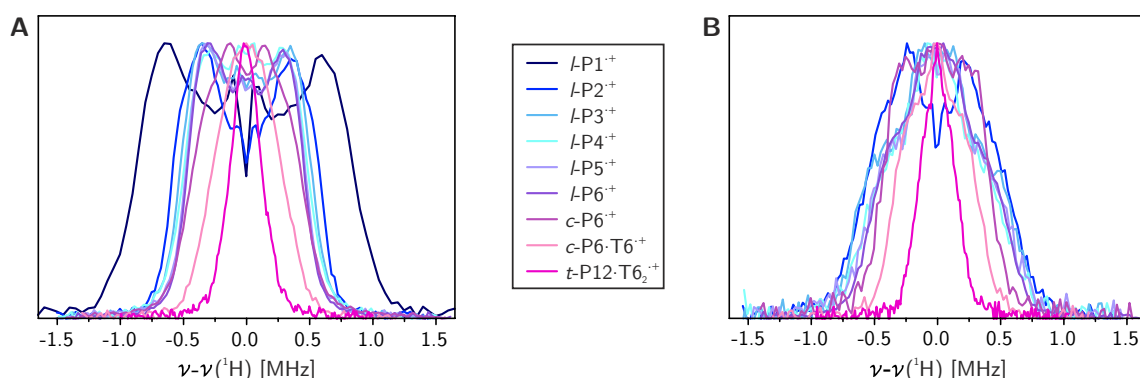


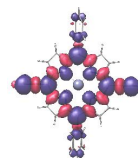
FIGURE 3.19: W-band ^1H Mims ENDOR spectra of l -P1 $^{+\bullet}$ to l -P6 $^{+\bullet}$, c -P6 $^{+\bullet}$, c -P6-T6 $^{+\bullet}$ and t -P12-T6 $^{+\bullet}$ in deuterated toluene:THF:CD $_2$ Cl $_2$ 1:1:1 generated by oxidation with ThSb recorded at 80 K at the field positions corresponding to $g_{\perp}$ (A) and to $g_{\parallel}$ (B). For l -P1 $^{+\bullet}$ the measurement was only performed on the maximum of the EPR signal and is shown in A.

increases. These results show that the electron is completely delocalised in the porphyrin dimer, but delocalisation seems to be limited in the longer oligomers.

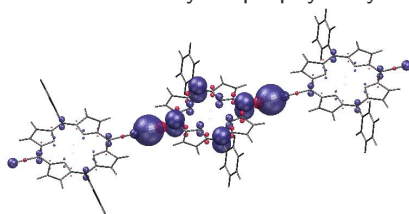
Continuous wave ENDOR measurements of the smaller porphyrin systems in liquid solution were attempted in collaboration with Prof. Damien Murphy at the University of Cardiff, to verify the validity of this type of analysis in a regime where it is known from continuous wave EPR data that the unpaired electron is completely delocalised. The measurements were unfortunately not successful.

A potential explanation for the absence of significant changes in the ENDOR spectra for the porphyrin oligomers with more than two porphyrin units would be an uneven spin density distribution. In order to verify this hypothesis, the simulations were performed considering different ratios of spin density on the different porphyrin units for the oligomers. The agreement between experiment and calculation for each of the considered spin density distributions was evaluated by calculating the root-mean-square deviations (RMSD) between the experimental and calculated ENDOR spectra, neglecting the central region of the spectrum (± 0.2 MHz). The average RMSD values for the ENDOR

- 1 Calculation of the spin density distributions in the radical cations by DFT at the B3LYP/EPRII level.

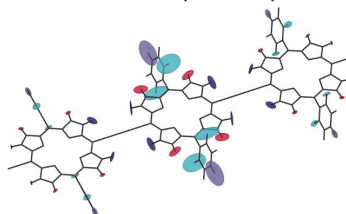


- 2 The spin densities are renormalised and evenly or unevenly distributed over π systems on the optimised geometries of the linear and cyclic porphyrin systems to give an array of positions and spin densities ρ_k .



- 3 The dipolar hyperfine tensors $\mathbf{T}$ are calculated based on a distributed point-dipole model according to:

$$\mathbf{T} = \frac{\mu_0}{4\pi\hbar} g_e \beta_e g_n \beta_n \sum_{k \neq N} \rho_k \frac{(3\mathbf{n}_k \tilde{n}_k - 1)}{R_k^3}$$



- 4 The isotropic hyperfine couplings a_{iso} are determined by extrapolation from the available DFT values according to the relationship:

$$a_{iso}(N) = \frac{a_{iso}(N=1)}{N}$$

in case of complete delocalisation or scaled according to the spin density on each porphyrin unit for uneven spin density distributions.

- 5 The hyperfine coupling tensors are used to calculate the ENDOR spectra in Easyspin.

FIGURE 3.20: Outline of the method used for the calculation of ENDOR spectra for different geometries and different distributions of spin density.

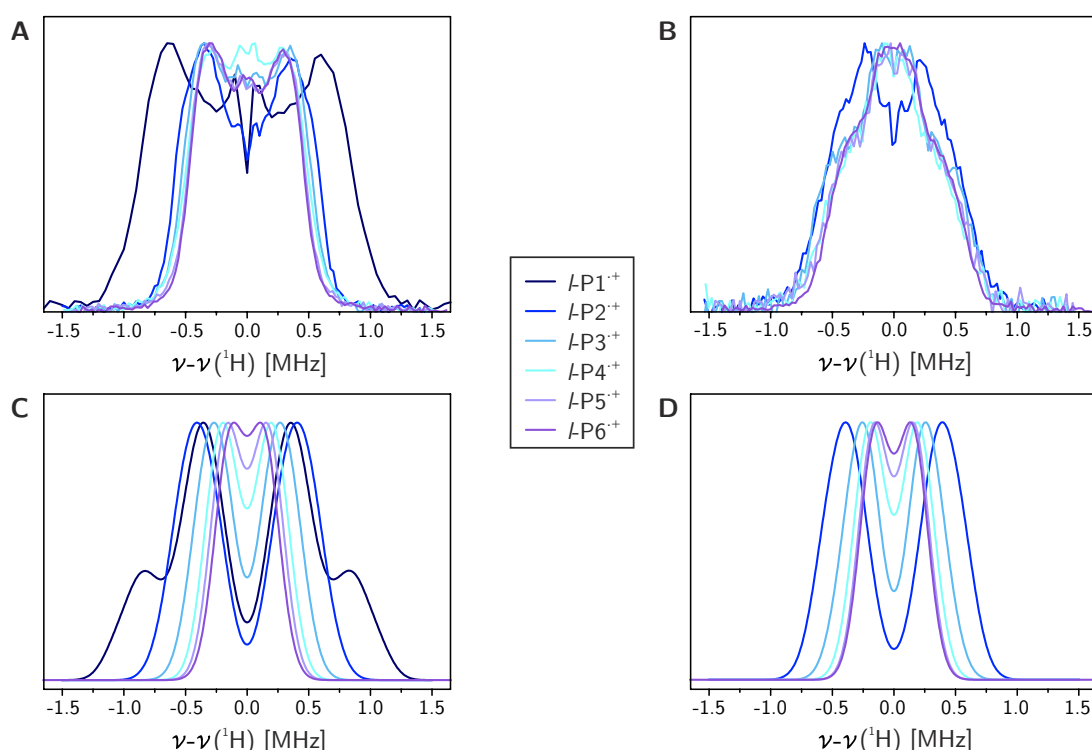


FIGURE 3.21: W-band ^1H Mims ENDOR spectra of the radical cations of the linear oligomers $l\text{-P1}^{\cdot+}$ to $l\text{-P6}^{\cdot+}$, generated by oxidation with ThSb, in deuterated toluene:THF: CD_2Cl_2 1:1:1 recorded at 80 K at the field positions corresponding to $g_{\perp}$ (A) and to $g_{\parallel}$ (B). The corresponding simulations based on the method described in the text and assuming complete delocalisation of the spin density over all the porphyrin units of the linear oligomers are shown in (C) and (D).

spectra recorded at the $g_{\perp}$ and $g_{\parallel}$ field positions are plotted as a function of the largest spin density on a single porphyrin unit in figure 3.22. The spin density distributions correspond to complete symmetric delocalisation on the left of each graph and localisation on a single unit or on two units for systems with an uneven and even number of porphyrin units, respectively, on the right. For all of the linear oligomers this analysis confirms that, in contrast to the results obtained at room temperature, in frozen solution the unpaired electron is not equally distributed over the whole porphyrin system. The spin density distribution giving the best agreement with experiment is depicted for $l\text{-P3}^{\cdot+}$ to $l\text{-P6}^{\cdot+}$ in figure 3.22 and corresponds to spin density mainly localised on two to three porphyrin units.

The ENDOR results for the linear oligomers indicate that the unpaired electron is not completely delocalised over the whole porphyrin system in frozen solution. The results can be interpreted in terms of localisation of the spin density on two to three porphyrin units, based on the observation of only minor changes in the ENDOR spectra of the longer oligomers, or in terms of uneven distributions of spin density. The insufficient resolution of the ENDOR data, due to the presence of many different protons with small hyperfine couplings, does not allow a distinction between the two situations. The two different cases could potentially be distinguished by selective deuteration of specific types of protons in the porphyrin molecule, to isolate the contributions of different types of protons to the ENDOR spectrum and observe their changes as a function of the number of porphyrin units.

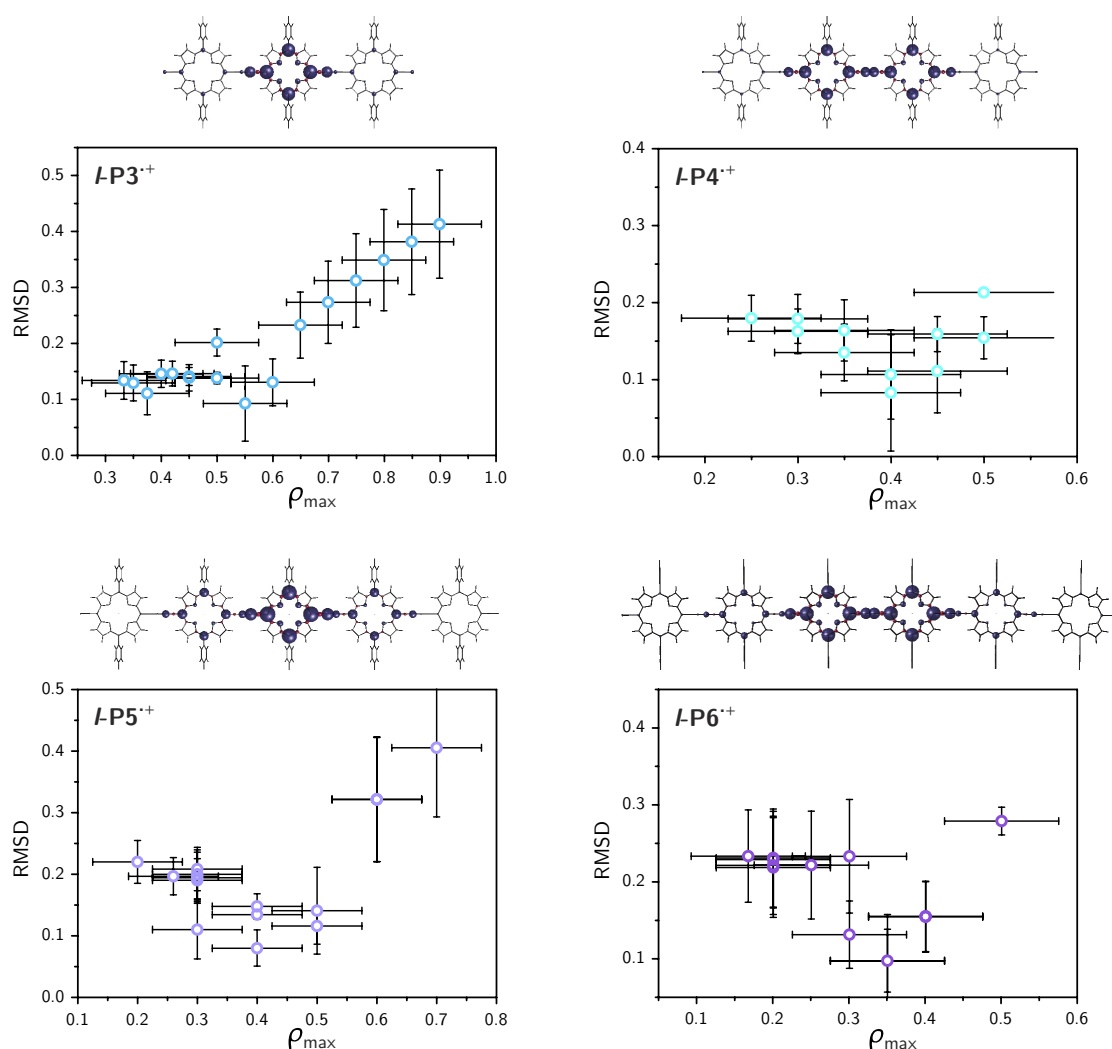


FIGURE 3.22: Root-mean-square deviations (RMSD) between the experimental ENDOR data and the calculated spectra with different spin density distributions, $\rho_{\max}$ refers to the largest relative spin density on a single porphyrin unit which determines the width of the ENDOR spectrum. The average RMSD for the two magnetic field positions was computed; the central region of the ENDOR spectrum (± 0.2 MHz) was not considered in this analysis. The error on the spin density was estimated based on the deviation of the real spectrum from the calculated spectrum for l-P2⁺ with a spin density distribution of 0.5:0.5. The spin density distribution with the lowest RMSD is depicted above the corresponding graphs.

A further narrowing and a change in the shape of the ENDOR spectra is observed for c-P6⁺ and c-P6-T6⁺. ENDOR simulations with hyperfine couplings calculated for spin density distributed over one to six porphyrin units in the optimised six-membered ring geometry are compared to the experimental spectra of the two ring systems in figure 3.23. In this case, the spin density distribution calculated for c-P6⁺ was used instead of the l-P1⁺ spin density for the calculation of the hyperfine tensors with the method described above. The best agreement between simulation and experiment is obtained for spin density localised on about three porphyrin units, suggesting that the extent of delocalisation in the cyclic systems might be slightly larger than in the linear arrays. It can nevertheless not be excluded that the differences observed in the ENDOR spectra of the linear and cyclic systems are due to changes in spin density distribution related to the difference in geometry, rather than to

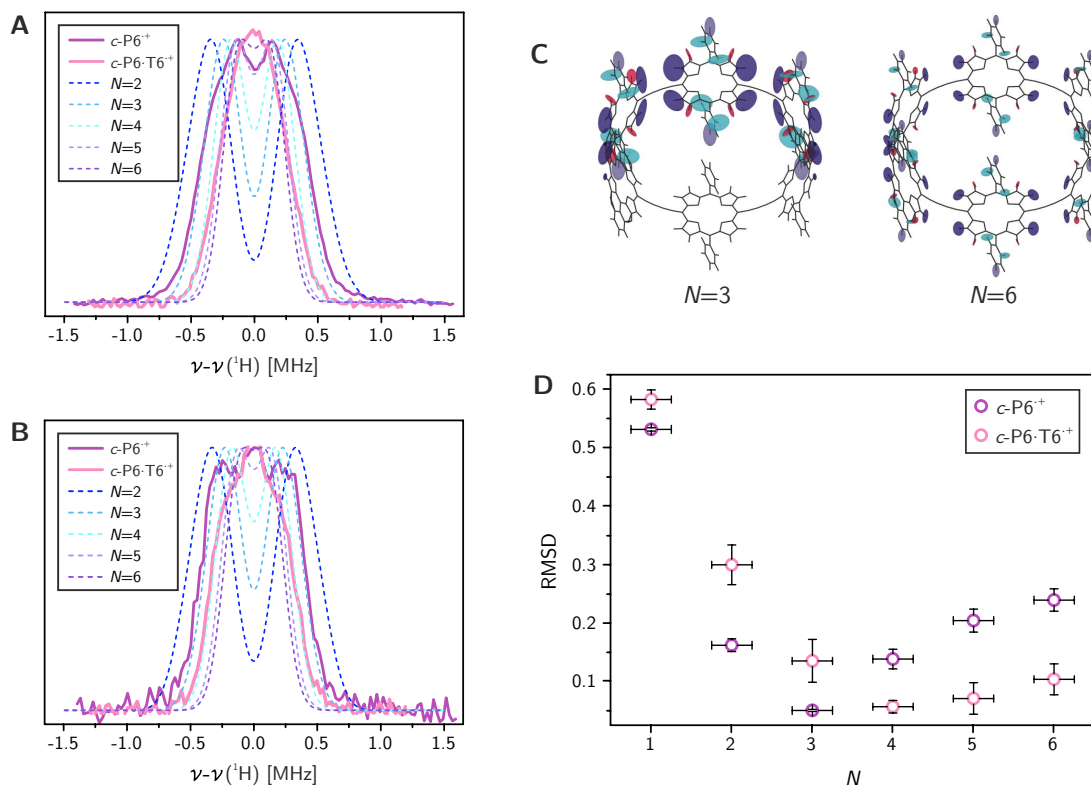


FIGURE 3.23: (A,B) Comparison of the experimental Mims ENDOR spectra recorded at the $g_{\perp}$ (A) and at the $g_{\parallel}$ (B) field positions at 80 K for $c\text{-P6}^{\bullet+}$ and $c\text{-P6}\cdot\text{T6}^{\bullet+}$ with simulations assuming spin density delocalised over N porphyrin units. (C) Proton hyperfine tensors calculated using the distributed point-dipole approximation for spin density distributed over three ($N=3$) and over all six ($N=6$) porphyrin units of $c\text{-P6}$. (D) Root-mean-square deviations (RMSD) between the experimental ENDOR data and the calculated spectra with N from 1 to 6. The average RMSD for the two magnetic field positions was computed; the central region of the ENDOR spectrum was not considered in this analysis.

different extents of electron delocalisation. The differences between the ENDOR spectra of $c\text{-P6}^{\bullet+}$ and $c\text{-P6}\cdot\text{T6}^{\bullet+}$ are more difficult to rationalise; they could be due again to changes in spin density distribution arising from small differences in geometry of the two types of ring systems. While $c\text{-P6}\cdot\text{T6}$ is held in a rigid circular ring shape by the template and is characterised by a high degree of symmetry due to the constraints imposed by the template, the ring without template has been shown to be more flexible by NMR, even though small-angle X-ray scattering data have shown that both rings are shape-persistent in solution [69]. Another possible interpretation of the experimental data could be slightly different extents of spin delocalisation due to the differences in shape and more importantly flexibility. ENDOR simulations for different distributions of spin density over the twelve porphyrin units in $t\text{-P12}\cdot\text{T6}_2^{\bullet+}$ were performed to determine the extent of delocalisation. The best agreement was obtained for spin density localised mainly on four porphyrin units, two on each of the porphyrin rings.

A quantification of the extent of delocalisation based on the ENDOR simulations for different spin density distributions over the multiporphyrin systems was attempted. A parameter ξ_{deloc} was defined as follows:

$$\xi_{deloc} = \sum_{i=1}^N \delta_{SD_i \geq 1/N} + \frac{1}{2} \sum_{i=1}^N \delta_{SD_i \geq 1/2N} \quad \text{where} \quad \delta_{SD_i \geq 1/nN} = \begin{cases} 1 & \text{if } SD_i \geq \frac{1}{nN} \\ 0 & \text{if } SD_i < \frac{1}{nN} \end{cases} \quad (3.8)$$

This parameter was computed for each of the spin density distributions corresponding to a simulation with an RMSD value falling below a cutoff defined as the minimum RMSD value +20% of the range of RMSD values. The parameter ξ_{deloc} should approximately reflect the extent of delocalisation and is plotted as a function of the number of porphyrin units in figure 3.24. In all cases the parameter is smaller than expected in case of complete delocalisation, indicating localisation of the unpaired electron on part of the multiporphyrin system.

Localisation of the radical cation in frozen solution has previously been reported for other multiporphyrin systems [68, 80, 81, 83], although in those cases the electron spin was localised on a single rather than two porphyrin units. Several reasons for charge localisation at low temperatures have been proposed, chiefly amongst them counter ion and solvent effects [68, 80, 110, 111]. In this investigation a weakly coordinating counter ion, $SbCl_6^-$, was used to minimise counter ion effects. Additionally, measurements on solutions with a large excess of electrolyte (tetrabutylammonium hexafluorophosphate) showed no significant changes in the ENDOR and W-band EPR spectra recorded at 80 K, excluding the counter ion as cause for the electron spin localisation observed at low temperatures. Localisation of the electron spin on a limited number of porphyrin units could also be driven by conformational dynamics of the linker. If conformations with the porphyrin units at right angles to each other are frozen in, this could cause localisation of the electron spin in only part of the porphyrin array. Since the barrier of rotation is much higher for the radical cation with respect to the

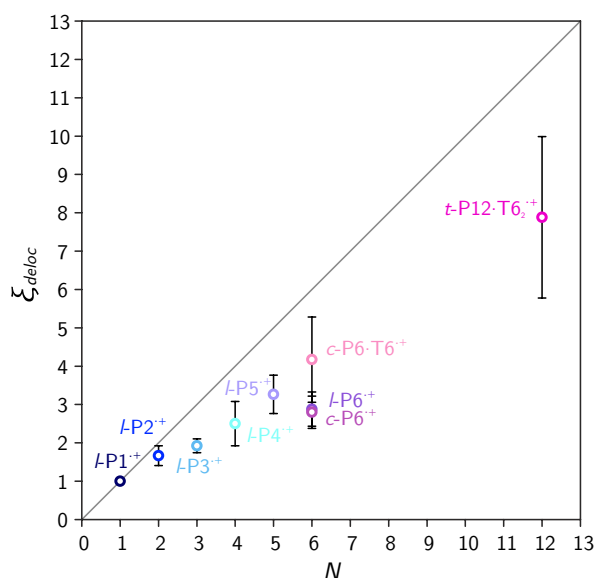


FIGURE 3.24: Plot of the parameter ξ_{deloc} determined based on equation 3.8 for each of the investigated multiporphyrin systems as a function of the number of porphyrin units N . The error bars were estimated from the distribution of solutions with RMSD values below a cutoff value defined in the text. In case of complete delocalisation the ξ_{deloc} values should be equal to N and therefore lie on the diagonal.

ground state of the neutral molecule (1.87 eV and 0.03 eV, respectively, based on DFT calculations) [67], this does, however, seem quite unlikely. Solvent reorganisation effects or aggregation have also been suggested as possible causes for the apparent hole localisation in frozen solution.

3.4.5 CHARACTERISATION OF THE NITROGEN HYPERFINE INTERACTION

The nitrogen hyperfine interactions in the radical cations were investigated using ^{14}N ESEEM and HSCORE at X-band and ^{14}N ENDOR at W-band. The results of HSCORE experiments performed on $l\text{-P1}^{+\bullet}$, $l\text{-P2}^{+\bullet}$, $l\text{-P3}^{+\bullet}$ and $c\text{-P6}^{+\bullet}$ are shown in figure 3.25. The results of ESEEM and ENDOR experiments are shown in figure 3.27 A and C.

The HSCORE data show that the nitrogen hyperfine couplings are reduced significantly from $l\text{-P1}^{+\bullet}$ to $l\text{-P2}^{+\bullet}$, as shown by the shift of the main signals from the $(-,+)$ to the $(+,+)$ quadrant of the HSCORE spectrum. No significant further changes are observed for $l\text{-P3}^{+\bullet}$ and $c\text{-P6}^{+\bullet}$.

In the HSCORE spectrum of $l\text{-P1}^{+\bullet}$ (see figure 3.25), the double-quantum peak positions at about $(-9.0, 12.5)$ MHz and $(-12.5, 9.0)$ MHz indicate an out-of-plane nitrogen hyperfine coupling of about 10.5 MHz. The isotropic hyperfine coupling was found to be 4.05 MHz from the simulations of the room temperature cw-EPR spectrum. The hyperfine and nuclear quadrupole interaction parameters predicted by DFT for the $^2\text{B}_1$ radical state agree quite well with the experimental results, while the calculations for the ^2A radical state predict much smaller nitrogen hyperfine couplings, confirming the assignment of the EPR data to the $^2\text{B}_1$ state (see figure 3.26 A and B).

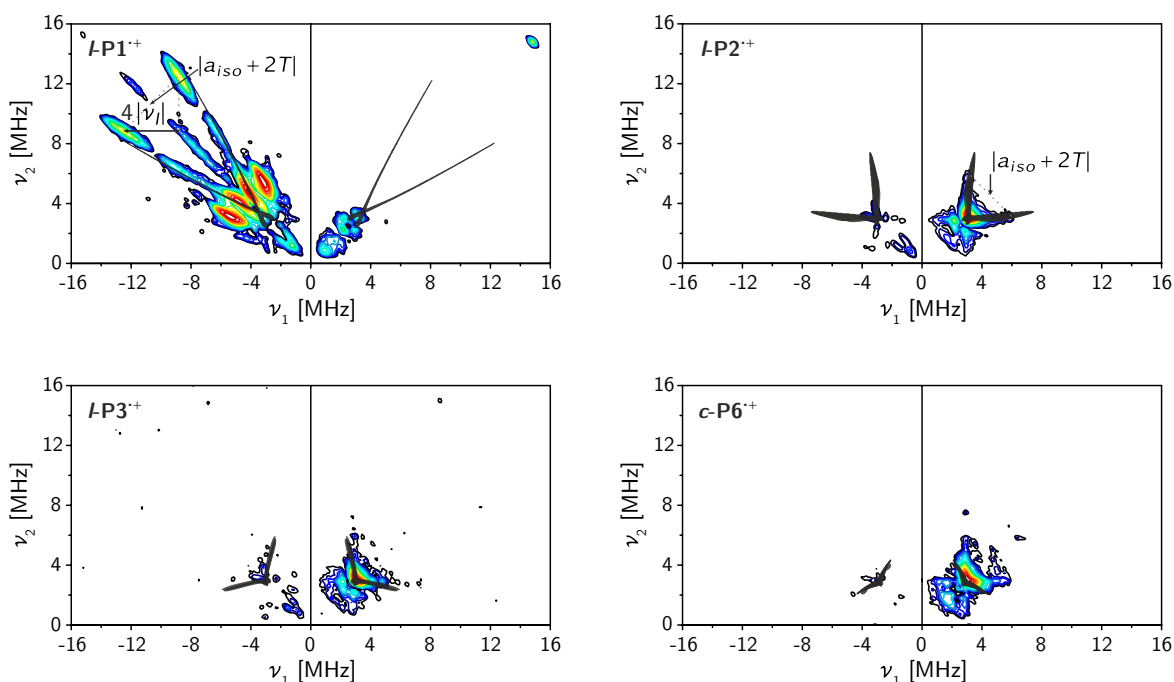


FIGURE 3.25: Experimental X-band ^{14}N HSCORE spectra recorded at 80 K for $l\text{-P1}^{+\bullet}$, $l\text{-P2}^{+\bullet}$, $l\text{-P3}^{+\bullet}$ and $c\text{-P6}^{+\bullet}$. The double-quantum cross-peak positions calculated as described in the text are superimposed in black.

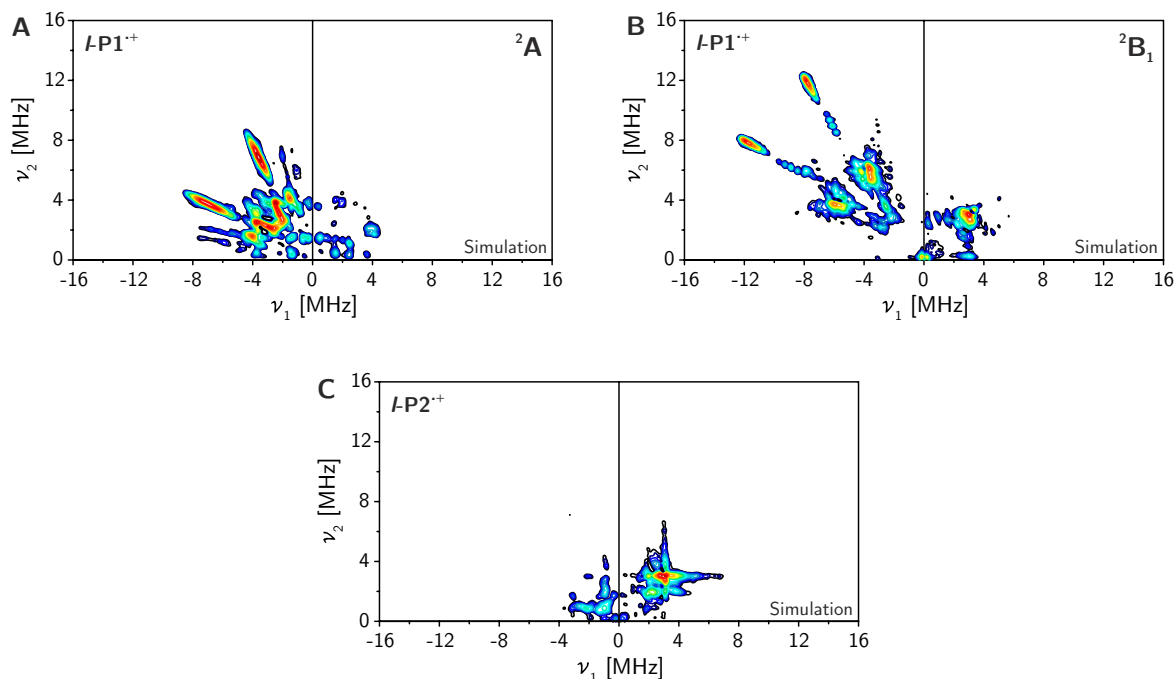


FIGURE 3.26: (A,B) HYSCORE simulations using the nitrogen hyperfine and nuclear quadrupole interaction parameters predicted by DFT for the 2A and 2B_1 radical states of $l\text{-P1}^{\bullet+}$. The simulation parameters are $A = [-1.49, -1.51, -6.25]$ MHz, $Q = 2.39$ MHz and $\eta = 0.47$ for the 2A state and $A = [-0.17, -0.44, 10.02]$ MHz, $Q = 2.13$ MHz and $\eta = 0.83$ for the 2B_1 state. (C) HYSCORE simulations using the nitrogen hyperfine and nuclear quadrupole interaction parameters predicted by DFT for $l\text{-P2}^{\bullet+}$. The simulation parameters are $A = [-0.09, -0.22, 4.77]$ MHz, $Q = 2.22$ MHz and $\eta = 0.69$ for the external nitrogen nuclei and $A = [-0.16, -0.30, -4.39]$ MHz, $Q = 2.22$ MHz and $\eta = 0.72$ for the internal nitrogen nuclei of the dimer molecule. The simulations were performed using EasySpin [23].

In the $l\text{-P2}^{\bullet+}$ HYSCORE spectrum, the main signals are shifted to the (+,+) quadrant, indicating a change from the strong to the weak coupling regime. The out-of-plane hyperfine coupling can again be estimated from the ends of the double-quantum ridges and amounts to about 4.5 MHz. The significant reduction in nitrogen hyperfine coupling suggests complete delocalisation of the unpaired electron over the two porphyrin units in the porphyrin dimer. The simulation performed based on the interaction parameters predicted by DFT for complete delocalisation in $l\text{-P2}^{\bullet+}$ are shown in figure 3.26 C and agree well with the experimental results.

The HYSCORE spectra of $l\text{-P3}^{\bullet+}$ and $c\text{-P6}^{\bullet+}$ are almost identical to that of $l\text{-P2}^{\bullet+}$, following the trend already observed for the ^1H ENDOR data. This is confirmed by comparison to simulations performed analogously to those described in the previous section. As the hyperfine couplings can be determined most easily from the double-quantum cross-peaks of the HYSCORE spectra, the experimental results are compared to the calculated double-quantum cross-peak positions in figure 3.25. There is a good agreement between the experimental and calculated peak positions for $l\text{-P1}^{\bullet+}$ and $l\text{-P2}^{\bullet+}$, however, for $l\text{-P3}^{\bullet+}$ and $c\text{-P6}^{\bullet+}$ lower transition frequencies are predicted, corresponding to smaller hyperfine couplings than those observed experimentally.

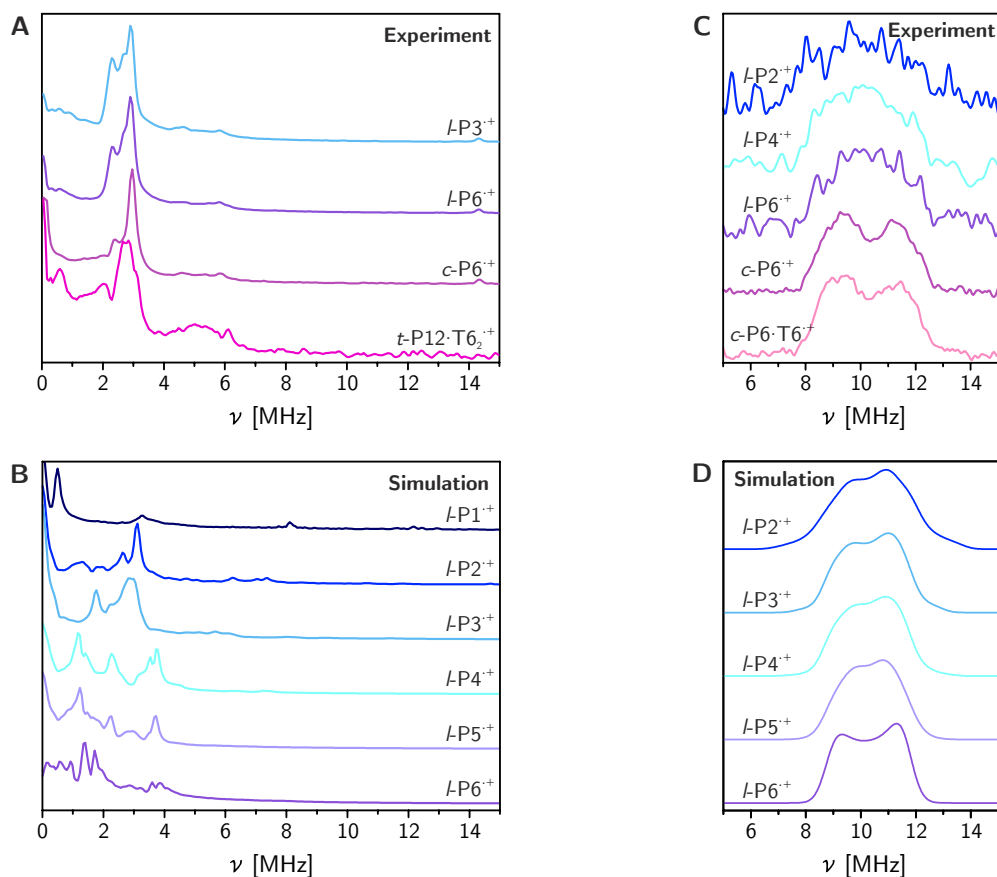


FIGURE 3.27: (A) FT spectra of experimental X-band ^{14}N three pulse ESEEM recorded at 80 K for $l\text{-P3}^+$, $l\text{-P6}^+$, $c\text{-P6}^+$ and $t\text{-P12}\cdot\text{T6}_2^+$. (B) Simulations of the ESEEM frequency domain spectra for the linear porphyrin arrays in case of complete delocalisation performed with the procedure described in the text. All ESEEM spectra are normalised to one. (C) W-band ^{14}N ENDOR spectra recorded at 60 K for $l\text{-P2}^+$, $l\text{-P4}^+$, $l\text{-P6}^+$, $c\text{-P6}^+$ and $c\text{-P6}\cdot\text{T6}^+$. (D) Simulations of the ENDOR spectra for the linear porphyrin arrays with two to six porphyrin units assuming complete delocalisation performed according to the procedure described in the text.

Three-pulse ESEEM was also recorded for $l\text{-P3}^+$, $l\text{-P6}^+$, $c\text{-P6}\cdot\text{T6}^+$ and $t\text{-P12}\cdot\text{T6}_2^+$, and the corresponding FT spectra are shown in figure 3.27 A. The ESEEM spectra are characterised by two well-defined peaks at around 2-3 MHz in all cases, with some broader low intensity peaks at lower frequencies. The additional low intensity peaks at higher frequencies (4-6 MHz) are likely combination peaks. The appearance of the spectra is characteristic of the weak-coupling regime (see section 2.2.5), where information on the hyperfine couplings is difficult to extract without simulations. Nevertheless, the fact that the position of the main peaks remains unchanged for $l\text{-P3}^+$, $l\text{-P6}^+$ and $c\text{-P6}\cdot\text{T6}^+$, with only small changes in relative intensity and in the low frequency region (figure 3.27 A), suggests that no significant change in hyperfine couplings occurs for oligomers with more than two porphyrin units. Slightly more pronounced changes are observed for $t\text{-P12}\cdot\text{T6}_2^+$ and would be in agreement with increased delocalisation, as also indicated by the ^1H ENDOR results. A more detailed analysis and interpretation is complicated by the additional presence of signals due to the nitrogen nuclei on the template.

Since the experimental data for the longer oligomers is not sufficiently well resolved to attempt fitting of the hyperfine and nuclear quadrupole couplings of multiple, potentially different, nitrogen nuclei, the experimental results were compared to simulations performed with the same procedure described in section 3.3.5 and used in the previous section for the simulation of ^1H ENDOR spectra. Three-pulse ESEEM simulations for the linear oligomers were performed assuming complete delocalisation of the unpaired electron over the whole porphyrin system (see figure 3.27 B). The simulations predict the occurrence of more significant changes in both peak positions and relative intensities with respect to those observed experimentally. The experimental spectra recorded for $l\text{-P3}^+$, $l\text{-P6}^+$ and $c\text{-P6}\cdot\text{T6}^+$ most resemble the simulations for delocalisation over two or three porphyrin units, in agreement with the results from the ^1H ENDOR experiments.

The ^{14}N Mims ENDOR measurements performed at W-band for $l\text{-P2}^+$, $l\text{-P4}^+$, $l\text{-P6}^+$, $c\text{-P6}^+$ and $c\text{-P6}\cdot\text{T6}^+$ are shown in figure 3.27 C. The low signal-to-noise ratio for the linear oligomers unfortunately makes an accurate analysis impossible, but it is still clear that no significant change, in particular of the width of the ^{14}N ENDOR spectrum, occurs as a function of oligomer size and geometry. Simulations were again performed for the linear oligomers assuming complete delocalisation and using a broadening leading to a reasonable agreement with the experimental data (see figure 3.27 D). The simulations show a slight narrowing as a function of oligomer size, but the differences lie within the noise of the experimental data and therefore the results of the W-band ^{14}N ENDOR measurements were inconclusive.

The analysis of the ^{14}N HYSCORE and ESEEM data of the linear oligomers suggests localisation of the spin density on mainly two to three porphyrin units, in agreement with the results of the previous section. Similar conclusions can be drawn regarding the cyclic porphyrin systems; the small changes in the experimental data between the linear oligomers and $c\text{-P6}^+$ or $c\text{-P6}\cdot\text{T6}^+$ are most likely due to slightly different spin density distributions in the planar and bent systems.

The effect of uneven spin density distributions was investigated using the same approach as described for the ^1H ENDOR results in the previous section. The results approximately mirror those obtained for the ^1H ENDOR data, however the differences in RMSDs between different spin density distributions are much smaller, making it harder to clearly identify a minimum. The reduced sensitivity of the experimental ^{14}N hyperfine data towards the spin density distribution is partly due to the presence of the nuclear quadrupole interaction, which determines the appearance of the spectra in the presence of only small hyperfine couplings. The analysis would be much simplified on ^{15}N isotope substituted porphyrin systems, as the changes in the spectra would then only depend on the hyperfine interaction. Selective isotope substitution for certain types of nitrogen nuclei could provide the possibility of investigating end-group effects in the linear porphyrin oligomers and could potentially allow determination of the spin density on each of the porphyrin units in larger systems, thus allowing the distinction between coherent delocalisation with an uneven spin density distribution and hole hopping in the investigated porphyrin arrays.

3.5 CONCLUSIONS

The extent of electron spin delocalisation in linear and cyclic *meso*-to-*meso* butadiyne linked porphyrin arrays was investigated at room temperature and in frozen solution by a combination of continuous wave and pulse EPR techniques. At room temperature, the electron delocalisation length was determined through the EPR linewidth, while at low temperatures ENDOR and ESEEM data provided a direct measure of the ^1H and ^{14}N hyperfine coupling parameters.

At room temperature, the electron is completely delocalised or hopping faster than the EPR timescale (ca. 10^7 s^{-1}) for all of the investigated systems containing up to six porphyrin units in a linear or cyclic arrangement. The low temperature data on the other hand, were interpreted in terms of limited delocalisation, either by localisation of the unpaired electron on two to three porphyrin units or by uneven spin density distributions with the largest contributions on only part of the porphyrin system.

The results of frozen solution W-band EPR, W-band ENDOR and X-band ESEEM measurements show only minor changes for the linear porphyrin systems containing more than two porphyrin units, indicating a similar extent of delocalisation as in the porphyrin dimer. Simulations based on hyperfine parameters determined from model spin density distributions derived from DFT results confirm that the observed hyperfine couplings correspond to spin densities localised mainly on two to three porphyrin units in all of the linear porphyrin arrays. Larger deviations are observed for the cyclic arrays of porphyrins, particularly in the W-band EPR spectra, where an inversion of the $g_{\parallel}$ and $g_{\perp}$ values occurs, and suggest changes occurring in the electron distribution as a result of the different geometry and absence of end-group effects. Simulations explicitly considering the differences in geometry lead to a similar conclusion as for the linear systems, i.e. localisation of the electron on about three of the six porphyrin units.

Further investigations would be required to determine the exact nature of the charge localisation. EPR measurements performed after selective isotope substitution could potentially provide more precise information on the mechanism of delocalisation. However, localisation on two rather than a single porphyrin unit would imply faster hole hopping and improved conjugation between the porphyrin units compared to previously investigated systems [68, 80, 81, 83].

This is the first extensive EPR study, including direct measurements of the hyperfine couplings through pulse ENDOR and ESEEM techniques, of a series of radical cations of porphyrin arrays with up to six units from room temperature down to cryogenic temperatures. The extent of electron delocalisation is reflected not only in the hyperfine values of proton and nitrogen nuclei, but also in the g -anisotropy that can be detected at high fields. The results suggest that, in order to obtain an accurate estimate of the electron delocalisation length in frozen solution, direct measurements of the hyperfine couplings using either ENDOR or ESEEM are desirable, as anisotropies and dynamical effects influence the low temperature EPR lineshape preventing an unequivocal correlation between the peak-to-peak linewidth and the extent of electron delocalisation.

The results have further shown that the available EPR techniques for the study of electron delocalisation lengths in materials proposed as molecular wires or in artificial supramolecular structures mimicking natural photosynthetic pigment arrays can be extended to pulse ENDOR and both one- and two-dimensional ESEEM techniques. These techniques can provide additional information that remains unresolved in the EPR spectra and are particularly useful for investigations of the low temperature behaviour of these types of systems.

4 TRIPLET STATE DELOCALISATION IN PORPHYRIN SYSTEMS

The investigation on the electron delocalisation in the radical cations of linear and cyclic porphyrin systems described in the previous chapter indicated complete delocalisation at room temperature, but localisation onto no more than two to three porphyrin units in frozen solution for all of the systems studied. Since significant differences in the extent of electron delocalisation in radical cations and in triplet states, with localisation of the triplet state on a single porphyrin unit, have previously been observed [112, 113], the investigation has been extended to the photoexcited triplet states of the same porphyrin systems already described in section 3.1.

4.1 EPR ON TRIPLET STATES OF PORPHYRIN SYSTEMS

In the past EPR has been used extensively to study triplet state delocalisation and energy transfer in photosynthetic reaction centres and their model systems [21, 114–123], as well as in linear arrays of π -conjugated organic molecules designed as molecular wires [113, 124–128]. The extent of triplet state delocalisation can be estimated either from the zero-field splitting (ZFS) interaction or the hyperfine interaction parameters. The zero-field splitting interaction is often approximated using a point-dipole model and can therefore be used to provide an estimate of the average inter-electron distance due to the r^{-3} dependence. The hyperfine couplings probe the spin density at specific points in the molecular system and should thus scale with respect to the monomer hyperfine couplings with the number of monomeric units the triplet state is delocalised over. The zero-field splitting interaction parameters can be obtained from the EPR spectrum, whereas the hyperfine couplings are generally not resolved in the EPR spectrum and need to be measured using pulse EPR techniques such as ENDOR and ESEEM.

Triplet state ENDOR investigations have demonstrated triplet state delocalisation over the special pair in the bacterial reaction centre of *Rhodobacter sphaeroides* based on a comparison of the ENDOR spectrum recorded on bacteriochlorophyll *a* *in vitro* with the spectrum recorded on the photosynthetic reaction centre. A decrease of the proton hyperfine couplings to about half in the special pair of bacteriochlorophyll molecules was observed [121]. Analogous investigations on plant reaction centres have shown no change in hyperfine couplings and it has been concluded that in that case the

triplet state is localised on a single chlorophyll unit of the special pair [123]. These results led to the conclusion that triplet state delocalisation is very sensitive to the relative position, in terms of both distance and relative orientation, of the single units, as has also been shown by further investigations on model systems consisting mainly of face-to-face dimers of porphyrin-type molecules [119, 122].

The triplet state delocalisation in linear π -conjugated porphyrins proposed to act as molecular wires has been investigated by studying the zero-field splitting interaction as a function of oligomer chain length [113, 126, 127]. The interpretation of the results was based on the point-dipole approximation and yielded average inter-electron distances that did not exceed the dimensions of a single monomeric unit. This led to the conclusion that the triplet state is localised on a single porphyrin in most of these systems; in contrast to the corresponding radical cations, which typically show more extensive delocalisation. Small changes in the zero-field splitting parameters D and E in porphyrin dimers with different linkers have prompted the proposal of an oblate-to-prolate spin transition, with a reorientation of the ZFS tensor, from monomers to polymer chains [113, 124]. The limitations of the point-dipole approximation for the determination of inter-electron distances were discussed in a recent paper by Riplinger et al. [129], where it was concluded that in systems with extensive delocalisation of the two unpaired electrons, the use of this model can lead to underestimation of the inter-electron distance. This observation suggests that triplet delocalisation in highly conjugated porphyrin systems can be more accurately determined through the measurement of hyperfine couplings rather than the zero-field splitting interaction.

4.2 EXPERIMENTAL METHODS

4.2.1 SAMPLE PREPARATION

The synthesised compounds were characterised and checked for purity by NMR, UV-Vis and analytical GPC (gel permeation chromatography) in Prof. H. Anderson's lab. The EPR measurements were performed on 50-200 μM solutions of the linear and cyclic porphyrin oligomers in MeTHF:pyridine 10:1. High concentrations (ca. 200 μM) were used for the short oligomers, while the concentration was lowered for the longer oligomers to prevent optical saturation. Measurements of porphyrin systems in the presence of templates were typically performed in toluene, as MeTHF is a coordinating solvent and prevents binding of the ligand to the Zn ion. In some cases, the measurements were performed in *o*-terphenyl, which allows the study of a wider range of conformations due to its higher glass transition temperature (246 K compared to 91 K for MeTHF and 117 K for toluene). The solutions were degassed by several freeze-pump-thaw cycles and shock-frozen in liquid N_2 .

4.2.2 TIME-RESOLVED EPR

The time-resolved EPR experiments were performed at X-band on a Bruker Elexsys 680 spectrometer equipped with a helium gas-flow cryostat from Oxford instruments. Laser excitation at 532 nm

was provided by the second harmonic of a Nd:YAG laser (Surelite Continuum) with a repetition rate of 10 Hz. Light depolarised with an achromatic depolariser was used unless otherwise stated. TR-EPR experiments were performed by direct detection with the transient recorder without lock-in amplification; the microwave power was 0.2 mW. The laser background signal was removed by 2D baseline-correction determined based on the off-resonance transients. The spectra were integrated over the first 2 μ s after the laser flash. Experiments were typically performed at 20 K, but no changes in the spectra were observed at temperatures between about 100 and 5 K.

Magnetophotoselection experiments were performed with polarised light from an Oportek Opolette Opto-parametric Oscillator (OPO) tuneable laser (20 Hz repetition rate) at wavelengths corresponding to the region of the Q bands in the UV-Vis spectra.

4.2.3 PULSE EPR

X-band pulse EPR measurements were performed on a Bruker Elexsys 680 spectrometer with a Bruker EN 4118X-MD4 resonator. The Q-band measurements were performed on a Bruker Elexsys 580 spectrometer in the National EPR facility at the University of Manchester. The measurements were performed at 20 K and with laser excitation as previously described. The repetition rate of the pulse experiments was determined by the laser repetition rate of 10 Hz (20 Hz for measurements with the OPO).

^1H Mims ENDOR spectra were recorded with the pulse sequence $\frac{\pi}{2} - \tau - \frac{\pi}{2} - T - \frac{\pi}{2} - \tau - \text{echo}$ with mw pulse lengths of $t_{\pi/2} = 24$ ns, $\tau = 120, 160, 200$ ns and a radiofrequency pulse with a length of 15 μ s applied during the delay T ; the rf power was adjusted based on a nutation experiment. The ENDOR spectra were recorded at the canonical field positions of the triplet state EPR spectrum; spectra were recorded for three different τ values (typically 120, 160 and 200 ns) and summed to prevent distortions by blind spots.

X-band three-pulse ESEEM experiments were performed with the pulse sequence $\frac{\pi}{2} - \tau - \frac{\pi}{2} - T - \frac{\pi}{2} - \tau - \text{echo}$ with mw pulses of length $t_{\pi/2} = 16$ ns and an initial T value of 64 ns with increments $dt = 8$ ns (1024 data points were collected). In the Q-band three-pulse ESEEM experiments, the pulse lengths were $t_{\pi/2} = 20$ ns and 512 data points were collected. A four-step phase cycle was used to remove unwanted echoes. The ESEEM traces were recorded for three different τ values (100, 160 and 200 ns) and the FT spectra were summed to avoid distortions due to blind spots. The experimental data were processed with a home-written Matlab routine, the time traces were baseline corrected with a stretched exponential, apodised with a Hamming window and zero-filled to 2048 data points. The frequency spectrum was calculated using the cross-term averaging procedure implemented in EasySpin [23, 87].

X-band HYSCORE experiments were performed using the pulse sequence $\frac{\pi}{2} - \tau - \frac{\pi}{2} - t_1 - \pi - t_2 - \frac{\pi}{2} - \tau - \text{echo}$ with mw pulses of lengths of $t_{\pi/2} = 16$ ns, $t_{\pi} = 16$ ns, $\tau = 120, 160$ and 200 ns and starting times

$t_1 = t_2 = 64$ ns with increments $dt = 16$ ns (data matrix 256×256). ^{14}N matched HYSCORE experiments were performed with matched pulses instead of the second and last pulse of the standard HYSCORE sequence. The matched pulses were applied at high microwave power and the pulse lengths were optimised for maximum modulation depth in three-pulse ESEEM experiments with varying pulse lengths (optimum length = 24 ns). Q-band HYSCORE was performed with pulse lengths of $t_{\pi/2} = 30$ ns, $t_{\pi} = 30$ ns, $\tau = 100$ and 212 ns, and starting times $t_1 = t_2 = 64$ ns with increments $dt = 16$ ns (data matrix 256×256). ^{14}N matched HYSCORE was performed at the Z^- field position with 74 ns matched pulses and 8 ns increments; τ values of 100 and 160 ns were used. A four-step phase cycle was used to remove unwanted echoes. The experimental data were processed in Matlab; the time traces were baseline corrected with a 3rd order polynomial, apodised with a Hamming window and zero-filled to 1024 points in each dimension. A 2D Fourier transform was applied and the absolute-value spectrum was calculated.

Q-band ENDOR was performed using the Davies sequence for the porphyrin monomer and the Mims sequence for the porphyrin dimer at the field positions corresponding to the out-of-plane orientation (1233.2 mT and 1235.0 mT, respectively). Davies ENDOR was performed with the $\pi - T - \frac{\pi}{2} - \tau - \pi - \tau - echo$ sequence with an 88 ns inversion pulse, a 16 ns - 32 ns detection sequence and a radiofrequency pulse of 40 μs applied during time T . Mims ENDOR was performed with the pulse sequence $\frac{\pi}{2} - \tau - \frac{\pi}{2} - T - \frac{\pi}{2} - \tau - echo$ with 20 ns microwave pulses and a 40 μs radiofrequency pulse applied during the delay T . Spectra were recorded with τ values of 100, 176, 252 and 328 ns and summed to avoid distortions due to blind spots.

ESE-detected kinetics were recorded using a two-pulse detection sequence after a variable delay after flash (DAF): flash - DAF - $\frac{\pi}{2} - \tau - \pi - \tau - echo$ with a $\frac{\pi}{2}$ pulse of 16 ns and a τ value of 120 ns.

4.2.4 SPECTRAL ANALYSIS

The spin-polarised triplet state spectra were simulated using EasySpin's *pepper* routine [23]. The zero-field splitting parameters D and E as well as the relative population probabilities at zero-field were determined by least-square fitting of the experimental time-resolved EPR data. The analysis was performed for an ordering of the triplet sublevels as $|Z\rangle > |Y\rangle > |X\rangle$ (see section 1.2.5).

The relative orientations of the zero-field splitting tensors and the optical transition dipole moments were determined based on the polarisation index calculated from the magnetophotoselection data. Magnetophotoselection is based on photoselection with polarised light and on selection of different orientations of the molecule in the magnetic field in different regions of the EPR spectrum (a detailed discussion can be found in section 4.3.2). The polarisation ratio is defined as [130, 131]:

$$P_i = \frac{I_i^{\parallel} - I_i^{\perp}}{I_i^{\parallel} + I_i^{\perp}} \quad (i = X, Y, Z) \quad (4.1)$$

where $I_i^{\parallel/\perp}$ are the intensities of the derivative EPR signal for excitation with light polarised parallel or perpendicular to the magnetic field at the field positions corresponding to the X, Y or Z orientation

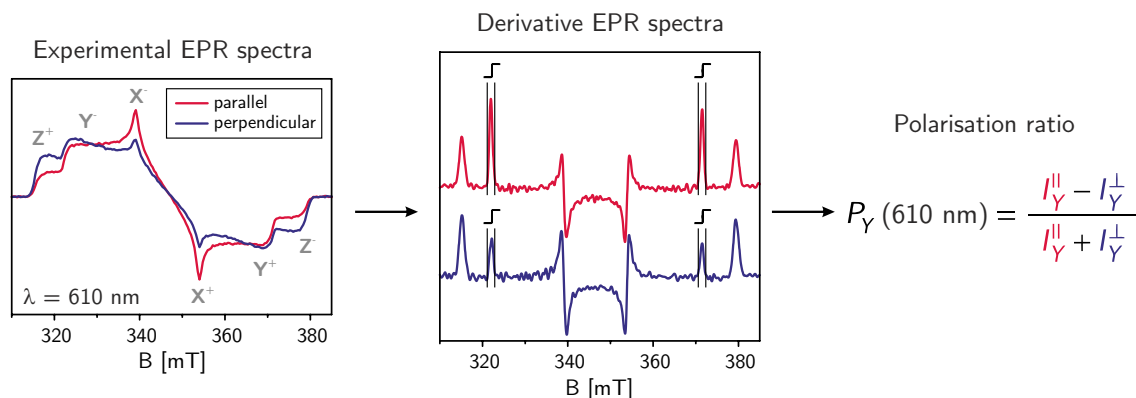


FIGURE 4.1: Schematic representation of the method used for the calculation of the polarisation ratio P_Y for the canonical Y transition (see equation 4.1). The derivative of the experimental EPR spectrum is calculated and integrated over the regions corresponding to the Y canonical orientations to give $I_Y^{\parallel}$ and $I_Y^{\perp}$.

of the ZFS tensor. The polarisation index was calculated for different wavelengths by integration of the low- and high-field canonical regions of the derivative spectra (see figure 4.1); standard deviations were estimated by considering different regions for the integration (derivative signal maximum ± 0.05 - 0.40 mT).

The values of the proton hyperfine couplings along the principal axes of the ZFS tensor were determined by Gaussian deconvolution of the ENDOR spectra. The deconvolutions were performed simultaneously for the low- and high-field canonical positions with the same hyperfine couplings and FWHMs.

The nitrogen hyperfine and nuclear quadrupole parameters were determined through simultaneous least-square fitting of the X- and Q-band three-pulse ESEEM (both time and frequency domain data), Q-band ENDOR and X- and Q-band HYSCORE data with a home-written Matlab routine. The ESEEM simulations were performed using EasySpin's *saffron* function [23, 24] to calculate the time-domain data, which were then treated in the same way as the experimental data to obtain the frequency domain ESEEM spectra. In analogy to the experiments, simulations were performed for three τ values and the corresponding FT spectra were summed. The ^{14}N ENDOR spectra were also simulated with EasySpin [23], using the *salt* function for Davies and the *saffron* function for Mims ENDOR. The relative intensities of the Davies ENDOR peaks were only correctly reproduced if hyperfine enhancement was also considered in the simulations. Since complete calculations of the HYSCORE spectra including intensities are very time consuming, just the cross-peak positions were calculated using a modified version of a Matlab routine written by Dr. Jeffrey Harmer, based on the exact diagonalisation of the complete Hamiltonian to determine the nuclear frequencies for every orientation of the magnetic field. I extended the program to triplet states by including the zero-field splitting interaction and by implementing the transition selection required for these systems.

The optimisation of the ^{14}N hyperfine and nuclear quadrupole parameters was performed through Marquardt-Newton-Gauss least-square fitting. The fitting routine simultaneously optimised the agreement with the X- and Q-band time and frequency domain three-pulse ESEEM data, the Q-band ENDOR spectrum and the X- and Q-band HYSCORE spectra. The residuals for the HYSCORE data were calculated using a method proposed by Dr. Jeffrey Harmer. The HYSCORE data were defined by an array of pairs of cross-peak frequencies determined by overlaying a grid on the experimental HYSCORE spectrum and selecting grid points corresponding to HYSCORE intensities above a certain cutoff value determined by inspection. The grid points assigned to double-quantum features were also saved separately to provide stronger constraints for these features, containing the most precise information on the hyperfine couplings. The unique pairs of cross-peak frequencies calculated for a certain set of hyperfine and nuclear quadrupole parameters were also saved in an array. The program then loops through the set of calculated frequency pairs for each of the experimental pairs of frequencies to find the closest match, which is then used to compute the residual.

The weighting of the different types of datasets used for the fitting was adjusted by normalisation of the root-mean-square deviations with respect to the initial simulation parameter set. A larger weighting was used for the ENDOR data and HYSCORE double-quantum cross-peak data (six and five times larger, respectively), as they contain the most precise information on the hyperfine and nuclear quadrupole parameters.

A distribution of hyperfine and nuclear quadrupole parameters was used in the simulations to reproduce the features observed in the experimental ENDOR and HYSCORE data (see discussion in section 4.3.4). A Gaussian weighting function centred on the mean value of each of the parameters and with a full width at half maximum of 2.5 MHz was used. Skewed weighting functions did not correctly reproduce the features observed in the experimental data, i.e. a broad peak in the ENDOR spectrum and ridges in the HYSCORE spectra. The centres and widths of the distributions of the three principal values of the hyperfine tensor and of the nuclear quadrupole parameters Q and η were optimised in the fitting routine. The orientations of the hyperfine and nuclear quadrupole tensors with respect to the zero-field splitting tensor were kept fixed at the values obtained from DFT calculations, which are in agreement with previous findings on similar porphyrin systems [132]. The error on the simulation parameters was evaluated by performing a grid search in the proximity of the optimised values and considering the root-mean-square deviation values as well as assessing the goodness of fit by visual inspection for the simulations with the lowest RMSD values.

4.2.5 COMPUTATIONAL METHODS

The ground-state optimised geometries described in the previous chapter were used as a starting point for triplet state geometry optimisations performed in ORCA [91] with the BP86 functional and the SV(P) basis set, using the RI (resolution of identity) approximation with the auxiliary SV/J basis set.

The THS groups were replaced by hydrogen atoms and the resulting structures were optimised without symmetry constraints. Calculations of the zero-field splitting interaction were performed according to the procedure published by Sinnecker et al. [5], using the B3LYP functional and the EPRII basis set [92] and calculating the spin-spin contribution to the ZFS using computed UNO (spin-unrestricted natural orbital) determinants. The results were compared to calculations with the BP86 and B3LYP functional to evaluate the extent of the self-interaction error and no significant difference was found. The spin-orbit part of the zero-field splitting interaction was initially also calculated, but the resulting contribution to the D -value was overestimated by orders of magnitude due to the difficulty in obtaining accurate results in spin-orbit calculations, hence only the spin-spin contributions were considered.

Time-dependent DFT calculations were performed on I -P1 to determine the nature of the first excited triplet state. The calculations were again performed with the B3LYP, BP86 and B3LYP functionals and the def2-TZVP and def2-TZVP/C basis sets [89] with the RI approximation. The calculations were performed with the Tamm-Dancoff approximation (TDA) [133]. In all cases the first excited triplet state was predicted to arise mainly from the HOMO $\rightarrow$ LUMO electronic transition (85 to 100%).

The hyperfine and nuclear quadrupole interaction parameters were calculated with the B3LYP functional and the EPRII basis set, purposely developed for the calculation of EPR hyperfine interaction values, for the C, N and H nuclei [92–94], and the 6-31G(d) basis set for Zn.

The effect of a coordinating pyridine molecule on the geometry of the porphyrin ring in P1 was investigated by performing a relaxed surface scan with fixed distances between the zinc ion of the porphyrin and the nitrogen atom of the pyridine molecule ranging from 2 to 4.4 nm with 0.2 nm increments. The hyperfine and nuclear quadrupole interaction parameters were calculated for each of the structures.

4.3 RESULTS AND DISCUSSION

4.3.1 TIME-RESOLVED EPR

The time-resolved EPR spectra of the porphyrin monomer (I -P1) and dimer (I -P2) obtained as an average of the transients up to 2 μ s after the laser flash are shown in figure 4.2. The spectra were recorded at 20 K, where the triplet state has a lifetime of the order of milliseconds. The zero-field splitting parameters and the relative sublevel populations, giving rise to the strongly spin polarised spectral shapes, were obtained by simulation and the results are summarised in table 4.1.

The zero-field splitting parameters determined for the porphyrin monomer are in agreement with the results of previous studies on similar molecules [126, 134]. The D -value of molecules with oblate symmetry such as porphyrins is typically positive and the Z -axis of the ZFS tensor corresponds to the out-of-plane orientation [135]. This has been confirmed for porphyrin monomer investigated here by magnetophotoselection experiments, which will be discussed below. The EPR spectrum is characterised by an $AAAE$ spin polarisation, indicating preferential population of the out-of-plane (Z)

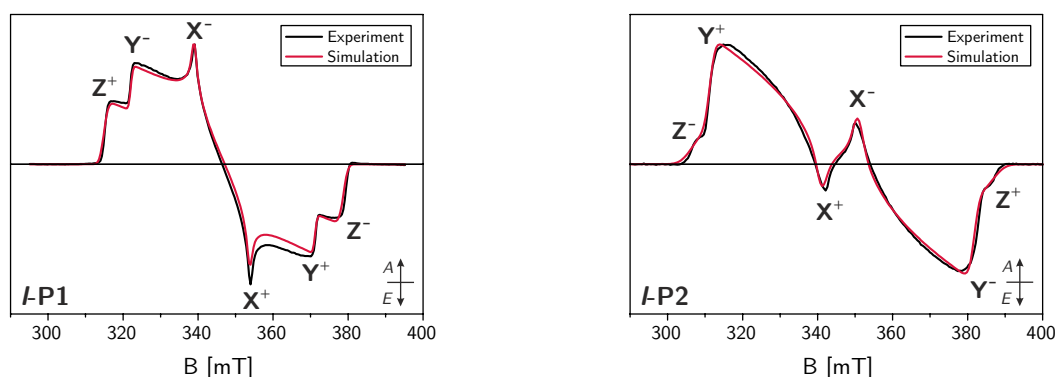


FIGURE 4.2: Experimental X-band time-resolved EPR spectra of the porphyrin monomer *l*-P1 and dimer *l*-P2 in MeTHF:pyridine 10:1 recorded as an average up to 2 μ s after the laser pulse at 20 K. Simulations with the parameters reported in table 4.1 are compared to the experimental data. The ordering of the triplet sublevels was chosen as $|Z\rangle > |Y\rangle > |X\rangle$ and the six canonical positions are indicated (A = absorption, E = emission).

triplet state sublevel. It is known from the literature that in free base porphyrin intersystem crossing (ISC) leads to preferential population of the in-plane triplet sublevels, while in the presence of zinc, the ISC mechanism changes and leads to an almost exclusive population of the out-of-plane sublevel [136–138]. The rates of triplet state sublevel population, as well as decay, are determined by two contributions, a Franck-Condon overlap integral, which is the same for all triplet state sublevels, and an electronic matrix element of the spin-orbit coupling Hamiltonian, which is characteristic for each sublevel and determines the selective populations of the triplet state sublevels [137, 139]. In free base porphyrins, as in aromatic hydrocarbons, the ISC mechanism is governed by Herzberg-Teller vibronic coupling and is selective for the in-plane sublevels, for which $\sigma\pi^*$ and $\pi\sigma^*$ states are admixed to the $\pi\pi^*$ states due to out-of-plane vibrations [137, 139]. In the presence of zinc, an additional direct coupling matrix element arises from the mixing of the Zn *d*-orbitals with the π system of the porphyrin [136] and determines the selective population of the out-of-plane sublevel.

The *D*-value of the porphyrin dimer is significantly larger than the value determined for the porphyrin monomer, in contrast to what would be expected in case of more extensive delocalisation due to the r^{-3} dependence (see section 1.2.5). The *E/D* ratio has also increased, indicating increased in-plane asymmetry. The spin polarisation of the porphyrin dimer spectrum is *AAEAE*E and is characteristic of

TABLE 4.1: Zero-field splitting parameters and relative zero-field sublevel populations for *l*-P1 and linear oligomers *l*-P2 to *l*-P6 determined through simulation of the time-resolved EPR spectra recorded after excitation at 532 nm and shown in figures 4.2 and 4.3.

	$ D $ [MHz]	$ E $ [MHz]	<i>E/D</i>	$P_X : P_Y : P_Z$
<i>l</i> -P1	898 ± 5	161 ± 2	0.18	0.00 : 0.05 : 0.95
<i>l</i> -P2	1117 ± 9	284 ± 2	0.25	0.00 : 0.90 : 0.10
<i>l</i> -P3	1169 ± 7	269 ± 2	0.23	0.00 : 0.53 : 0.47
<i>l</i> -P4	1195 ± 8	273 ± 2	0.23	0.00 : 0.47 : 0.53
<i>l</i> -P5	1201 ± 8	254 ± 2	0.21	0.00 : 0.24 : 0.76
<i>l</i> -P6	1199 ± 9	260 ± 3	0.21	0.00 : 0.26 : 0.74

a preferential population of the Y triplet sublevel. A potential explanation for the observed changes is a transition from an oblate to a prolate spin distribution, occurring due to delocalisation of the triplet state over both porphyrin units in the dimer. The occurrence of such a transition has been proposed before for molecular wire-type systems based on a variation of the E/D ratio as a function of the size of the system [113, 125, 128]. This would lead to a change of the sign of the D -value and a reorientation of the ZFS tensor with the axis of maximum dipolar coupling Z now lying in-plane along the long axis of the molecule. Since the magnitude of the D -value of a triplet state with a prolate spin distribution is twice as large as that of an oblate spin distribution for the same average inter-electron separation, this would explain the observed increase in D . The change of the out-of-plane sublevel from Z in the monomer to Y in the dimer would also explain the observed spin polarisation, assuming the ISC is still promoted by the zinc spin-orbit coupling.

The time-resolved EPR spectra of the porphyrin trimer (l -P3), tetramer (l -P4), pentamer (l -P5) and hexamer (l -P6) obtained as an average of the transients up to $2 \mu\text{s}$ after the laser flash are shown in figure 4.3. The zero-field splitting parameters and the relative sublevel populations are also summarised in table 4.1. The triplet yields of l -P1 and l -P2 are high and thus strong EPR signals are observed, but the triplet yields of the larger linear and cyclic systems decrease significantly [140],

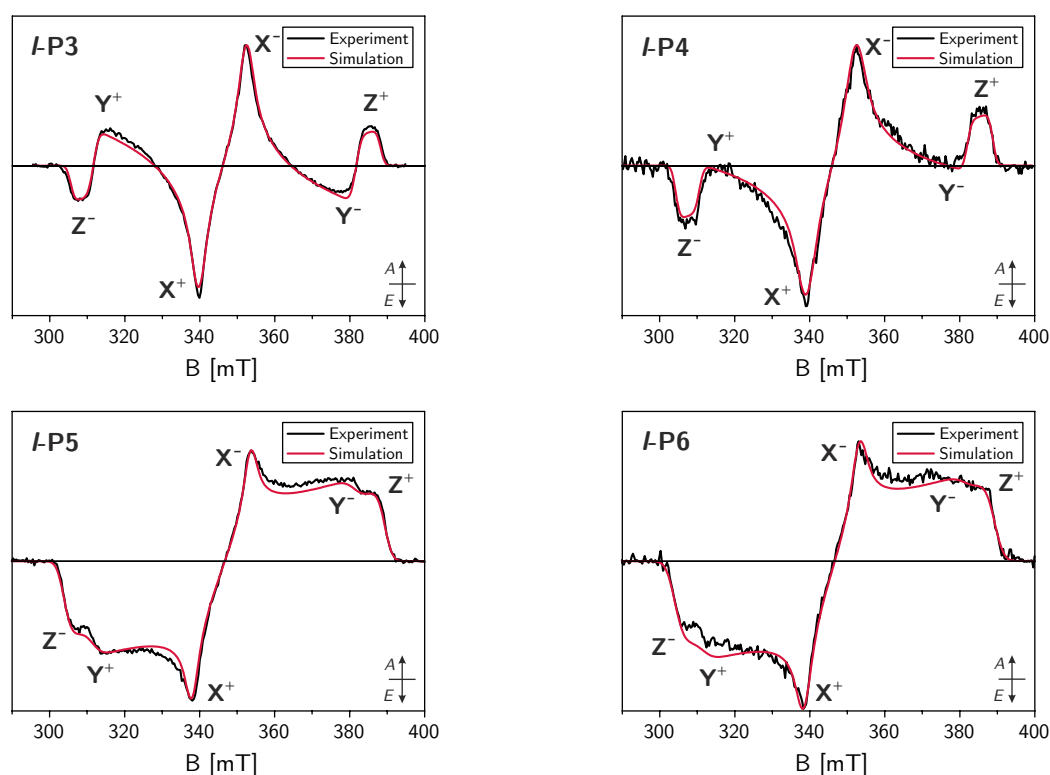


FIGURE 4.3: Experimental X-band time-resolved EPR spectra of the porphyrin trimer (l -P3), tetramer (l -P4), pentamer (l -P5) and hexamer (l -P6) in MeTHF:pyridine 10:1 recorded as average up to $2 \mu\text{s}$ after the laser pulse at 20 K. Simulations with the parameters reported in table 4.1 are compared to the experimental data. The ordering of the triplet sublevels was chosen as $|Z\rangle > |Y\rangle > |X\rangle$ and the six canonical positions are indicated (A = absorption, E = emission).

complicating the EPR measurements considerably. The lower triplet yields have been attributed to faster radiative and non-radiative decay of the first excited singlet states in the longer oligomers.

The D -values of the longer oligomers are similar to those of the porphyrin dimer with only slight increases of 5-7%. The E -values are also very similar, indicating a similar degree of asymmetry in the plane perpendicular to the Z axis of the ZFS tensor. The spin polarisations do, however, change significantly; in l -P3 the spin polarisation is characteristic of almost equal sublevel populations for the Y (out-of-plane) and Z (long axis) sublevels and for the longer oligomers the relative population of the out-of-plane sublevel decreases further, while it increases accordingly for the long axis Z sublevel.

The zero-field splitting parameter D has often been used to estimate the extent of triplet state delocalisation by exploiting a description of the ZFS interaction based on the point-dipole approximation [113, 124–128]. The calculation of the average inter-electron distance from the experimental D -values yields estimates of about 3.5 Å for l -P1 and about 4.1 Å for l -P2 to l -P6 (the *meso*-to-*meso* distance of the porphyrin amounts to about 6.9 Å). This would lead to the conclusion that the triplet state is localised on a single porphyrin unit. A study on nitroxide biradicals by Riplinger et al. [129] investigated the limitations of the point-dipole approximation in the description of electron-electron dipole couplings and came to the conclusion that it is not applicable in case of extensive delocalisation and unsaturated linkers. Distributed point-dipole approximation approaches or quantum mechanical calculations were suggested as better methods for the interpretation of zero-field splitting parameters in such systems.

The calculation of ZFS parameters for the triplet states of aromatic molecules with a distributed point-dipole approximation, as proposed by Visser and Groenen [141], is based on the assumption that the triplet wavefunction can be described as a linear combination of the atomic p -orbitals, modelled by two half-point spins separated by 61 pm from the nucleus on both sides of the porphyrin plane. The ZFS D -value can then be calculated as a sum of two-centre terms with weights given by the coefficients of the molecular orbitals occupied by the two unpaired triplet spins. This method has been further refined and modified to include spin polarisation effects for the study of fullerene triplet states by van Gastel [142, 143]. The D -values calculated using this method for the porphyrin monomer and dimer with the Mulliken spin populations of the HOMO and LUMO of the corresponding ground states, obtained by DFT calculations performed in ORCA [91], correctly reproduce the change in sign and the increase in magnitude from l -P1 to l -P2, but the predicted values deviate quite significantly from the experimental results.

The ZFS interaction parameters of l -P1 and l -P2 were also calculated by DFT, as described in section 4.2.5. Based on the results of time-dependent DFT calculations performed for l -P1, which assigned the first excited triplet state mainly to the HOMO $\rightarrow$ LUMO transition, the ZFS interaction was calculated for this electron configuration. The D - and E -values calculated for l -P1 at the B3LYP/EPRII level are 492 MHz and 120 MHz, for l -P2 they are equal to -609 MHz and -72 MHz. DFT correctly predicts the change in sign and orientation of the ZFS tensor and the calculated E/D ratio is relatively close to the experimental one. The absolute values, however, differ quite significantly, by almost a factor of two. The reasonable agreement between the calculated and experimental hyperfine couplings

(discussed below) provides a validation of the DFT wavefunction; hence the observed disagreement seems to be related to the calculation of the ZFS interaction. A similar discrepancy between experimental and calculated D -values was observed for a series of polyacenes [5, 144] and was attributed to the limitation of DFT to predict ZFS interaction parameters for large aromatic systems accurately. Better agreement was in that case obtained using CASSCF (Complete Active Space Self-Consistent Field) calculations with the inclusion of static π -electron correlation. Given the multi-configurational nature of porphyrins, a description at CASSCF level would certainly be more accurate, however, currently the computational cost for performing this type of calculation for the systems investigated here is significant and seems unwarranted given that DFT still correctly predicts the relative changes in ZFS parameters: experimentally an increase of the magnitude of D by 26% from l -P1 to l -P2 is observed and DFT predicts an increase of 24 %.

For the longer oligomers, DFT predicts uneven spin density distributions with increased spin density on the central porphyrin units (see figure 4.4) and the D -values decrease slightly with respect to l -P2, e.g. for l -P3 and l -P4 the D -values predicted with different functionals correspond to 75-86% of the l -P2 D -value.

The changes of the relative sublevel populations from l -P2 to l -P6 indicate a difference in the mechanism of triplet state formation, leading to increased population of the Z in-plane sublevel corresponding to the tensor axis collinear with the long axis of the porphyrin arrays. Possible explanations for the change in selectivity of triplet state sublevel populations would be a change in the ISC mechanism or triplet state formation through either intra- or intermolecular triplet-triplet energy transfer. The spin polarisation of a triplet state arising from triplet-triplet energy transfer can be predicted based on the sublevel populations of the donor triplet state, P_i^D ($i = X, Y, Z$), and the relative orientation of donor and acceptor due to the conservation of spin angular momentum [145–147]. The

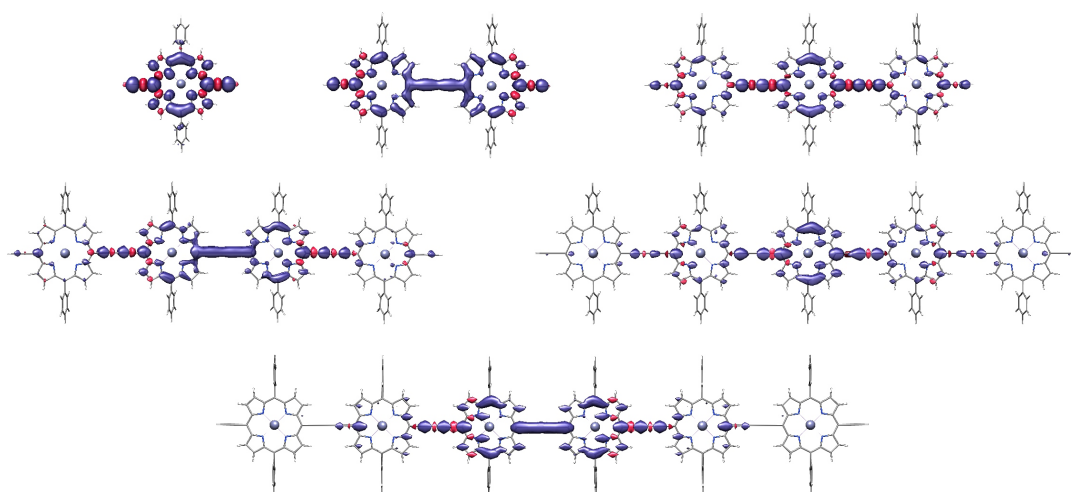


FIGURE 4.4: Spin density distributions in the first excited triplet state calculated with B3LYP/EPRII for the optimised geometries of l -P1 to l -P6.

sublevel populations of the acceptor, P_i^A ($i = X, Y, Z$), can be calculated as [145, 146]:

$$P_i^A = \sum_j \cos^2 \theta_{ij} P_j^D \quad (4.2)$$

where θ_{ij} is the angle between the principal axis j of the zero-field splitting tensor of the donor and the zero-field splitting axis i of the acceptor. The observed spin polarisations in *l*-P3 to *l*-P6 could not be reproduced by considering intramolecular energy transfer between adjacent porphyrin units at varying angles with respect to each other or intermolecular energy transfer between stacked porphyrin oligomers.

In terms of ISC, an alternative mechanism driving population mainly into the sublevel corresponding to the long axis of the molecule and becoming more dominant as the oligomer length increases, would explain the observed spin polarisations. In order to verify the likeliness of this hypothesis, time-resolved EPR measurements were performed on the free base oligomers, where ISC is not affected by the direct spin-orbit coupling contribution of the zinc ion. Magnetophotoselection and ENDOR experiments were performed on the free base porphyrins to determine whether the same changes as

TABLE 4.2: Zero-field splitting parameters and relative sublevel populations of the zinc and free base porphyrins determined through simulation of the time-resolved EPR spectra recorded at wavelengths corresponding to the planar conformations (645, 750, 800 and 830 nm for the zinc porphyrins and 680, 740, 780 and 810 nm for the free base porphyrins).

	Zinc porphyrin			Free base porphyrin		
	D [MHz]	E [MHz]	$P_X : P_Y : P_Z$	D [MHz]	E [MHz]	$P_X : P_Y : P_Z$
<i>l</i> -P1	898 ± 5	161 ± 2	0.00 : 0.05 : 0.95	1024 ± 3	144 ± 2	0.53 : 0.47 : 0.00
<i>l</i> -P2	1117 ± 9	284 ± 2	0.00 : 0.88 : 0.12	1053 ± 3	311 ± 4	0.29 : 0.00 : 0.71
<i>l</i> -P3	1169 ± 7	269 ± 2	0.00 : 0.46 : 0.54	1087 ± 3	321 ± 2	0.08 : 0.00 : 0.92
<i>l</i> -P4	1195 ± 8	273 ± 2	0.00 : 0.28 : 0.72	1116 ± 6	308 ± 5	0.04 : 0.00 : 0.96

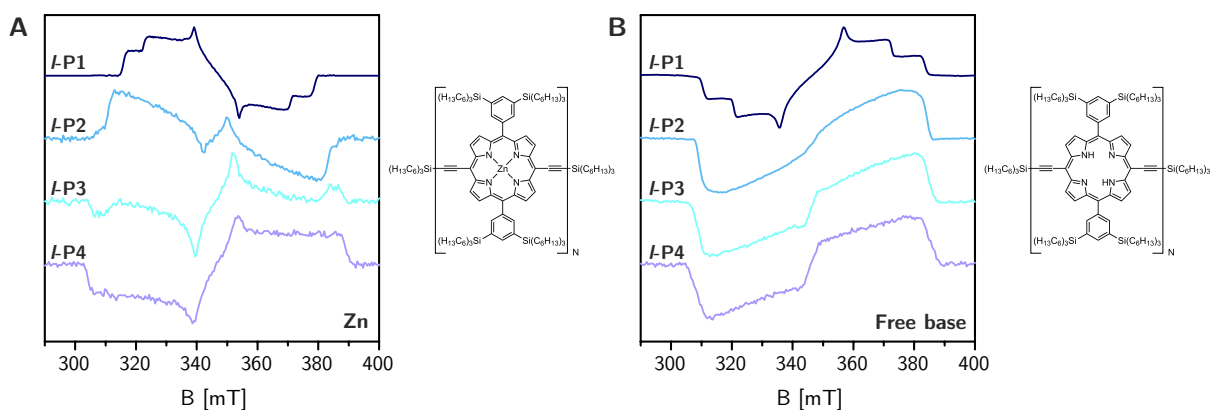


FIGURE 4.5: Experimental X-band time-resolved EPR spectra of the zinc (A) and free base (B) porphyrins with one to four units in MeTHF:pyridine 10:1 recorded as average up to 2 μ s after the laser pulse at 20 K. The spectra were recorded after excitation at wavelengths corresponding to the planar conformations (645, 750, 800 and 830 nm for the zinc porphyrins and 680, 740, 780 and 810 nm for the free base porphyrins).

observed for the zinc porphyrins occur as a function of the number of porphyrin units and are summarised in appendix A. As will be discussed in section 4.3.5, different conformations contribute to the EPR spectrum for excitation at 532 nm, therefore the spectra recorded after excitation at wavelengths selecting the planar conformations are considered here. The time-resolved EPR spectra recorded for the zinc and free base porphyrin oligomers with one to four units are compared in figure 4.5 and the relative sublevel populations determined through simulation are reported in table 4.2.

The spin polarisation of the free base monomer corresponds to almost equal population of the two in-plane sublevels, as reported in the literature for other free base porphyrins, and is characteristic of ISC mediated by vibronic coupling [137, 148]. Magnetophotoselection and ENDOR measurements reported in appendix A confirm that an analogous change from an oblate to a prolate spin distribution from the free base monomer to the longer oligomers, as proposed for the zinc porphyrin systems, occurs. The progressive increase of the long axis sublevel population from the free base monomer to the oligomers reflects the analogous increase observed for the zinc porphyrins and supports the hypothesis of a competing ISC mechanism (see figure 4.6). The sublevel populations of the zinc porphyrins can be calculated as linear combinations of the free base populations, arising from the vibronic contribution of the porphyrin ring to ISC, and the theoretical populations obtained assuming the direct zinc spin-orbit coupling to be the only populating mechanism and leading to population of only the out-of-plane sublevel ($P_Z = 1, P_X, P_Y = 0$ for *l*-P1 and $P_Y = 1, P_X, P_Z = 0$ for the longer oligomers). The relative contribution of the two mechanisms determined by least-square fitting is reported in figure 4.6 C.

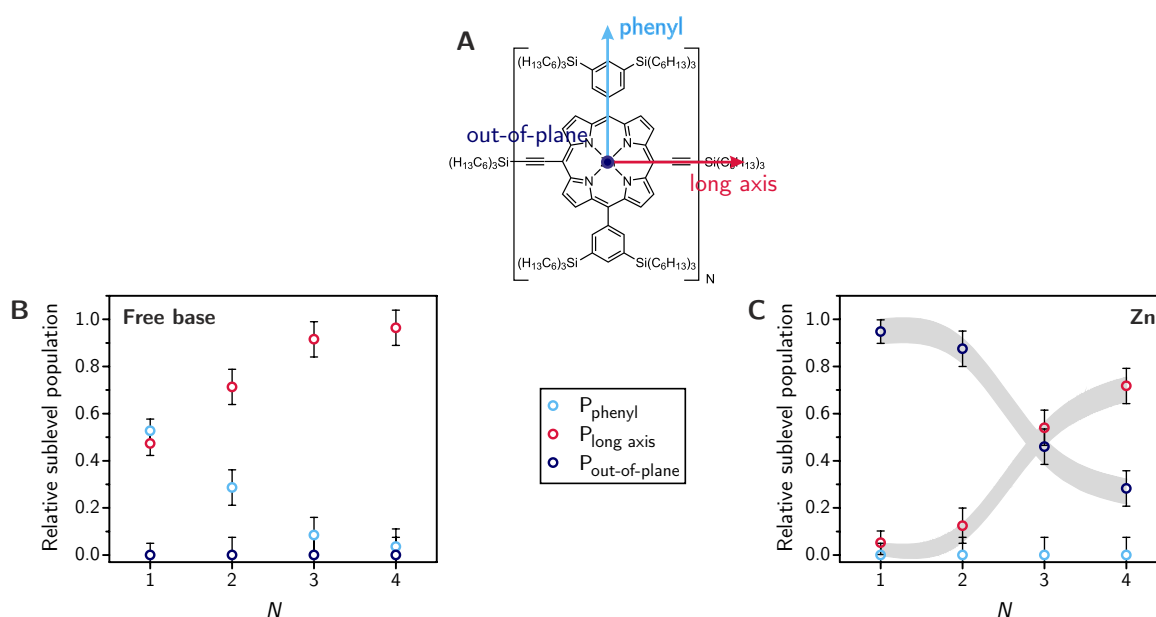


FIGURE 4.6: Relative triplet state sublevel populations for the free base (B) and zinc porphyrins (C) determined from the experimental EPR data as a function of the number N of porphyrin units. The populations correspond to the three principal axes of the zero-field splitting tensor: out-of-plane, along the phenyl rings and along the long axis of the molecule as shown in (A). The shaded area in the graph for the zinc porphyrins represents the populations calculated as linear combinations of the free base populations, originating from the vibronic spin-orbit coupling mechanism, and the populations predicted for the direct zinc spin-orbit coupling mechanism. The contribution of the vibronic mechanism to the ISC would correspond to 0.05, 0.13, 0.56 and 0.73 for *l*-P1, *l*-P2, *l*-P3 and *l*-P4, respectively.

The contribution of the porphin ring to the ISC populating mechanism is promoted by admixture of $n\pi^*$ and $\sigma\pi^*$ character to the $\pi\pi^*$ triplet state induced by out-of-plane vibrations [137, 149]. The increasing vibrational freedom of longer porphyrin oligomers could thus explain the increasing importance of this vibronic spin-orbit coupling contribution to the ISC mechanism and why it seems to be favoured over the direct zinc spin-orbit coupling contribution in the zinc porphyrins.

The spin polarisation of the triplet state EPR spectra of the porphyrin oligomers was found to also depend on the solvent system as shown in figure 4.7. These results show that the population of the out-of-plane sublevel with respect to the long axis sublevel is very sensitive to the porphyrin environment. Excitation wavelength-dependent measurements in MeTHF and *o*-terphenyl on *l*-P3 have shown that the difference persists at wavelengths corresponding to the planar conformations (see section 4.3.5), ruling out the contribution of different conformations as the principal cause for the observed changes. If the spin polarisation of the longer oligomers is determined by vibronic spin-orbit coupling, changes in solvation and zinc coordination could influence the relative importance of the two ISC mechanisms and explain the sensitivity of the relative sublevel populations to changes in experimental conditions. However other factors cannot be excluded and most likely a combination of solvent interactions, solvent-dependent spectral shifts, potential aggregation or long-range order, in addition to changes in the ISC mechanism, contribute to the observed solvent dependence.

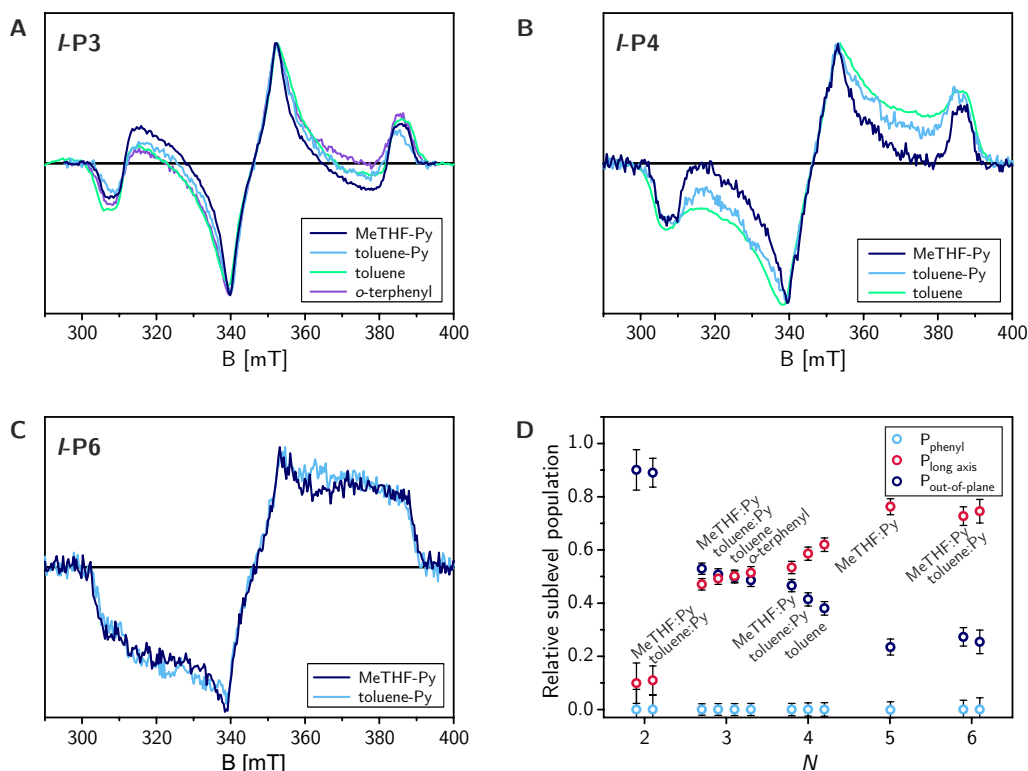


FIGURE 4.7: Time-resolved EPR spectra recorded at 20 K after excitation at 532 nm for *l*-P3 (A), *l*-P4 (B) and *l*-P6 (C) in different solvent mixtures. The corresponding relative sublevel populations determined through simulation are shown in (D).

4.3.2 MAGNETOPHOTOSELECTION EXPERIMENTS

In the discussion of the time-resolved EPR spectra so far it has been assumed that the D -value is positive for the porphyrin monomer and it has been proposed that the sign changes in the longer oligomers due to delocalisation. In order to verify this assumption and to provide clear experimental proof for the proposed reorientation of the zero-field splitting tensor, magnetophotoselection experiments were performed.

Magnetophotoselection combines magnetoselection provided by the orientational selectivity in EPR and photoselection using linearly polarised light in order to determine the relative orientation of the principal axes of the ZFS tensor and the optical transition moments [130, 131, 150, 151]. Light polarised parallel or perpendicular to the magnetic field excites a distribution of molecular orientations with a probability of $\cos^2 \theta$, where θ is the angle between the axis of the optical transition moment and the light polarisation vector. The triplet state EPR spectrum then shows intensity enhancement at magnetic field values corresponding to the excited orientations of the molecule with respect to the magnetic field vector as illustrated in figure 4.8. For example, assuming that for the porphyrin monomer the optical transition moment Q_y is aligned with the Y axis of the ZFS tensor, excitation with light polarised parallel to the magnetic field will select orientations of the molecule with the triplet Y axis aligned with the magnetic field (see figure 4.8, left). This leads to enhancement of the Y transitions compared to the EPR spectrum recorded with depolarised light. Excitation with light

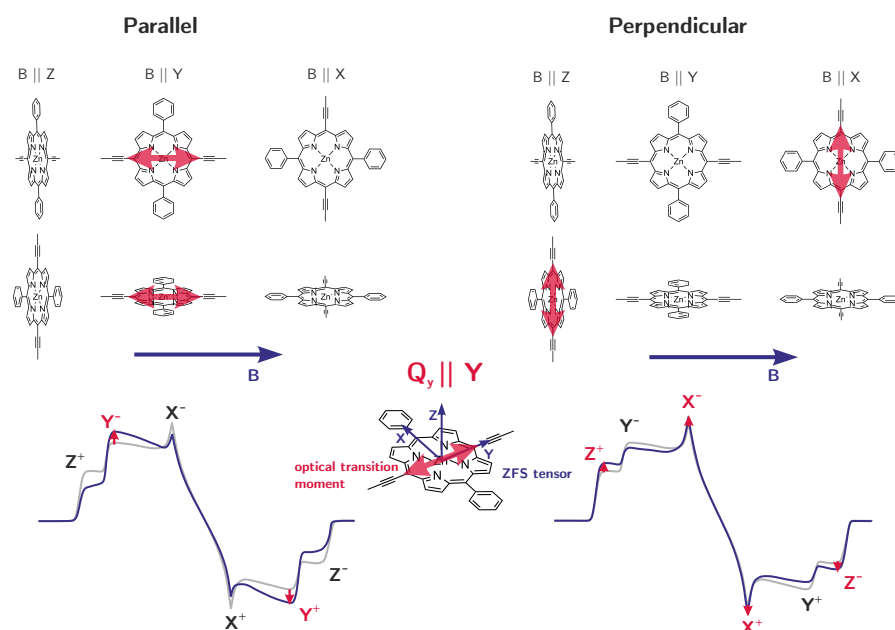


FIGURE 4.8: Schematic illustration of the principle of magnetophotoselection for the porphyrin monomer excited at a wavelength corresponding to the Q_y optical transition for the relative orientation of the ZFS tensor (X, Y, Z) and the optical transition moment indicated in the inset. Linearly polarised light only excites a subset of orientations as indicated by the arrows for light polarised parallel or perpendicular to the magnetic field. This leads to a relative enhancement of the corresponding lines in the EPR spectrum and attenuation of the lines belonging to the other orientations with respect to the depolarised spectrum (shown in grey).

polarised perpendicular to the magnetic field selects different orientations of the molecule (see figure 4.8, right), with the triplet X or Z axes aligned with the field. Hence the corresponding transitions in the EPR spectrum will be enhanced, while the Y transition, corresponding to orientations not selected with the perpendicularly polarised light, will be attenuated. A comparison of the two EPR spectra shows that in case of alignment of the optical transition moment with a triplet axis, the corresponding transitions are expected to be enhanced for excitation with light polarised parallel to the field and attenuated for excitation with light polarised perpendicular to it.

In order to determine the relative orientation of the ZFS tensor and the optical transition moments, a polarisation index P_i is defined for each canonical orientation i ($i = X, Y, Z$) as described in equation 4.3 [130].

$$P_i = \frac{I_i^{\parallel} - I_i^{\perp}}{I_i^{\parallel} + I_i^{\perp}} = \frac{3r_i - 1}{1 + \frac{4}{3}k + r_i} \quad (4.3)$$

where $I_i^{\parallel/\perp}$ are the intensities of the derivative EPR signal for excitation with light polarised parallel or perpendicular to the magnetic field, r_i is the component of the polarisation of the optical transition moment along the i th axis of the ZFS tensor and k is a constant introduced to include the percentage of depolarised light present or other depolarising effects [130]. In principle the exact angle between each ZFS tensor axis and the optical transition moment could be determined based on equation 4.3, however uncertainty in the value of the constant k and difficulties in exciting a single optical transition often prevent this. Nevertheless, some simple rules based on this equation have been devised for aromatic molecules, which typically have optical transition moments lying in the plane of the molecule (XY plane) [130, 150]. In that case, three situations can be distinguished [150]:

- $P_j > 0$ and $P_{i \neq j} < 0$: The ZFS axis j lies within $0 - 35^\circ$ of the optical transition moment.
- $P_j > 0$, $P_i > 0$ and $P_Z < 0$: The ZFS axis j lies within $35 - 55^\circ$ of the optical transition moment (and the axis i within $125 - 145^\circ$).
- $P_j < 0$, $P_i > 0$ and $P_Z < 0$: The ZFS axis j lies within $55 - 90^\circ$ of the optical transition moment.

The UV-Vis spectra of the porphyrin monomer and oligomers are shown in figure 4.9. The UV-Vis spectra of porphyrins are characterised by two groups of absorption bands, the B or Soret bands around 450 nm and the Q bands at higher wavelengths. The appearance of porphyrin UV-Vis spectra was first explained by a four-orbital model by Gouterman [152, 153]. The HOMO and HOMO-1 (a_{1u} and a_{2u} for D_{4h} symmetry) as well as the LUMO and LUMO+1 (e_g for D_{4h} symmetry) levels are usually degenerate, or close to degenerate, depending on the symmetry of the system. Due to the near degeneracy of the configurations arising from the transitions between these orbitals, strong configuration interaction occurs. This causes constructive and destructive combinations of the transition dipoles giving rise to the strong allowed B bands and the weak forbidden Q bands, respectively.

The UV-Vis spectra of the longer oligomers show a red-shift, broadening and intensification of the Q band absorption and a broadening of the B band absorption. The broadening has been attributed to the contribution of different conformations based on Kasha's model for exciton coupling [62]. The

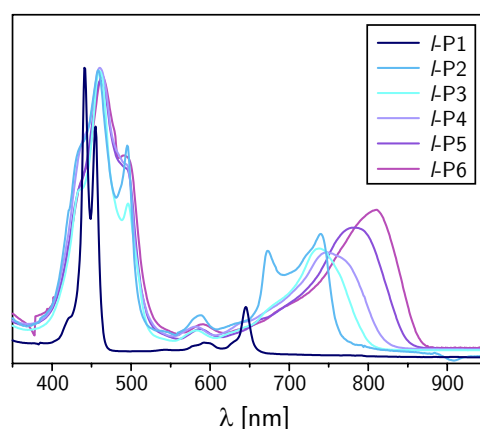


FIGURE 4.9: Room temperature UV-Vis spectra of *l*-P1, *l*-P2, *l*-P3, *l*-P4, *l*-P5 and *l*-P6 in MeTHF:pyridine 10:1.

red-shift and the intensification of the Q band has been attributed to the increased conjugation length in the oligomer systems [154, 155]. In addition to this, due to changes in energy and symmetry of the frontier orbitals, the Q band transitions are not forbidden or quasi-forbidden anymore [155].

The time-resolved EPR spectra recorded after excitation with linearly polarised light at wavelengths corresponding to both the Q_x and Q_y transitions for *l*-P1 and *l*-P2 and only to the Q_y transition for *l*-P3 and *l*-P4 are shown in figure 4.10. As previously discussed, for optical transition moments lying within $0-35^\circ$ of a triplet axis, the EPR intensity of the corresponding triplet transition will be enhanced for excitation with light polarised parallel to the field and attenuated for light polarised perpendicular to it.

This qualitative rule indicates that in *l*-P1 the triplet Y axis is approximately aligned with the optical Q_y transition ($\lambda = 645$ nm) and the triplet X axis is approximately aligned with the optical Q_x transition ($\lambda = 600$ nm). This identifies the X and Y axes of the ZFS tensor as the in-plane axes and hence confirms the assumption of a positive D -value with the out-of-plane axis as the axis of maximum dipolar coupling, Z. The results of a more quantitative analysis are reported in figure 4.11 A, where the polarisation indices obtained from equation 4.3 for the three canonical orientations of the ZFS tensor are plotted as a function of excitation wavelength. In the wavelength region corresponding to the Q_y transition, the polarisation indices P_y are positive, while the other two are negative, indicating that the triplet Y axis lies within $0-35^\circ$ of the Q_y transition moment. The assignment is less clear in the wavelength region corresponding to the Q_x transition. However, the presence of a vibronic progression for the Q_y transition causes an overlap of the absorption bands of the two Q transitions, which probably explains the positive values of P_y . The polarisation indices P_z are negative in the whole wavelength region, indicating that no optical transition moment is aligned with the triplet Z axis.

For the longer oligomers, excitation at a wavelength corresponding to the maximum of the Q_y transition (long wavelength Q transition) always leads to enhanced intensity of only the Z EPR transitions for excitation with light polarised parallel to the magnetic field, indicating that the corresponding optical transition moment is approximately aligned with the triplet Z axis. Since even in the larger systems the optical transition moments are confined to the plane of the porphyrin molecule, this

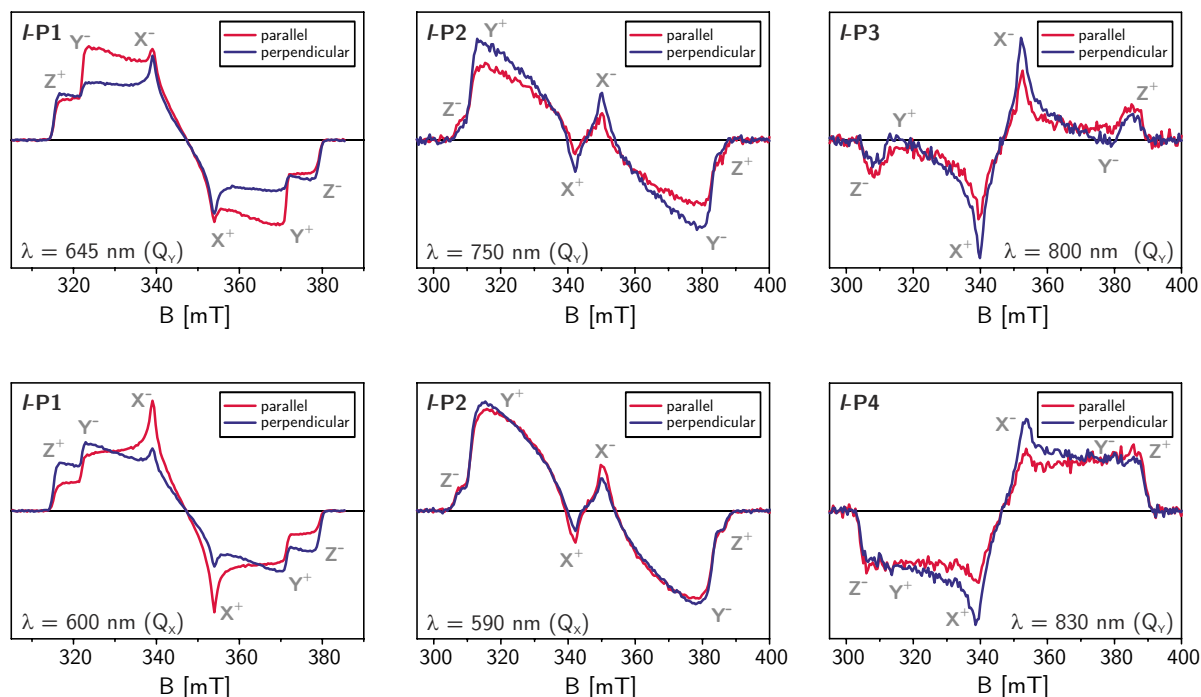


FIGURE 4.10: Time-resolved EPR spectra of *l*-P1, *l*-P2, *l*-P3 and *l*-P4 recorded with light polarised parallel and perpendicular to the magnetic field. The excitation wavelengths are indicated and assigned to the Q_x or Q_y optical transitions.

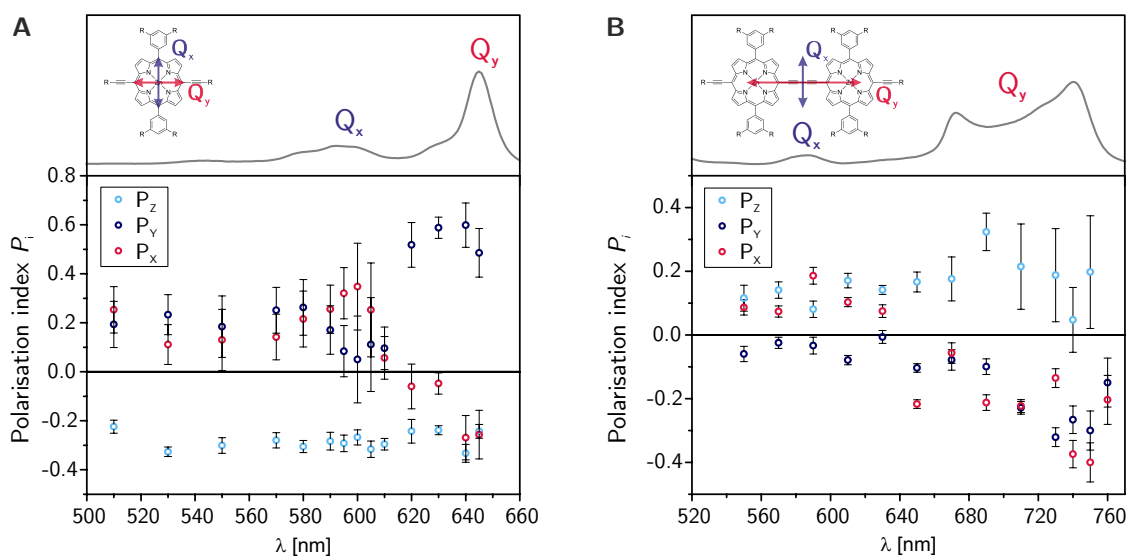


FIGURE 4.11: Polarisation indices for the three canonical orientations calculated for *l*-P1 (A) and *l*-P2 (B) as a function of excitation wavelength in the Q band region of the porphyrin absorption spectrum. The corresponding regions of the room temperature UV-Vis spectra are shown above the graphs and the orientations of the optical transition moments in the molecular frame are depicted in the insets. The polarisation indices were calculated as described in section 4.2.4.

demonstrates that the Z axis now lies in the plane of the porphyrin. Optical studies on the porphyrin oligomers investigated here have shown the Q_y transition to be long-axis polarised [155]. The results of the magnetophotoselection studies therefore provide the first experimental evidence for a reorientation of the ZFS tensor and a sign change of D from the porphyrin monomer to longer oligomers.

The porphyrin dimer *l*-P2 has also been investigated in more detail across the whole region of the Q bands and the results are shown in figure 4.11 B. The polarisation index P_z is positive over the whole investigated wavelength region, clearly identifying the triplet Z axis as the long axis of the dimer molecule. The polarisation index P_x becomes positive at wavelengths around 600 nm, the position of the Q_x absorption band, indicating alignment of the triplet X axis and the Q_x transition moment. In analogy to the results for *l*-P1, overlap of different absorption bands leads to a residual positive value for P_z at this wavelength. The polarisation indices P_y are negative over the whole Q band region, identifying Y as the new out-of-plane axis of the ZFS tensor, in agreement with the assignment made in the previous section based on the spin polarisation of the EPR spectrum.

4.3.3 CHARACTERISATION OF THE PROTON HYPERFINE INTERACTION

The zero-field splitting parameters determined from the time-resolved EPR spectra do not provide a sufficiently accurate estimate of the extent of triplet state delocalisation. Information on the delocalisation of the spin density in the photoexcited triplet state can also be obtained from the hyperfine couplings, therefore the proton hyperfine couplings were investigated using ENDOR spectroscopy. In addition to this, the nitrogen hyperfine and nuclear quadrupole couplings were also determined using a combination of X- and Q-band ENDOR, ESEEM and HYSCORE and will be discussed in the next section.

^1H HYPERFINE COUPLINGS IN *l*-P1

The ^1H Mims ENDOR spectra recorded for *l*-P1 at the six canonical field positions are shown in figure 4.12 C. As the D -value for *l*-P1 is known to be positive from magnetophotoselection, the sign of the proton hyperfine couplings can be assigned and is indicated above the corresponding peaks in figure 4.12 C. The values of the hyperfine couplings along the different axes of the ZFS tensor were determined through deconvolution as described in section 4.2.4 and the results are shown in the frequency-field plot in figure 4.12 D. A negative hyperfine coupling, of about -3 MHz, is observed at the field positions corresponding to the X and Z orientations, while only smaller couplings are observed for the Y orientation. The peak at -3 MHz in the ENDOR spectrum recorded at the high-field Y position arises from the Z transition, which still contributes significantly at this field position, as can be seen in figure 4.12 A.

A comparison with the results of a DFT calculation on the excited triplet state of *l*-P1 allowed assignment of the hyperfine couplings to specific protons and determination of the orientation of the ZFS tensor in the molecular frame. DFT calculations predict a large negative hyperfine coupling for the β protons (H_1) close to the acetylene bonds in the out-of-plane direction and in the in-plane direction

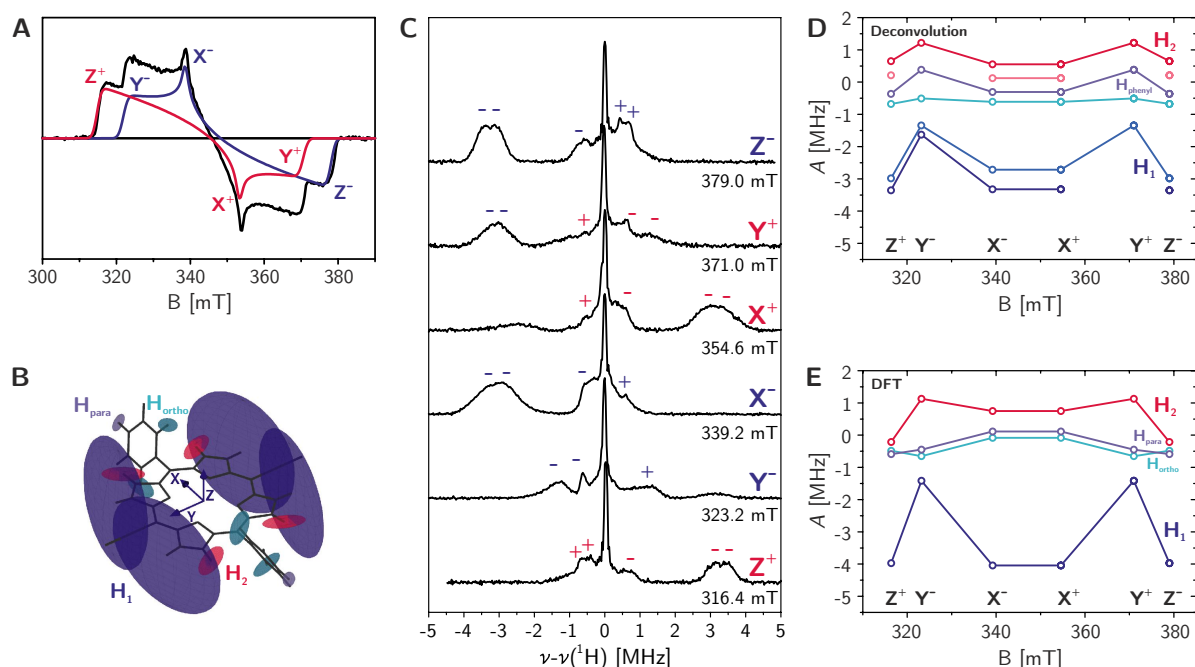


FIGURE 4.12: (A) Experimental time-resolved *l*-P1 EPR spectrum (black) and simulations for the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions. (B) Proton hyperfine tensor ellipsoids calculated for the porphyrin monomer using B3LYP/EPRII and orientation of the ZFS tensor in the molecular frame. (C) Experimental Mims ENDOR spectra of the porphyrin monomer recorded at the canonical field positions. The signs of the hyperfine coupling constants of the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions are shown above the corresponding ENDOR peaks. (D) Field-frequency plots for the peak positions obtained by deconvolution of the experimental ENDOR spectra as described in section 4.2.4. The hyperfine couplings were tentatively assigned to different types of protons based on comparison with DFT calculations. (E) Plot of the hyperfine couplings along the ZFS tensor axes as predicted by B3LYP/EPRII calculations on *l*-P1.

aligned with the phenyl substituents and a smaller coupling in the direction perpendicular to it (see figure 4.12 B). The excellent agreement of the trends in the changes of proton hyperfine couplings predicted by DFT and determined experimentally, which can be seen by comparison of the frequency-field plots in figure 4.12 D and E, confirms assignment of the Z axis as the out-of-plane axis and allows assignment of the X axis as the in-plane axis directed along the phenyl substituents and of the Y axis as the second in-plane axis perpendicular to it, directed along the acetylene bonds. Further comparison of the experimental and calculated results allows assignment of the positive hyperfine couplings to the second type of β proton of the porphyrin ring (H_2). The small hyperfine couplings close to the strong nuclear Larmor peak are not sufficiently well resolved for an assignment to the different types of phenyl protons.

While DFT predicts a single hyperfine coupling for the two types of protons on the porphyrin ring, experimentally the peaks are found to be split into two, as can be seen most clearly in the ENDOR spectra corresponding to the Z transitions. A similar behaviour has been observed before in free base porphyrin, where it has been attributed to specific solvent-solute interactions which render these protons inequivalent [156].

^1H HYPERFINE COUPLINGS IN *l*-P2

Analogous ENDOR measurements were performed for *l*-P2 and the results of the experiments and the analysis are shown in figure 4.13. In figure 4.13 C the ENDOR spectra of *l*-P2 are compared to those of *l*-P1 recorded at similar magnetic fields. A comparison of the two sets of spectra leads to the following observations:

- The largest hyperfine coupling for *l*-P2 amounts to about 1.5 MHz, while it was about 3 MHz for *l*-P1.
- The peaks corresponding to the largest hyperfine coupling are observed for the Y and X transition in *l*-P2, while they correspond to the Z and X orientations in *l*-P1.
- The peaks observed at comparable field positions (e.g. the high-field X position) are on opposite sides of the nuclear Larmor frequency.

The reduction of the hyperfine couplings by a factor of about two from *l*-P1 to *l*-P2 indicates delocalisation of the triplet state over both porphyrin units in *l*-P2. The observation of a shift of the peaks

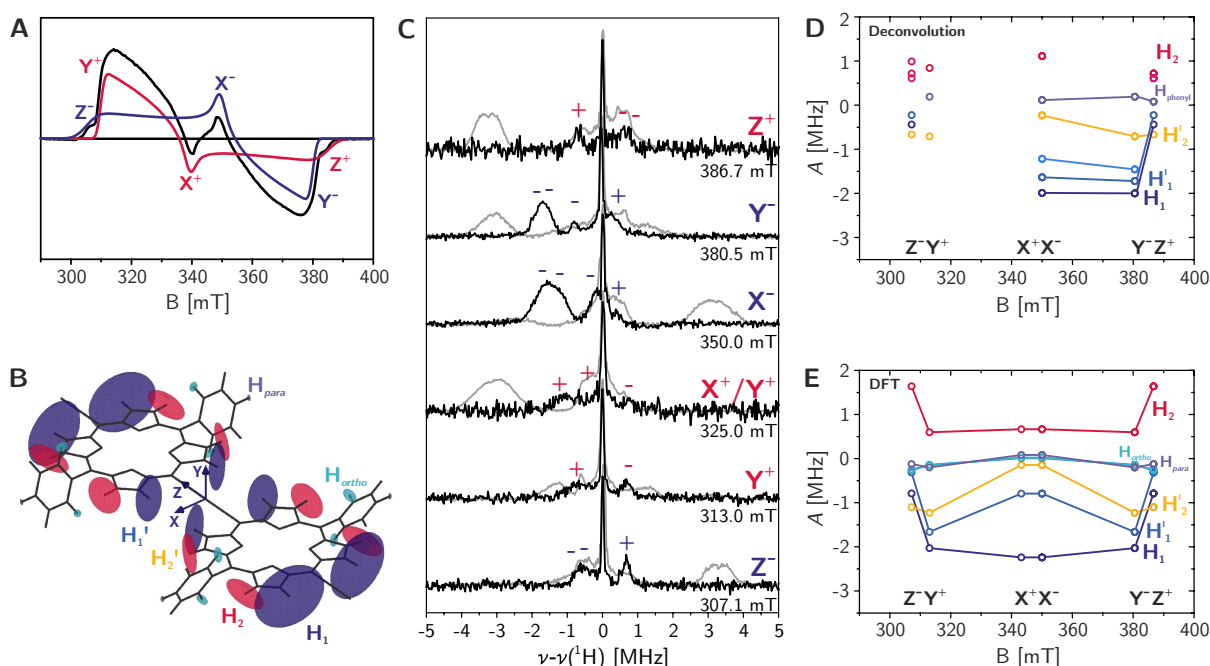


FIGURE 4.13: (A) Experimental time-resolved *l*-P2 EPR spectrum (black) and simulations for the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions. (B) Proton hyperfine tensor ellipsoids calculated for the porphyrin dimer using B3LYP/EPRII and orientation of the ZFS tensor in the molecular frame. (C) Experimental Mims ENDOR spectra of the porphyrin dimer recorded at the canonical field positions. The signs of the hyperfine coupling constants of the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions are shown above the corresponding ENDOR peaks. The ENDOR spectra of the porphyrin monomer from figure 4.12 are shown in the background for comparison. (D) Field-frequency plots for the peak positions obtained by deconvolution of the experimental ENDOR spectra as described in section 4.2.4. The hyperfine couplings were tentatively assigned to different types of protons based on comparison with DFT calculations. Exact assignments in the low-field part are unclear. (E) Plot of the hyperfine couplings along the ZFS tensor axes as predicted by B3LYP/EPRII calculations on *l*-P2.

corresponding to the largest proton couplings from Z and X to Y and X is in agreement with a reorientation of the ZFS tensor in *l*-P2 already predicted based on the results of the magnetophotoselection experiments. The ENDOR data allow the assignment of the Y triplet axis as the new out-of-plane axis, while Z becomes the axis aligned with the central butadiyne bond in *l*-P2. The change in the position of the ENDOR peaks with respect to the Larmor frequency demonstrates that the reorientation of the ZFS tensor occurs with a change of the sign of the *D*-value, which must thus be negative for *l*-P2. Comparison of the hyperfine couplings along the ZFS axes determined by deconvolution of the experimental spectra with the results of DFT calculations again show a good agreement in the relative changes of hyperfine couplings as a function of field (figure 4.13 D and E). The widths of the ENDOR peaks suggest the presence of multiple similar hyperfine couplings, which is a sign of a reduction of symmetry for the molecule in solution, analogous to the results for *l*-P1.

¹H HYPERFINE COUPLINGS IN *l*-P3

The ENDOR spectra recorded at three field positions corresponding to the three principal directions of the ZFS tensor for *l*-P1, *l*-P2 and *l*-P3 are compared in figure 4.14. The spin polarisation of the spectrum and the decreasing triplet yield for *l*-P3 prevented measurements with sufficient signal-to-noise for all six canonical field positions. The ENDOR spectra of the porphyrin trimer are similar to those of the dimer, as they both have the largest hyperfine couplings in the X and Y orientations and as the peaks appear at the same side of the Larmor peak. These similarities indicate that the orientation of the ZFS tensor is the same in *l*-P2 and *l*-P3, with the Z axis parallel to the vector connecting the porphyrin units and a negative *D*-value. The positions of the major ENDOR peaks are however shifted towards larger hyperfine couplings in the porphyrin trimer with respect to the dimer. The hyperfine couplings along the ZFS tensor axes were determined by deconvolution and are shown in the frequency-field plot in figure 4.14 C. The results are in good agreement with the hyperfine couplings predicted by DFT (see figure 4.14 B and D).

The DFT calculations for the porphyrin trimer predict an uneven spin density distribution with increased spin density on the central porphyrin unit. The predicted ratio of spin densities on the three porphyrin units depends on the amount of exact Hartree-Fock exchange included in the DFT functional. A calculation with the GGA (generalised gradient approximation) functional BP86 predicts a spin density distribution of 0.26 : 0.48 : 0.26 over the three porphyrin units, while the hybrid functional B3LYP, containing 20% of exact exchange and 80% of DFT exchange, predicts a distribution of 0.19 : 0.62 : 0.19. Variation of the contribution of exact exchange in the B3LYP functional in the range from 10% to 40% leads to variations of the relative spin density on the central porphyrin unit from 0.54 to 0.74. The best agreement of calculated and experimental hyperfine couplings is obtained for 20% exact exchange, corresponding to the standard B3LYP functional. This amount of exact exchange almost corresponds to the optimal mixing ratio of exact and DFT exchange believed to yield results close to chemical accuracy (25%) [157].

The uneven spin density distribution explains the increase in hyperfine couplings with respect to the porphyrin dimer, where the spin density is equally distributed over both porphyrin units. The ratio of

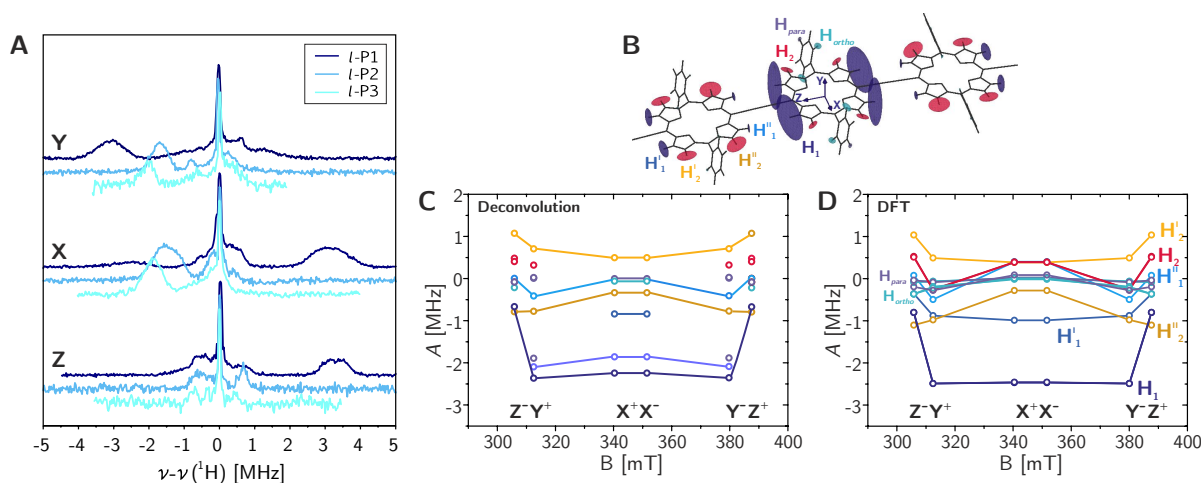


FIGURE 4.14: (A) Experimental Mims ENDOR spectra of *l*-P1, *l*-P2 and *l*-P3 recorded at field positions corresponding to the three canonical orientations of the ZFS tensor (Y^+ , X^+ and Z^+ for *l*-P1 and Y^- , X^- and Z^- for *l*-P2 and *l*-P3). (B) Proton hyperfine tensor ellipsoids calculated for the porphyrin trimer using B3LYP/EPRII and orientation of the ZFS tensor in the molecular frame. (C) Field-frequency plots for the peak positions obtained by deconvolution of the experimental ENDOR spectra as described in section 4.2.4. The data points at the low-field Y and high-field Z positions were added by symmetry as the corresponding experimental spectra could not be recorded with sufficient signal-to-noise to be used for the deconvolution analysis. (D) Plot of the hyperfine couplings along the ZFS tensor axes as predicted by B3LYP/EPRII calculations on *l*-P3.

spin densities on the three porphyrin units can also be estimated by comparison of the largest hyperfine coupling in *l*-P3, corresponding to the H_1 protons on the central porphyrin unit, with the hyperfine coupling of the same protons in the porphyrin monomer. A comparison of the spin density distribution for *l*-P1 and on the central porphyrin unit in *l*-P3 (see figure 4.4) shows that the differences are small and thus a comparison of the hyperfine couplings is possible. The ratio of the *l*-P3 and *l*-P1 hyperfine couplings is approximately 0.60, predicting a spin density distribution of 0.20 : 0.60 : 0.20, close to the results of the DFT calculation. The H_1 protons on the external rings would then give a hyperfine coupling of about -0.63 MHz in the X orientation and there is a broad peak at that position in the experimental spectrum. Overlap with other small hyperfine couplings prevents a definite assignment and experimental confirmation of the proposed uneven spin density distribution.

¹H HYPERFINE COUPLINGS IN THE LONGER OLIGOMERS

The analysis of the proton hyperfine couplings in the longer oligomers has been limited to the ENDOR spectra recorded at the high-field X position, the position of the highest echo intensity; the corresponding experimental data are shown in figure 4.15 A. The hyperfine couplings determined from the position of the main hyperfine peak in the ENDOR spectra are plotted as a function of oligomer size in figure 4.15 B and compared to the theoretically predicted hyperfine couplings for complete delocalisation.

The hyperfine coupling determined experimentally for *l*-P2 corresponds exactly to the predicted value, since the spin density is equally distributed over both porphyrin units in the system. Deviations from the theoretical prediction do, however, occur for the longer oligomers: as previously discussed, the

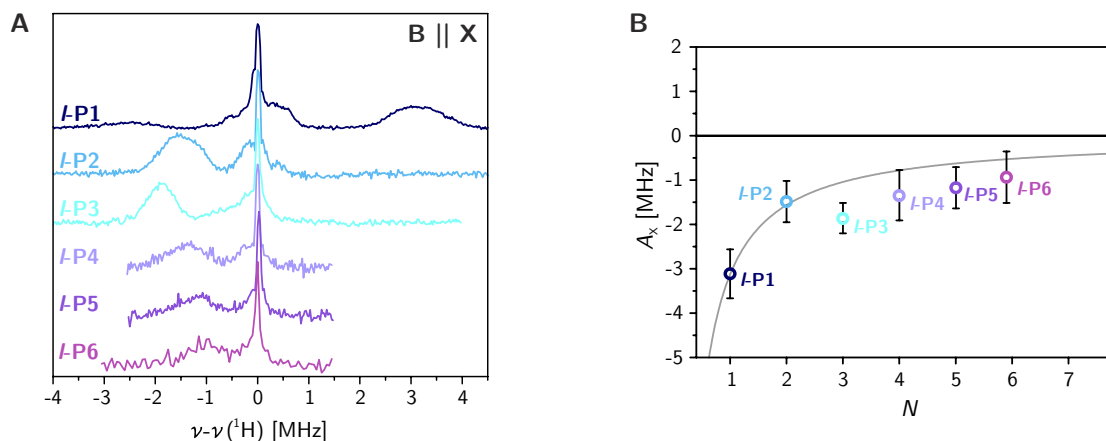


FIGURE 4.15: (A) Experimental Mims ENDOR spectra of *l*-P1 to *l*-P6 recorded at the high-field X position at 20 K. (B) Hyperfine couplings of the H_1 protons along the X axis of the ZFS tensor (A_x) determined by Gaussian fitting of the principal hyperfine peak in the experimental ENDOR spectra as a function of oligomer size; the error bars indicate the full width at half maximum (FWHM). The grey line corresponds to the theoretical N^{-1} relationship for the hyperfine couplings in case of complete delocalisation.

hyperfine coupling of *l*-P3 is 1.25 times larger than for *l*-P2, the hyperfine couplings of *l*-P4, *l*-P5 and *l*-P6 correspond to 0.90, 0.79 and 0.67 times the *l*-P2 hyperfine coupling, respectively. The results for *l*-P3 were interpreted in terms of an uneven spin density distribution and the same argument also explains the changes observed for *l*-P4, *l*-P5 and *l*-P6. DFT also predicts uneven spin density distributions for *l*-P4, *l*-P5 and *l*-P6, with the spin density mainly localised on the two central porphyrin units for even N and on the central porphyrin unit for odd N . The ratios of spin densities resulting from B3LYP/EPRII calculations (20% exact exchange) correspond to 0.10 : 0.40 : 0.40 : 0.10 for *l*-P4, 0.04 : 0.19 : 0.54 : 0.19 : 0.04 for *l*-P5 and 0.02 : 0.10 : 0.38 : 0.38 : 0.10 : 0.02 for *l*-P6. The BP86 functional again predicts different spin density distributions, which are slightly more spread out over the porphyrin array due to the self-interaction error affecting DFT calculations with functionals only containing DFT exchange [158].

A similar procedure as already described for *l*-P3, based on the comparison of hyperfine couplings, has been used to predict the amount of spin density on the central porphyrin units in *l*-P4, *l*-P5 and *l*-P6. For *l*-P5, a relative spin density contribution of 0.39 on the central porphyrin unit is predicted. For *l*-P4 and *l*-P6 the spin density distributions on the two central porphyrin units resemble the dimer spin density more than the monomer spin density, hence the dimer hyperfine couplings have been used for comparison, yielding a relative spin density contribution of 0.45 on the two central porphyrin units in *l*-P4 and of 0.34 in *l*-P6 (the values obtained based on the monomer hyperfine couplings only deviate by 0.02 from the reported values). The hyperfine couplings on the external porphyrin rings are too small to be clearly identified.

The changes in hyperfine couplings in the porphyrin oligomers with three to six units were so far interpreted in terms of uneven spin density distributions. While predictions based on this hypothesis agree reasonably well with the experimental results, other causes cannot be excluded. The possibility

of distributions of different conformations with different extents of triplet state delocalisation giving rise to the observed experimental results were investigated by wavelength-dependent EPR and EN-DOR measurements that will be discussed in section 4.3.5. Overall, the results show an increase in delocalisation with the number of porphyrin units, even though it is slower than the increase expected for complete delocalisation.

4.3.4 CHARACTERISATION OF THE NITROGEN HYPERFINE INTERACTION

The conclusions on the delocalisation of the triplet state wavefunction based on the analysis of the proton hyperfine couplings were verified by measurements of the ^{14}N hyperfine couplings using X- and Q-band ESEEM and HYSCORE as well as Q-band ENDOR. The analysis of the experimental data was complicated by the additional presence of the nuclear quadrupole interaction for this $I = 1$ nucleus.

^{14}N HYPERFINE COUPLINGS IN *l*-P1

The three-pulse ESEEM spectra recorded at the six canonical field positions for *l*-P1 are shown in figure 4.16. The echo is strongly modulated by the nitrogen interaction and significant differences are observed between ESEEM traces recorded at different field positions. The differences in the ESEEM traces for the low- and high-field positions corresponding to the same orientation of the ZFS tensor are due to the contributions of nuclear frequencies from different manifolds. The ESEEM spectra obtained by Fourier transform all show a strong peak between 3 and 4 MHz, which only shifts slightly as a function of field position. A small peak at twice this frequency is also visible in all of the spectra and has been assigned to a combination peak. The weak orientation-dependence of the main peak in the ESEEM spectra identifies it as the double-quantum peak arising from the $m_S = 0$ manifold. For some of the orientations, peaks at smaller frequencies, the sum of which corresponds to the frequency of the double-quantum peak, can be identified and assigned as the single-quantum nuclear

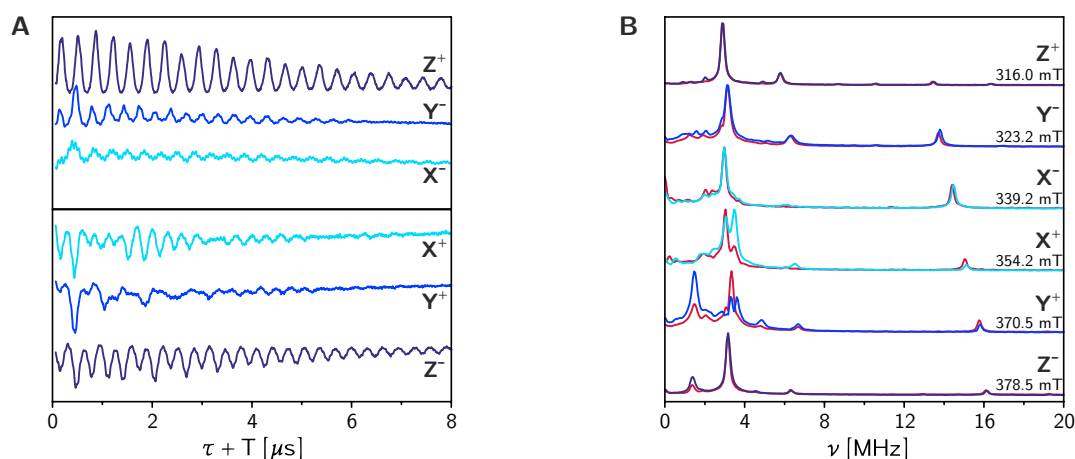


FIGURE 4.16: (A) Experimental X-band three-pulse ESEEM time-domain traces recorded at the six canonical field positions for *l*-P1 in MeTHF:pyridine 10:1 (traces recorded with a τ value of 160 ns are shown). (B) Corresponding ESEEM spectra for the six canonical field positions (sum of FT spectra with $\tau = 120, 160$ and 200 ns). The spectra shown in red were recorded in pure MeTHF.

frequencies of the $m_S = 0$ manifold. The proton matrix peak is also present in all of the recorded spectra.

The spectra corresponding to the X and Y orientations in some cases show two peaks with similar intensities. Most of the pulse experiments on these porphyrin systems were performed in MeTHF with pyridine, as the pyridine binds to the zinc and reduces aggregation. The pyridine nitrogen can give rise to additional peaks and hence the measurements were repeated in a MeTHF solution without pyridine (red spectra in figure 4.16 B). The complete removal of pyridine proved to be difficult as it binds tightly to the porphyrin, but no evidence of remaining pyridine was detected by NMR on the sample used for the final EPR measurements. The changes in the ESEEM spectra without pyridine identify some of the peaks as arising mainly from pyridine nuclear frequencies.

The main signals in the ESEEM spectra seem to arise from the $m_S = 0$ manifold and only contain information on the nuclear quadrupole interaction. Bowman and Norris [159] investigated the ^{14}N nuclear quadrupole interaction in phenazine triplet states by ESEEM and based their analysis on analytical expressions for the nuclear frequencies in the $m_S = 0$ manifold determined using the methods described by Muha [160, 161]. The parameters of the nuclear quadrupole interaction for the porphyrin nitrogen nuclei were estimated by fitting of the analytical expressions to the peak positions of the maxima, resulting in $Q \approx 2.4$ MHz and $\eta \approx 0.9$. The tensor orientations were kept fixed at the values obtained by DFT and shown in figure 4.21. No clear double-quantum peaks from the other two manifolds, which would provide information on the nitrogen hyperfine couplings, could be identified, as they are probably too broad. The cross-suppression effect [47], leading to suppression of shallow modulations in the presence of deep modulations at a different frequency, might also play a role in the difficulty of detecting the double-quantum peaks.

Double-quantum peaks of ^{14}N systems are typically characterised by high intensities in HYSCORE experiments. Further, the lineshapes in three-pulse ESEEM are affected by inhomogeneous hyperfine broadening, which often prevents the detection of broad peaks arising from distributions of hyperfine couplings. The inhomogeneous broadening is refocused by the mixing pulse in the HYSCORE experiment [3], facilitating the detection of broad peaks. The results of HYSCORE experiments on *l*-P1 are shown in figure 4.17.

The strongest cross-peaks observed in the HYSCORE spectra have been attributed to double-quantum cross-peaks. As previously discussed, information on the nitrogen hyperfine couplings can be most easily obtained from the positions of the double-quantum cross-peaks in the HYSCORE spectrum, since they do not depend on the nuclear quadrupole interaction to first order [38]. In almost all of the spectra, strong peaks on the diagonal are observed, which are mostly due to non-ideal mixing pulses. The HYSCORE spectra recorded at field positions corresponding to the two triplet state transitions for the X direction of the ZFS tensor (X^+ and X^-) exhibit strong cross-peaks at about (6.1, 3.2) MHz and (3.2, 6.1) MHz for X^- and cross-peaks very close to the diagonal for X^+ . This agrees with predictions for the appearance of triplet state HYSCORE spectra for $I = 1$ nuclei close to the cancellation regime. The double-quantum cross-peaks for one of the triplet transitions would lie close to the diagonal, as the nuclear frequencies of both manifolds connected by it will be similar, one being only determined

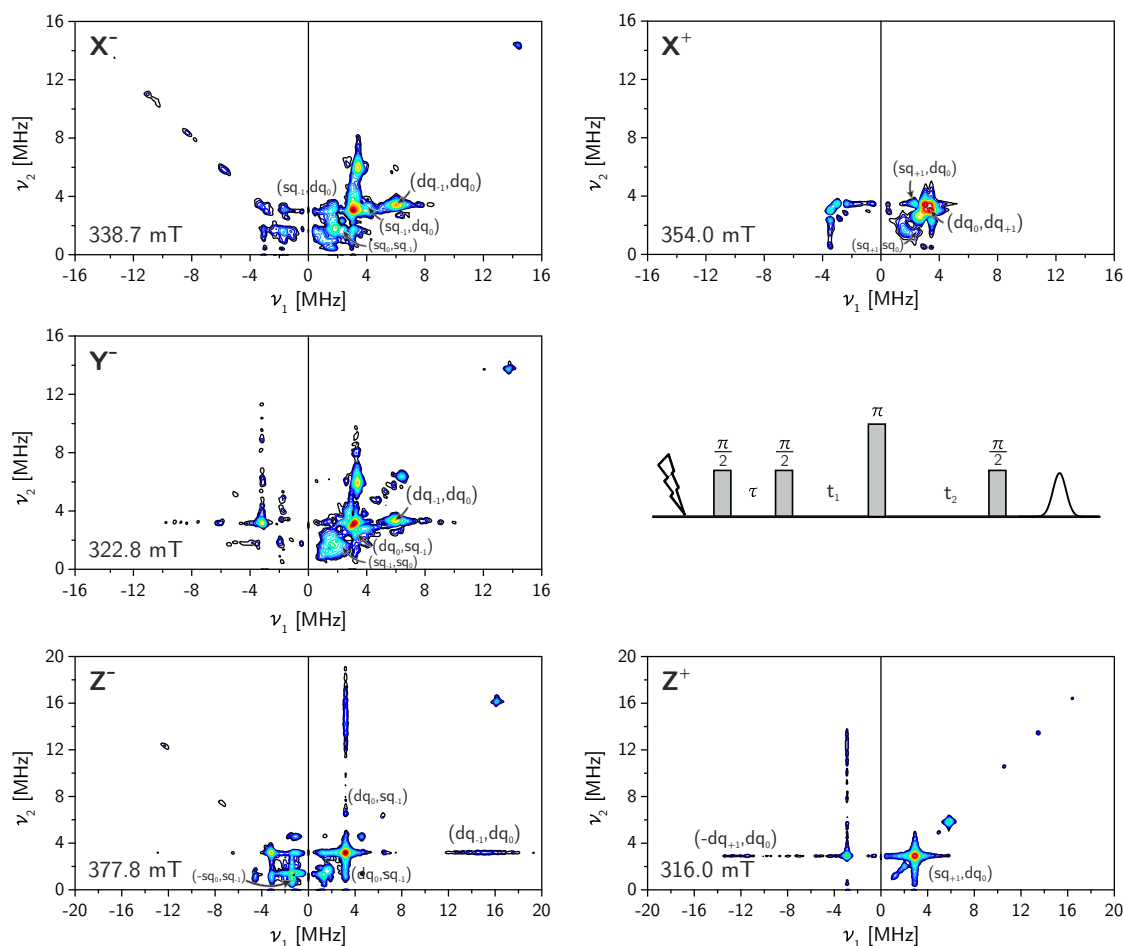


FIGURE 4.17: Experimental X-band HYSORE spectra recorded at 20 K for five canonical field positions for *l*-P1 in MeTHF:pyridine 10:1. The Z^- spectrum was recorded using ^{14}N matched HYSORE. The experimental details are described in section 4.2.3. The assignment of some of the cross-peaks to nuclear frequencies of the three triplet state manifolds is also shown.

by the nuclear quadrupole interaction and one by both the nuclear quadrupole and the nuclear Zeeman interaction. In this regime, the difference between the positions of the two double-quantum peaks in the HYSORE spectrum arising from the other triplet transition corresponds approximately to two times the hyperfine coupling for that orientation. Based on calculations, the error of neglecting the contribution of the nuclear quadrupole coupling amounts to an underestimate of the hyperfine coupling by about 20%. The nitrogen hyperfine coupling in the X direction of the ZFS tensor can thus be estimated to about 1.7 MHz. The sign of the hyperfine coupling is positive, since the larger nuclear frequencies are observed for the X^- transition and the D -value of the porphyrin monomer is positive (see section 2.2.6). The HYSORE spectrum recorded at the Y^- field position is very similar to the HYSORE spectrum of the X^- field position, with double-quantum peaks at approximately the same frequencies. Hence the hyperfine coupling in the second in-plane orientation of the ZFS tensor also amounts to approximately 1.7 MHz.

The HYSORE spectra recorded for the out-of-plane orientation are more difficult to interpret (figure 4.17, bottom). First of all, the double-quantum cross-peaks for the Z^- transition could only be

clearly resolved using ^{14}N matched HYSORE (standard HYSORE data not shown), which leads to an increase in the intensities of multiple quantum features [37]. Secondly, the broad ridges parallel to the frequency axes observed in the HYSORE spectrum for the Z^- transition are unexpected for a triplet state, where single-crystal like spectra are anticipated due to orientation selection, which is particularly strong for the Z orientation. The hyperfine coupling estimated from the centre of these ridges is about 6.5 MHz, which at X-band is far from the cancellation regime, where HYSORE spectra are easier to interpret. The estimated hyperfine couplings indicate an approximately axial hyperfine interaction tensor with a large hyperfine coupling in the out-of-plane orientation, as also previously observed for a free base porphyrin [132] and as predicted by the DFT calculations. In order to confirm the assignment of the broad ridges as double-quantum features and to shed more light on their origin, the HYSORE measurements for the out-of-plane orientation of the ZFS tensor were repeated at Q-band, closer to the cancellation regime, and the resulting spectra are shown in figure 4.18.

At Q-band the broad ridges for the Z^- transition were again only visible using ^{14}N matched HYSORE, but are more clearly defined with respect to the X-band data. For the Z^+ transition, some ridges closer to the diagonal are present, in agreement with the features expected closer to the cancellation regime. The Q-band HYSORE spectra are in agreement with a positive average hyperfine coupling of about 6.5 MHz. This result is also confirmed by Q-band ENDOR (figure 4.22, bottom left), where the single-quantum transitions of the $m_S = 0$ manifold give rise to two sharp lines, while the single quantum transitions of the $m_S = -1$ manifold give rise to a considerably broadened peak centred at about 11 MHz. X-band ^{14}N ENDOR measurements were also attempted, but did not yield sufficiently well-resolved peaks to allow determination of additional information on the hyperfine or nuclear quadrupole couplings.

The ridges in the HYSORE spectra and the broad peak in the ENDOR spectrum can be explained by a distribution of hyperfine couplings. Similar observations were reported for the out-of-plane nitrogen hyperfine couplings of cobalt corrins and also explained by a distribution of hyperfine couplings in this direction attributed to either inequivalence of the ^{14}N nuclei or A-strain [102, 162]. A principal hyperfine tensor axis shifted with respect to the ZFS out-of-plane axis and the conformational flexibility of the porphyrin plane have been investigated as possible origins for the observed distributions. If the out-of-plane hyperfine axis would be rotated away from the Z axis of the ZFS tensor, this would lead

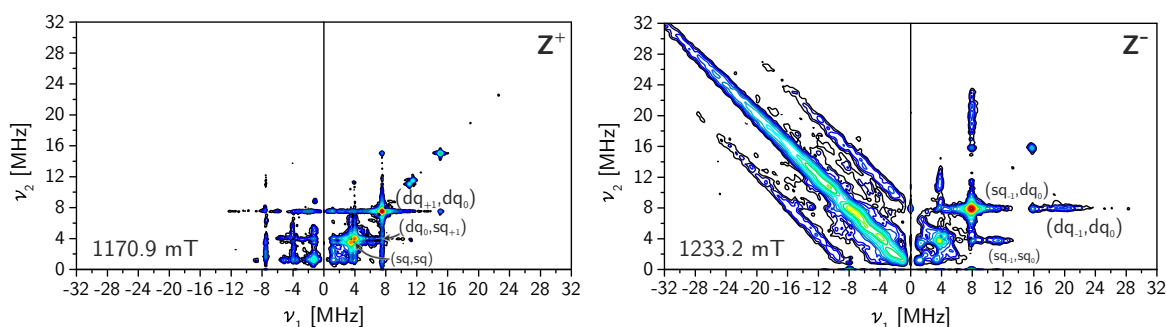


FIGURE 4.18: Experimental Q-band HYSORE spectra for the Z^+ and Z^- field positions for l -P1. The Z^- spectrum was recorded using ^{14}N matched HYSORE. The experimental details are described in section 4.2.3.

to significant changes in hyperfine couplings over a small range of orientations, especially given the pronounced axially of the nitrogen hyperfine tensors. The angle between the out-of-plane hyperfine and ZFS axes is predicted to be 9° by DFT. However much larger deviations, of about 25° would be required to produce ridges similar to those observed experimentally and give a worse agreement of the simulations for the X and Y orientations with the experimental data. Another possible origin for the observed distribution of out-of-plane hyperfine couplings could be the influence of pyridine, coordinated to the Zn ion of the porphyrin, on the geometry of the porphyrin plane. A potential energy surface scan performed by DFT for different Zn-pyridine distances shows increasing saddle-like distortions of the porphyrin plane of *l*-P1 as the pyridine molecule approaches the zinc ion (see figure 4.19). The nitrogen hyperfine tensors were calculated for each of the structures and the out-of-plane component was found to vary from about 6 to 8 MHz, leading to a distribution in close agreement with experimental observations. HYSCORE spectra recorded at the Z^- field position for *l*-P1 in MeTHF without pyridine and in pure toluene are almost identical to those recorded in the presence of pyridine. This would mean that both coordinating solvents such as MeTHF as well as non-coordinating solvents have similar effects on the conformation of the porphyrin plane. In coordinating solvents, the origin of the conformational distribution can be similar as for pyridine, as confirmed by a DFT calculation of *l*-P1 with a coordinated MeTHF molecule yielding analogous distortions of the porphyrin plane. Non-coordinating solvents such as toluene have been previously shown to give rise to specific solvent-solute interactions with porphyrins affecting the proton hyperfine couplings [156], potentially by inducing distortions of the porphyrin plane.

The ESEEM spectra recorded for the X and Y orientations of the ZFS tensor changed slightly in the absence of pyridine, therefore the HYSCORE spectra were also recorded under these conditions and compared to the previous results (data shown in figure 4.20). The intensities of the double-quantum peaks are reduced with respect to the spectra recorded in the presence of pyridine, but they are still present. This would seem to suggest that both the porphyrin and the pyridine nitrogen nuclei

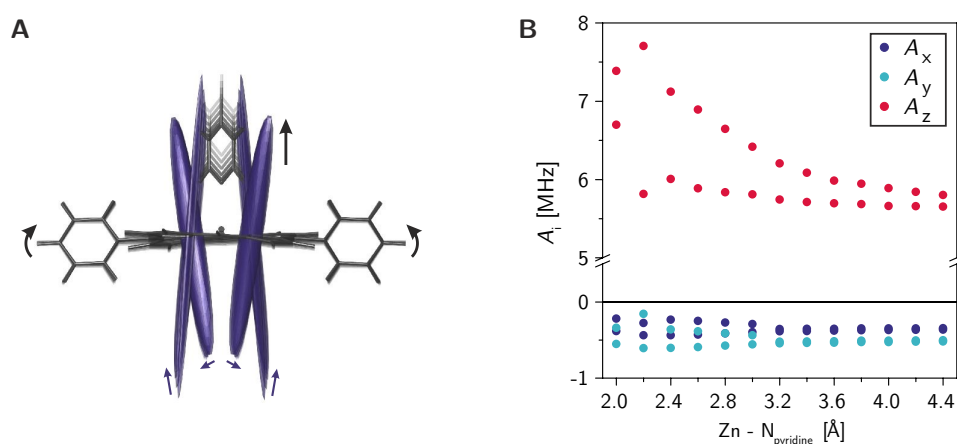


FIGURE 4.19: (A) DFT geometries and nitrogen hyperfine tensors calculated for different Zn-pyridine distances. The arrows indicate changes in geometry and hyperfine tensors for increasing distances between the Zn ion and pyridine. (B) Principal values of the hyperfine coupling tensor as a function of the distance between the Zn ion and the nitrogen of the coordinated pyridine molecule obtained from B3LYP/EPRII calculations.

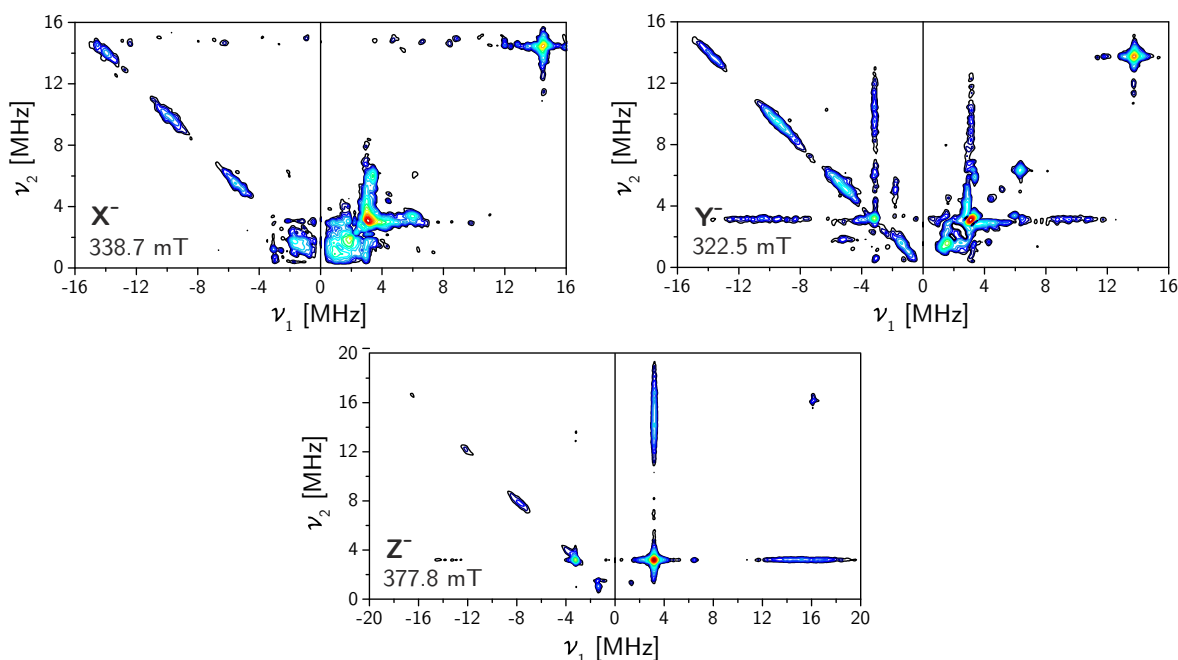


FIGURE 4.20: Experimental X-band HYSCORE spectra for the X^- , Y^- and Z^- field positions for *l*-P1 in MeTHF without pyridine recorded at 20 K. The Z^- spectrum was recorded using ^{14}N matched HYSCORE.

have similar in-plane hyperfine couplings. It can, however, not be excluded that small amounts of pyridine are still present, especially since measurements performed after two and after three purification cycles show differences in the intensities of these peaks. Any remaining amounts of pyridine could not be detected by UV-Vis or NMR spectroscopy, which would make it seem unlikely that they would contribute so much to the HYSCORE and ESEEM spectra, especially considering that MeTHF, as a coordinating solvent, competes with pyridine for binding at the zinc ion [163].

In order to determine the best ^{14}N hyperfine and nuclear quadrupole parameters, a simultaneous fitting of the available data, i.e. X- and Q-band ESEEM, X- and Q-band HYSCORE and Q-band ENDOR, was performed as described in section 4.2.4. The parameter optimisation was performed assuming that the double-quantum features in the HYSCORE spectra were determined by both the porphyrin and the pyridine nitrogen and also assuming that the double-quantum peaks are solely determined by pyridine hyperfine couplings. The pyridine nitrogen hyperfine couplings were kept fixed at the values predicted by DFT for different zinc-pyridine distances to reduce the fitting variables. This was considered to be acceptable since the predicted hyperfine couplings were in good agreement with the peaks in ESEEM, ENDOR and HYSCORE spectra that could be clearly identified as belonging to pyridine. The simulations were performed assuming four equivalent nitrogen nuclei, since no clear evidence for the contribution of more than one set of nuclei could be determined from the experimental data. The differences, if present, would be small due to the symmetry of the molecule and are taken into account by using distributions of hyperfine couplings. The tensor orientations were fixed at the values obtained by the DFT calculations (figure 4.21). The fitting results obtained in both cases were reasonable, particularly considering the significant amount of different data, and thus constraints,

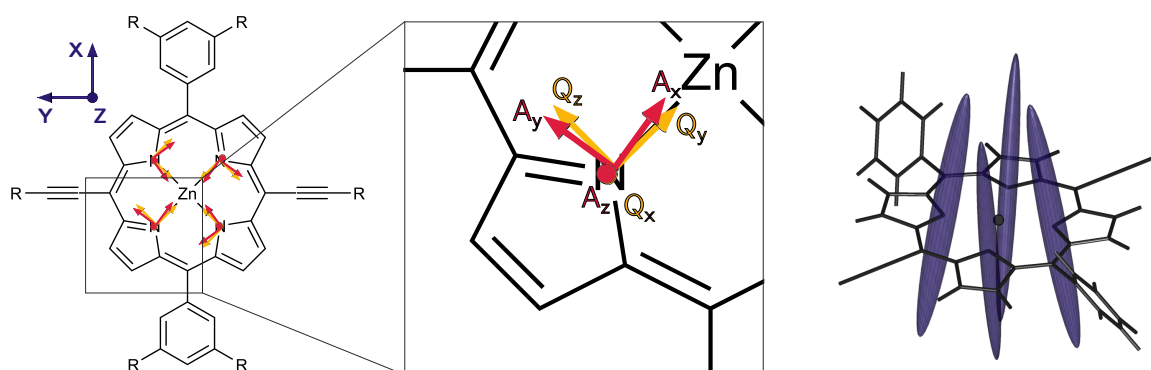


FIGURE 4.21: Diagram of the porphyrin monomer indicating the orientations of the ZFS tensor and the hyperfine and nuclear quadrupole interaction tensors predicted for the nitrogen nuclei by DFT calculations with B3LYP/EPRII. The nitrogen hyperfine tensor ellipsoids are also depicted on the right. The Euler angles defining the orientation of the hyperfine tensors with respect to the ZFS tensor are $\alpha = -145^\circ$, $\beta = 9^\circ$ and $\gamma = -161^\circ$, while the orientation of the nuclear quadrupole tensors are defined by $\alpha = 9^\circ$, $\beta = 90^\circ$ and $\gamma = 45^\circ$ (angles for one ^{14}N nucleus, the other ones are related by symmetry).

used in the fitting. For the ESEEM data, the peak positions agree quite well with the experimental data, while the intensities in some cases do not match, but correctly reproducing experimental ESEEM and HYSCORE intensities is notoriously difficult [34, 46]. The optimised parameters are reported in table 4.3 and the simulations are shown in figure 4.22 and in figure B.2 of appendix B.

The uncertainty on the in-plane hyperfine couplings is considerable, mainly due to the difficulty of reliably assigning the peaks in the HYSCORE spectra to different nuclei. The nuclear quadrupole interaction parameters and the centre and width of the distribution of hyperfine couplings in the out-of-plane orientation are, however, well defined by the ENDOR and HYSCORE data. This means that the out-of-plane ^{14}N hyperfine coupling can provide information on the extent of triplet state delocalisation. The experimental methods best suited for the determination of this hyperfine coupling are Q-band ENDOR and X- or Q-band HYSCORE. The ESEEM spectra on the other hand are dominated by

TABLE 4.3: ^{14}N hyperfine and nuclear quadrupole couplings for *l*-P1 and *l*-P2 determined by fitting of the experimental data as described in the text. The two sets of values for *l*-P1 correspond to an optimisation assuming that the double-quantum features in the HYSCORE spectra of the X and Y orientations are due to both porphyrin and pyridine nitrogen nuclei (01) and assuming that they are only determined by pyridine nitrogen nuclei (02). The standard deviations indicate the distribution of values used in the simulations shown in figures 4.22/B.2 and 4.25/B.5. N_{ext} and N_{int} for *l*-P2 refer to the external and internal nitrogen nuclei of each porphyrin unit (figure 4.26). There is an additional error on the centre of the distribution of about 0.3 MHz for A_x and A_y , of 0.1 MHz for A_z , of 0.05 MHz for Q and of 0.1 for η . The tensor orientations used in the simulations are derived from DFT calculations (see figure 4.21).

		A_x [MHz]	A_y [MHz]	A_z [MHz]	Q [MHz]	η
<i>l</i> -P1	01	1.8 ± 0.2	2.1 ± 0.25	6.8 ± 2.8	2.30 ± 0.01	0.79 ± 0.18
<i>l</i> -P1	02	0.4 ± 0.3	0.6 ± 0.3	6.8 ± 2.8	2.30 ± 0.01	0.79 ± 0.18
<i>l</i> -P2	N_{ext}	0.8 ± 0.2	1.1 ± 0.2	3.5 ± 1.2	2.25 ± 0.01	0.75 ± 0.20
<i>l</i> -P2	N_{int}	0.5 ± 0.2	0.6 ± 0.2	1.9 ± 1.2	2.35 ± 0.01	0.80 ± 0.20
<i>l</i> -P2	02	-0.6 ± 0.2	-0.2 ± 0.2	3.4 ± 1.4	2.30 ± 0.01	0.70 ± 0.20
<i>l</i> -P2	02	-0.6 ± 0.2	0.4 ± 0.2	1.8 ± 1.4	2.20 ± 0.01	0.80 ± 0.20

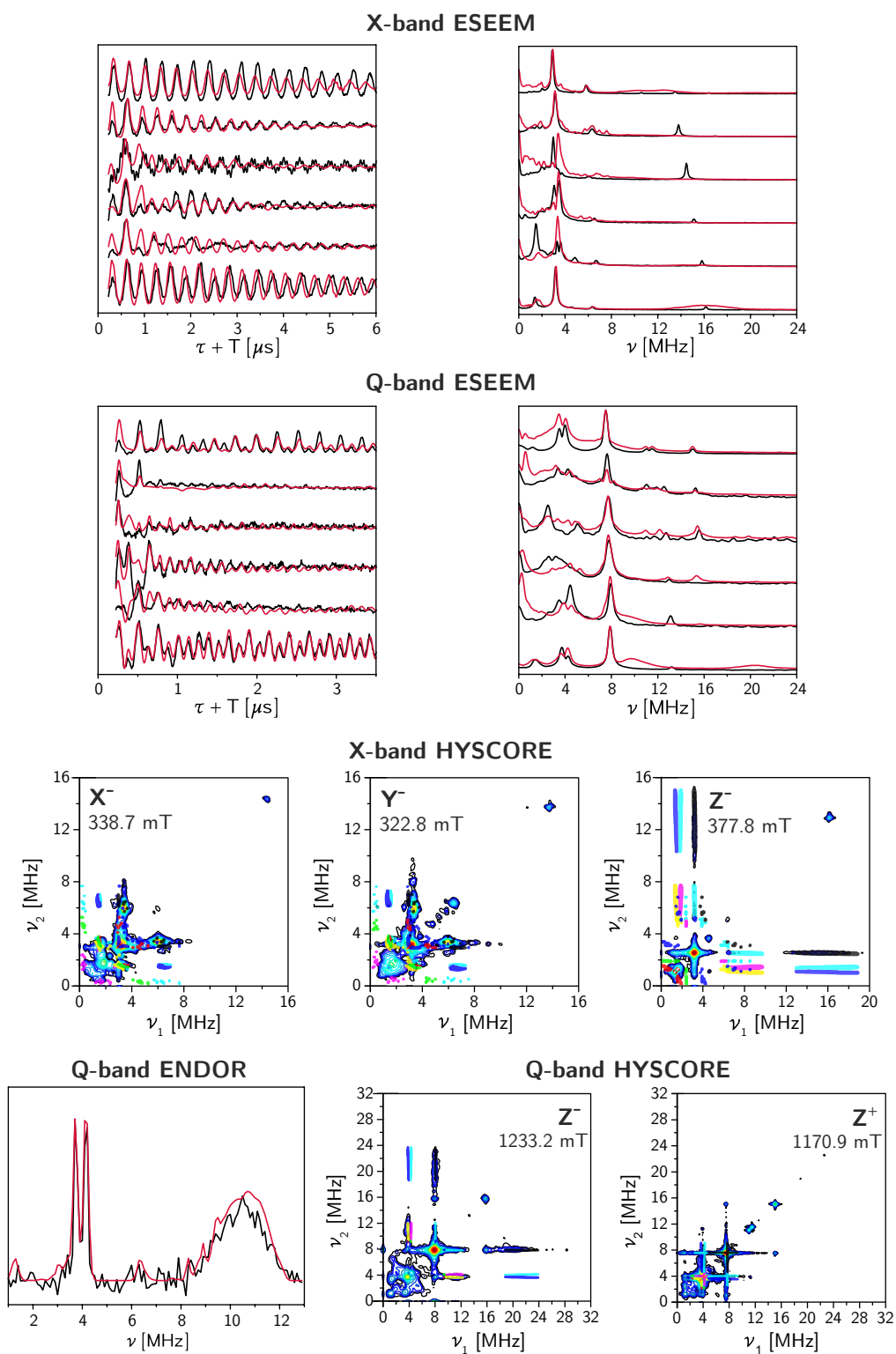


FIGURE 4.22: Experimental and simulated I -P1 ^{14}N ESEEM and ENDOR data and calculated HYSCORE cross-peak positions for the first set of optimised parameters of table 4.3 (O1, optimisation assuming that both porphyrin and pyridine nitrogens contribute to the strongest cross-peaks in the HYSCORE spectra of the X and Y positions). All simulations except the time-domain ESEEM simulations include the contribution of pyridine nitrogens based on DFT calculations for a distribution of different pyridine-zinc distances. The full set of HYSCORE spectra is shown in appendix B. The calculated HYSCORE cross-peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

the nuclear frequencies of the $m_S = 0$ manifold and do not allow the determination of the nitrogen hyperfine couplings on their own.

Analysis of the nitrogen hyperfine couplings would be much simplified by isotope substitution with ^{15}N , an $I = \frac{1}{2}$ nucleus. The absence of nuclear quadrupole interaction would considerably simplify the analysis of the experimental data and would most probably allow a more accurate determination of both the out-of-plane and in-plane hyperfine couplings.

^{14}N HYPERFINE COUPLINGS IN *l*-P2

Analogous measurements were performed on *l*-P2 to determine whether the nitrogen hyperfine couplings show the same trend observed for the ^1H ENDOR data, thus confirming the conclusion of complete delocalisation of the triplet state over both porphyrin units. The results of three-pulse ESEEM measurements are shown in figure 4.23. A comparison with the ESEEM spectra recorded for *l*-P1 shows that the positions of the main peaks are very similar, indicating a similar nuclear quadrupole interaction. On the other hand, the spectra do show significant differences in the low frequency region. Again no double-quantum peaks could be reliably identified, complicating the analysis of the spectra in terms of hyperfine couplings.

X-band HYSCORE measurements were performed at three field positions of the X^- , Y^- and Z^- transitions and the results are compared to the corresponding spectra of *l*-P1 in figure 4.24. Since the hyperfine couplings were shown to be positive for the porphyrin monomer, these transitions are expected to provide the most information on the nitrogen hyperfine couplings. Measurements at the Y^+ and Z^+ field positions confirmed this assumption, but are not shown here.

The largest nitrogen hyperfine couplings, assigned to the out-of-plane orientation, again shift from the Z orientation in *l*-P1 to the Y orientation in *l*-P2. The sign of the out-of-plane hyperfine couplings is positive, as expected, since the double-quantum features are observed for the $m_S = -1 \rightarrow m_S = 0$ transition. The magnitude of the nitrogen hyperfine couplings, estimated based on the distance between the positions of the double-quantum peaks, is reduced by a factor of about two from *l*-P1 to *l*-P2, as indicated by the shift of the double-quantum peaks to lower frequencies for the two in-plane orientations (X and Y for *l*-P1 and X and Z for *l*-P2). This conclusion is independent on whether the corresponding peaks are assigned to porphyrin or pyridine nitrogen nuclei, as the same decrease in hyperfine couplings is expected for both in case of delocalisation of the triplet state over the two porphyrin units in *l*-P2. For the out-of-plane orientation (Z^- in *l*-P1 and Y^- in *l*-P2), in addition to a reduction in hyperfine couplings, a splitting of the double-quantum features in the HYSCORE spectra and of the single-quantum peak in the Q-band ENDOR spectrum into two is observed (see figure 4.26), which is indicative of a different spin density distribution around the external and internal nitrogen nuclei in *l*-P2. This is found to be in agreement with DFT calculations, predicting smaller couplings for the internal nitrogen nuclei and larger couplings for the external nitrogen nuclei, which are more similar in terms of hyperfine anisotropy and nuclear quadrupole interaction to those of *l*-P1. In this case, Q-band HYSCORE measurements did lead to improved spectra for the out-of-plane orientation.

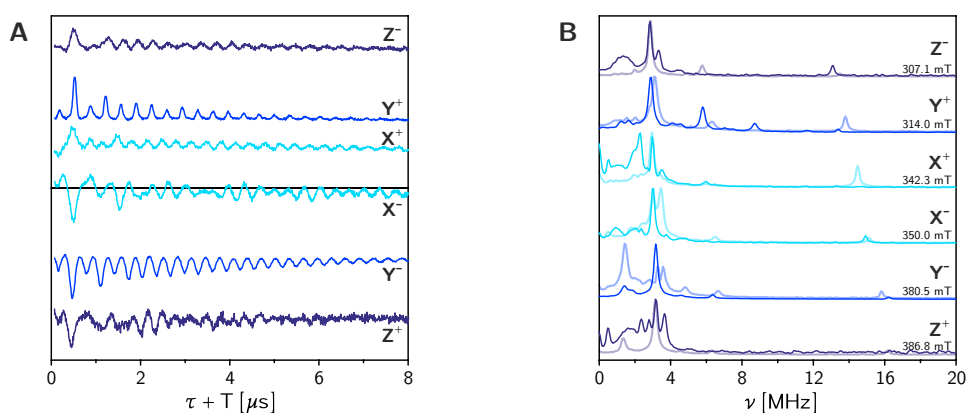


FIGURE 4.23: (A) Experimental X-band three-pulse ESEEM time-domain traces recorded at the six canonical field positions for *l*-P2 (traces recorded with a τ value of 160 ns are shown). (B) Experimental ESEEM spectra for the six canonical field positions (sum of FT spectra with $\tau = 120, 160$ and 200 ns). The ESEEM spectra of *l*-P1 are shown in the background for comparison.

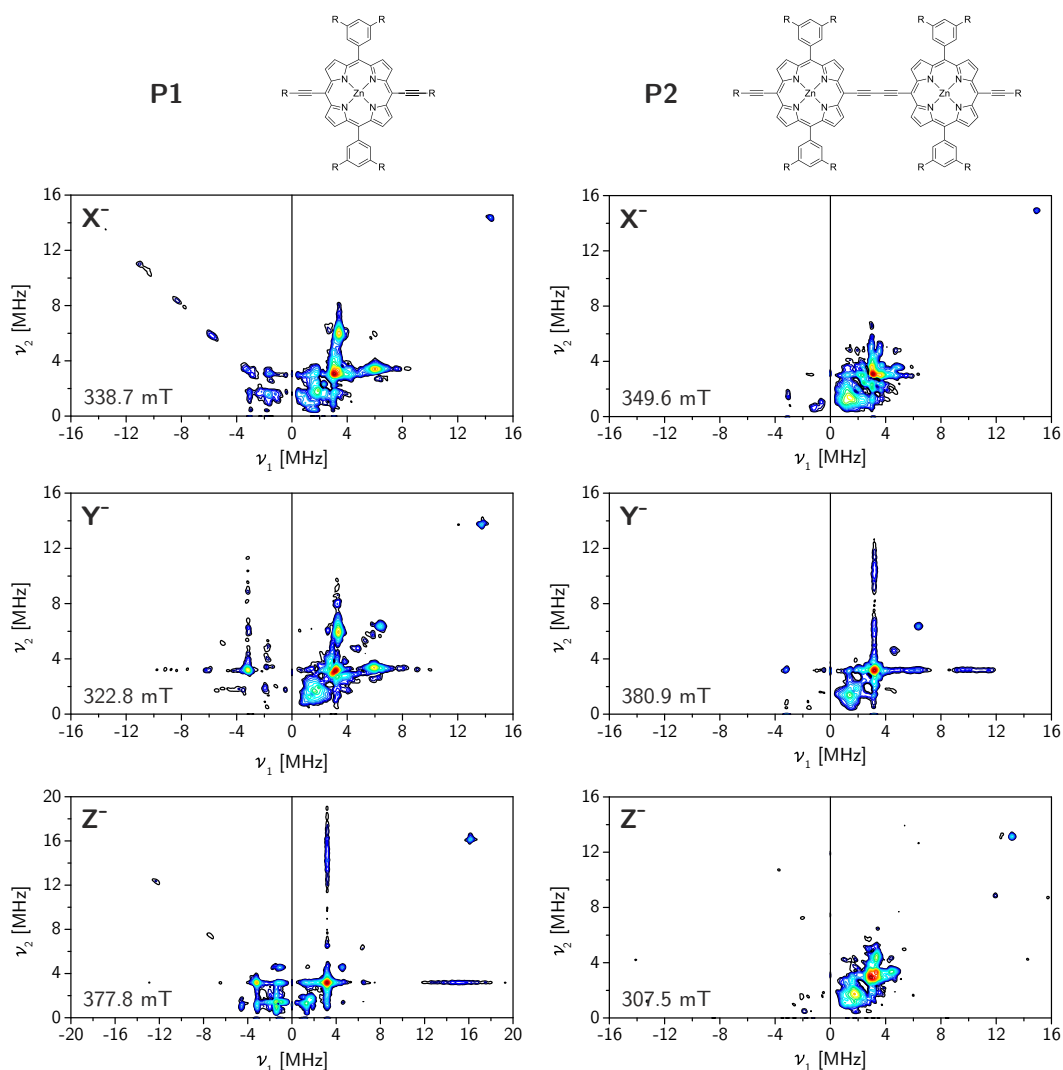


FIGURE 4.24: X-band HYSCORE spectra recorded at the X^- , Y^- and Z^- field positions for *l*-P1 and *l*-P2 at 20 K. The Z^- spectrum for *l*-P1 was recorded using ^{14}N matched HYSCORE. The experimental details are described in section 4.2.3.

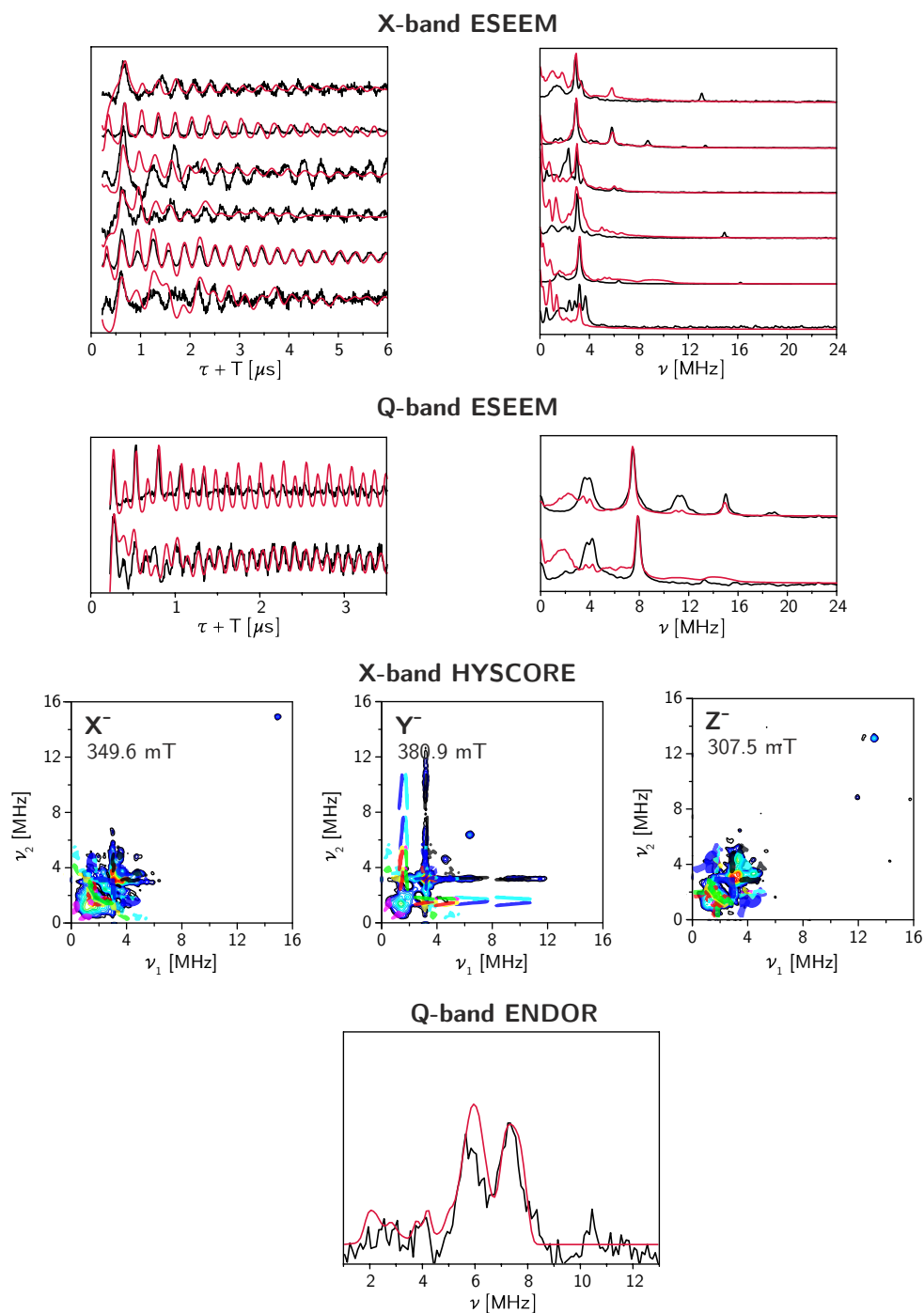


FIGURE 4.25: Experimental and simulated l -P2 ^{14}N ESEEM and ENDOR data and calculated HYSCORE cross-peak positions for the first set of optimised parameters of table 4.3 (01, optimisation assuming both porphyrin and pyridine nitrogens contribute to the strongest cross-peaks in the HYSCORE spectra of the X and Z positions). All simulations except the time-domain ESEEM simulations include the contribution of pyridine nitrogens based on DFT calculations for a distribution of different pyridine-zinc distances. The full set of HYSCORE spectra is shown in appendix B. The calculated HYSCORE cross-peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

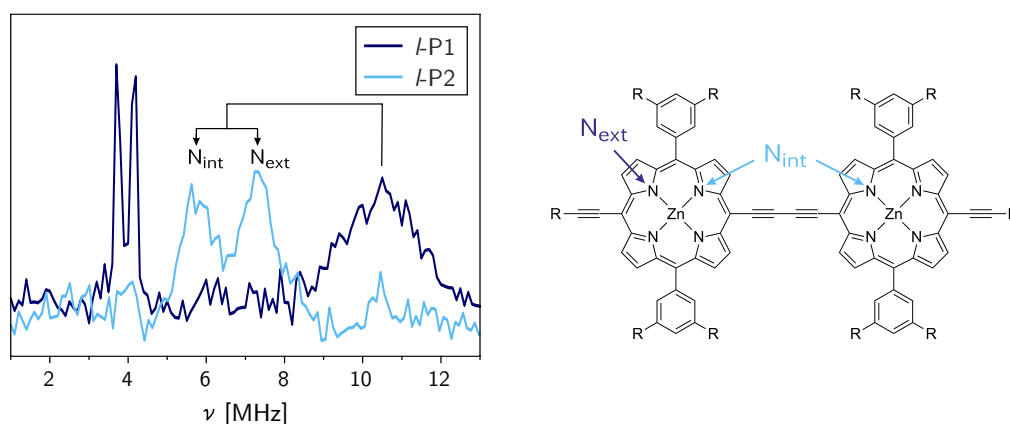


FIGURE 4.26: Comparison of the experimental Q-band Mims ENDOR spectra recorded at 20 K at the Z^- field position (1233.2 mT) for *l*-P1 and at the Y^- field position (1235.0 mT) for *l*-P2. The two peaks in the *l*-P2 ENDOR spectrum have been assigned to the external and internal nitrogen nuclei in the porphyrin dimer, indicated on the right.

The porphyrin nitrogen hyperfine couplings determined by simultaneous fitting of the available experimental data are reported in table 4.3 and the simulations for the two sets of optimised parameters are shown in figure 4.25 and figure B.5 in appendix B.

^{14}N HYPERFINE COUPLINGS IN THE LONGER OLIGOMERS

The change in nitrogen hyperfine couplings between *l*-P1 and *l*-P2 indicates delocalisation over the two porphyrin units and the splitting into two peaks for *l*-P2 indicates different environments for the internal and external nitrogen nuclei. The largest hyperfine coupling in the out-of-plane orientation in *l*-P2 amounts to almost exactly one half of the corresponding hyperfine coupling in *l*-P1, hence it could be used to estimate the extent of delocalisation in the longer oligomer systems.

The three-pulse ESEEM and HYSCORE spectra recorded for the porphyrin trimer are compared to the corresponding results for monomer and dimer in figure 4.27. Both the time and frequency domain ESEEM data are almost identical between *l*-P2 and *l*-P3, even in the low-frequency region, indicating that both hyperfine and nuclear quadrupole interaction parameters are probably similar. More detailed information could potentially be obtained from the HYSCORE spectrum, but the decreasing triplet yield complicates the data acquisition. The double-quantum features are visible in the HYSCORE spectrum at similar positions as for *l*-P2, but are not very well defined. If the centre of the visible ridges is used to estimate the hyperfine coupling, a slightly smaller value with respect to the porphyrin dimer is obtained. While this would be in agreement with the hyperfine couplings calculated by DFT using B3LYP/EPRII (standard B3LYP with 20% exact exchange, found to give the best agreement with the proton hyperfine data), the experimental signal-to-noise ratio is not sufficient to absolutely confirm this. Similar measurements on the longer oligomers were unfortunately prevented by the further decrease in triplet yield, excessively increasing the measurement time needed to obtain reasonable HYSCORE data.

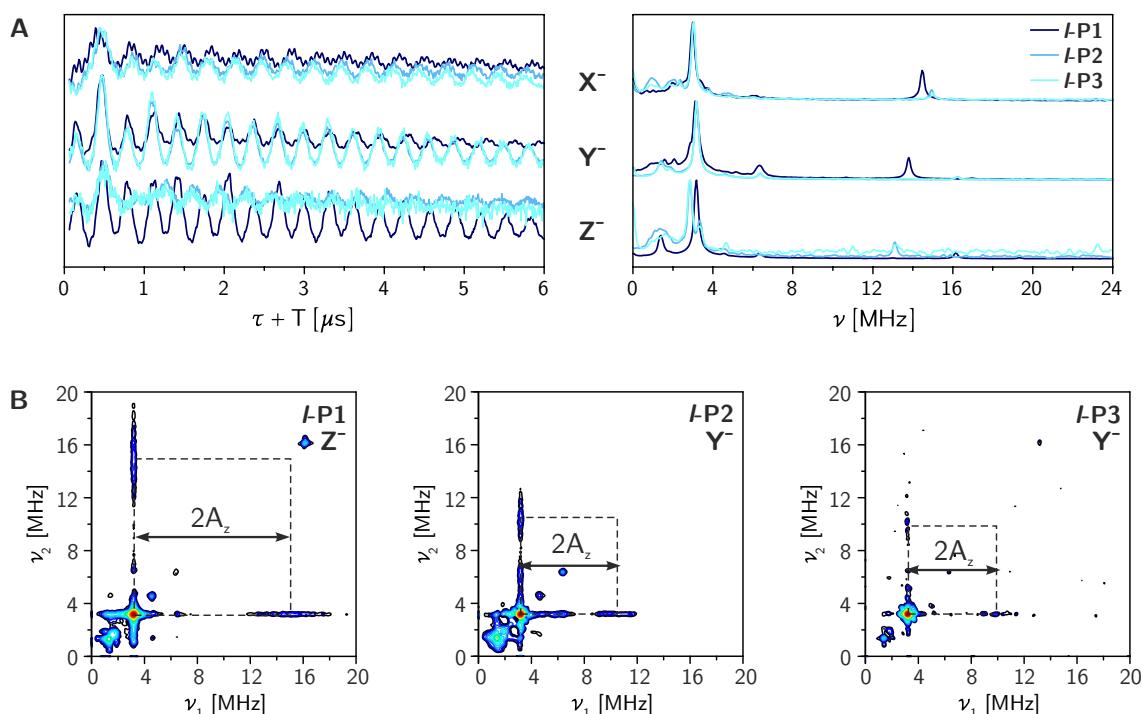


FIGURE 4.27: (A) Experimental three-pulse ESEEM time and frequency domain data for *l*-P1, *l*-P2 and *l*-P3 at the canonical field positions X⁻, Y⁻ and Z⁻. (B) ¹⁴N matched HYSCORE spectra recorded at the field position corresponding to the $m_S = 0 \rightarrow m_S = -1$ transition of the out-of-plane orientation for *l*-P1 (Z⁻), *l*-P2 (Y⁻) and *l*-P3 (Y⁻). The out-of-plane hyperfine coupling can be estimated from the distance between the double-quantum features as indicated in the graphs.

4.3.5 EXCITATION WAVELENGTH DEPENDENCE

The triplet delocalisation in porphyrin oligomers could be limited by the conformational flexibility, leading to conformations with porphyrin units at right angles to each other, thus disrupting conjugation. Previous optical studies on the porphyrin dimer have shown that the planar and twisted conformations can be selectively excited in different wavelength regions [67, 164]. The conformational flexibility and its influence on triplet state delocalisation was investigated by variable temperature optical studies performed by Dr. Patrik Neuhaus and Mr. Martin Peeks and is studied here by excitation wavelength-dependent EPR measurements.

EXCITATION WAVELENGTH DEPENDENCE IN *l*-P2

The UV-Vis absorption spectrum of the porphyrin dimer is characterised by two types of absorption bands, the Q bands in the wavelength region from about 550 nm to 800 nm and the B or Soret bands centred at about 450 nm. Optical studies have shown that different conformations of the porphyrin dimer, with a different torsional angle around the connecting bond, contribute to the absorption spectrum in different wavelength regions, as indicated in figure 4.28 A [67, 164]. The barrier of rotation around the central butadiyne bond was determined by DFT potential energy surface scans to amount to about 0.03 eV for the ground state ($k_B T$ at 25°C is 0.0257 eV). This means that at room temperature almost all conformations will be significantly populated, explaining the appearance of the room

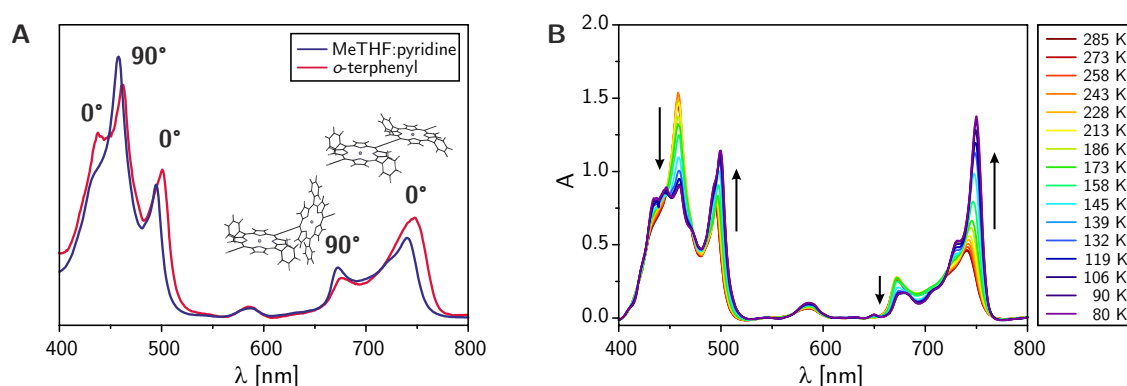


FIGURE 4.28: (A) Room temperature UV-Vis spectrum of *l*-P2 in MeTHF:pyridine 10:1 and spectrum recorded at 238 K in *o*-terphenyl. The absorption bands in the spectra have been assigned to the planar and twisted conformations of the porphyrin dimer according to reference [67]. (B) Variable temperature UV-Vis spectra of *l*-P2 in MeTHF:pyridine 10:1. (The data shown in this figure were recorded by Dr. Patrik Neuhaus and Mr. Martin Peeks).

temperature absorption spectrum. Upon cooling, the UV-Vis absorption spectrum changes as shown in figure 4.28 B. In particular, the part of the Q-band at about 740 nm increases in intensity, while the band at 670 nm decreases in intensity. A similar behaviour is also shown in the Soret bands. The changes were interpreted in terms of planarisation of the porphyrin dimer at lower temperatures. In a preliminary analysis performed by Dr. Patrik Neuhaus and Mr. Martin Peeks, the rotational barrier was estimated based on this data and was found to be in agreement with the theoretical predictions. The UV-Vis spectrum at 80 K was attributed to the planar porphyrin dimer by comparison with spectra recorded using ligands forcing the dimer into a coplanar arrangement of the two porphyrin units [67]. The peak at 680 nm is still present at 80 K and has been assigned to the vibrational structure of the principal peak of the planar conformation based on the measurement of the emission spectrum and on time-dependent DFT (TDDFT) calculations performed by Mr. Martin Peeks. The planar conformation of the porphyrin dimer will thus contribute in the whole wavelength region of the Q bands.

Since the EPR measurements described above were performed in frozen solutions of MeTHF (freezing temperature around 130 K), the EPR signals will be almost exclusively due to the planar conformation of the porphyrin dimer. In order to study the twisted conformation, measurements were performed in *o*-terphenyl (freezing point at 333 K, glass transition temperature of 246 K). The UV-Vis spectrum recorded in *o*-terphenyl at 238 K is compared to the room temperature spectrum recorded in the MeTHF:pyridine mixture in figure 4.28. The UV-Vis spectrum in *o*-terphenyl remains unchanged in the region from 173 to 238 K (data not shown), indicating the presence of a wide range of conformations.

Time-resolved EPR spectra were recorded after excitation at different wavelengths in the Q band region in both MeTHF and *o*-terphenyl and the resulting spectra are shown in figure 4.29. The spectra recorded in MeTHF only show small changes as a function of excitation wavelength, but in *o*-terphenyl more significant changes are observed. In particular, the intensity of the X transitions decreases from 530 nm to about 660 nm and then increases again and reaches a maximum at 760 nm, and the Y transitions broaden towards the centre of the spectrum with the largest broadening observed around

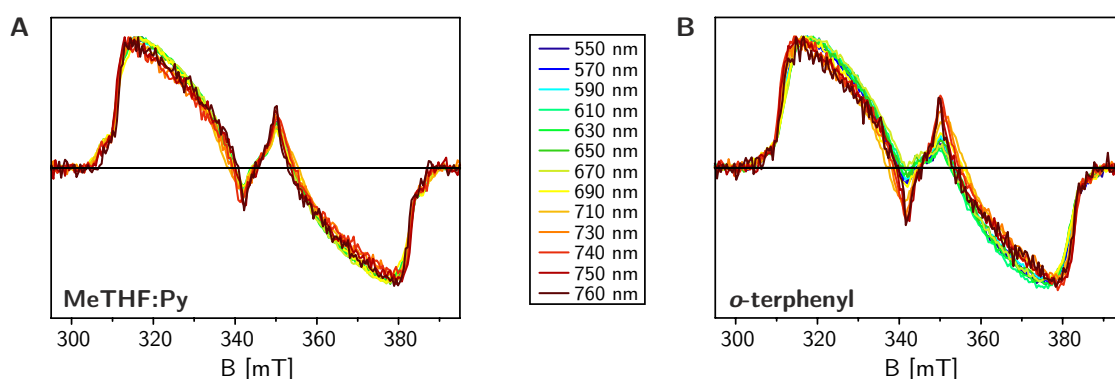


FIGURE 4.29: Time-resolved EPR spectra recorded at 20 K for *l*-P2 in MeTHF:pyridine 10:1 (A) and in *o*-terphenyl (B) using different excitation wavelengths. The spectra were recorded using light polarised parallel or perpendicular to the magnetic field and the depolarised spectra shown here were reconstructed as $\frac{1}{3}(I_{||} + 2I_{\perp})$.

640-660 nm. The most significant changes in the EPR spectrum occur at the wavelengths attributed to the absorption of the twisted dimer conformation. Due to the overlap of the absorption band of the twisted conformation with the vibrational structure of the absorption band attributed to the planar conformation, the two spectra will be superimposed and based on the small changes observed in the experimental time-resolved EPR spectra it would seem that the spectrum of the planar conformation prevails. In order to exclude other possible causes for the observed changes, the same measurements were also performed on *l*-P1 and no wavelength-dependent changes were observed.

Excitation wavelength-dependent ENDOR measurements were also performed at 379 mT, corresponding to the high-field Y transition for the porphyrin dimer and the high-field Z transition for the porphyrin monomer (see figures 4.12 and 4.13 for the corresponding ENDOR spectra recorded after excitation at 532 nm). As expected, no wavelength-dependent changes are observed in the ENDOR spectra of *l*-P2 recorded in MeTHF:pyridine and the peak observed at about -1.6 MHz corresponds to the out-of-plane hyperfine coupling of the H₁ protons for a triplet state delocalised over both porphyrin units. In *o*-terphenyl, an identical spectrum is obtained for excitation at 750 nm, however

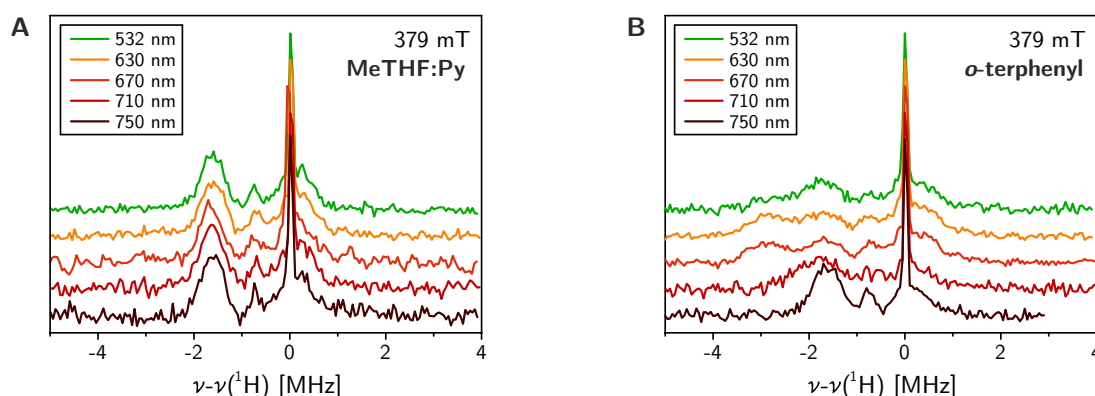


FIGURE 4.30: Mims ENDOR spectra recorded at 20 K at a magnetic field of 379 mT (high field Y transition) for *l*-P2 in MeTHF:pyridine 10:1 (A) and in *o*-terphenyl (B) using different excitation wavelengths.

excitation at lower wavelengths leads to significant broadening of the same peak and to the appearance of an additional peak at around -3 MHz for excitation in the region from 630-670 nm. The position of this peak corresponds almost exactly to the ENDOR peak observed at the same field position in the porphyrin monomer (see figure 4.12), except for a small shift towards the Larmor frequency. The appearance of this monomer-like peak at wavelengths corresponding to the absorption of the twisted conformation of the porphyrin dimer indicates localisation of the triplet state on a single porphyrin unit with the porphyrin planes at approximately right angles to each other. The broadening of the peaks towards larger hyperfine couplings at lower and higher wavelengths can be interpreted in terms of contributions of different intermediate conformations with varying hyperfine couplings.

The ENDOR results are a clear indication of the localisation of the triplet state on a single porphyrin unit for a perpendicular arrangement of the two porphyrin units in *I*-P2, although the changes observed in the time-resolved EPR spectra at the same wavelengths are not straightforward to interpret. The expectation would be that a monomer-like EPR spectrum will be superimposed on the dimer EPR spectrum, but no clear additional peaks are present. In order to interpret the changes in the time-resolved EPR spectra as well as the ENDOR spectra, model calculations were performed to reproduce and explain the observed changes.

The simulation of the contribution of different conformations of the porphyrin dimer at different wavelengths requires the knowledge of the "pure" EPR and ENDOR spectra corresponding to each conformation. Due to the extensive overlap of their absorption bands, the experimental determination of these spectra was prevented and they were instead estimated based on DFT calculations and the experimental results obtained for the planar conformation of *I*-P2 and for *I*-P1, as a model of the twisted conformation with localisation of the triplet state on a single unit, as outlined in figure 4.31.

A potential energy surface scan was performed by DFT by changing the dihedral angle between the two porphyrin units in 10° increments and by optimising the geometries at B3LYP/6-31G(d) level. Single-point calculations with the EPRII basis set were performed for each conformation to determine the zero-field splitting and hyperfine parameters. The DFT calculations predict equal spin densities on the two porphyrin units for a dihedral angle of 0° and a gradual shift of the spin density onto one of the porphyrin units as the dihedral angle increases. The *D*-value decreases gradually for increasing dihedral angles, but the sign of the *D*-value and the orientation of the zero-field splitting tensor are predicted to change suddenly in the interval of dihedral angles from 50° to 60° (spin density distributions over the two porphyrin units of 0.87 : 0.13 and 0.90 : 0.10, respectively). The *D*- and *E*-values predicted for the conformation with the two porphyrin units at right angles to each other correspond approximately to the values calculated by DFT for the porphyrin monomer.

In order to calculate the time-resolved EPR spectra corresponding to the different conformations, the calculated zero-field splitting parameter *D* was scaled based on the experimentally determined values for the planar conformation. This was based on the assumption that DFT predicts the relative changes in ZFS as a function of inter-porphyrin angle correctly, while it severely underestimates the magnitudes. The *E*-values were calculated from the *D*-values using the *E*/*D* ratios obtained by linear interpolation of the experimental *E*/*D* ratios of the porphyrin monomer (*E*/*D*=0.18) and dimer

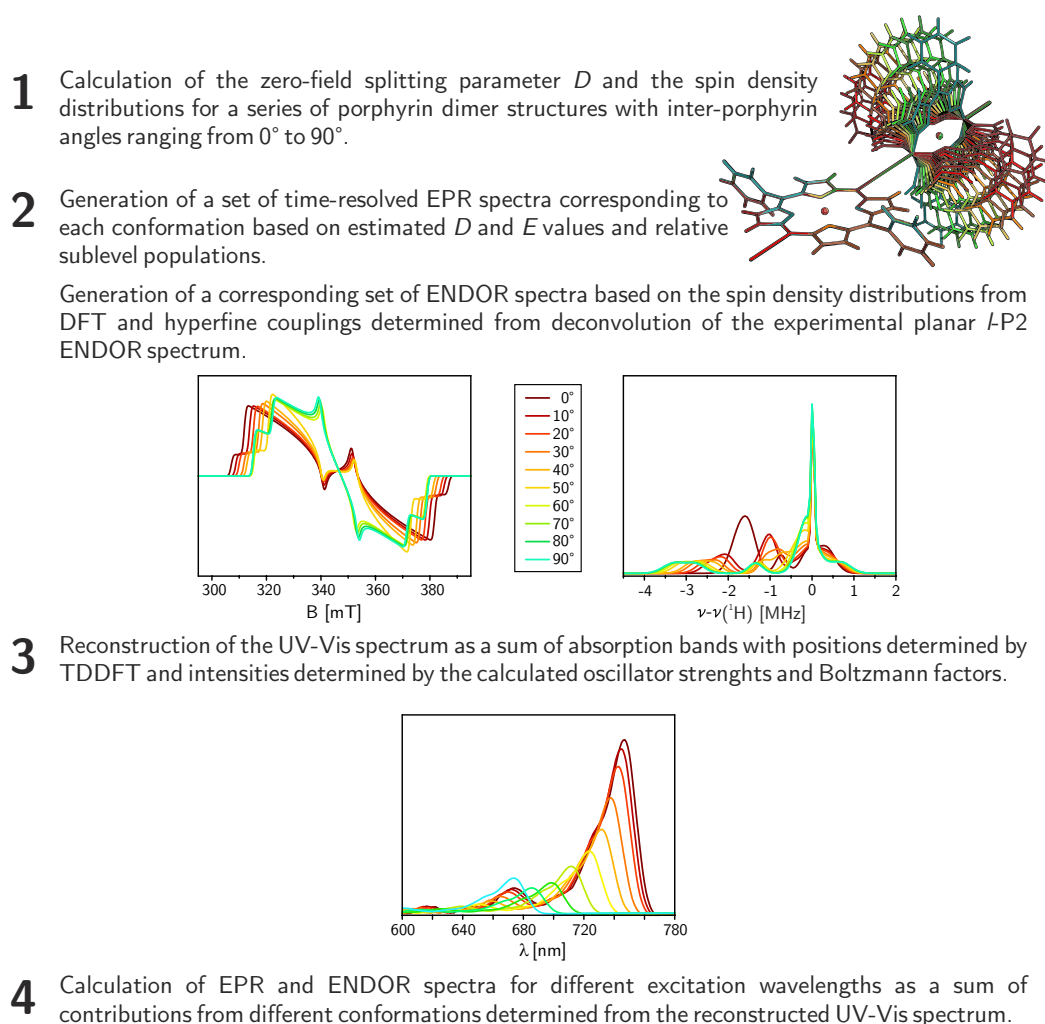


FIGURE 4.31: Outline of the procedure used for the simulation of wavelength-dependent EPR and ENDOR spectra for *l*-P2.

($E/D=0.25$). This led to a better agreement with experiment with respect to a scaling of the D - as well as the E -values determined by DFT. The relative sublevel populations were predicted by assuming population to occur exclusively for the out-of-plane orientation of each porphyrin unit, as the direct zinc spin-orbit coupling contribution determines ISC, and by projecting the corresponding axes onto the zero-field splitting tensor axis system. The projections were weighted by the relative amount of spin density on each porphyrin unit. In order to predict the ENDOR spectra for the different conformations, the hyperfine couplings in the out-of-plane orientation for the porphyrin dimer were determined by deconvolution of the experimental spectrum corresponding to the planar conformation and scaled based on the ratio of spin densities on the two porphyrin units predicted by DFT.

The contribution of the spectra corresponding to the different conformations of the porphyrin dimer at different wavelengths was predicted based on a combination of the results from TDDFT calculations reported in reference [67] and experimental results of UV-Vis measurements (figure 4.32 A). The positions of the absorption bands for different dihedral angles were predicted by TDDFT calculations [67].

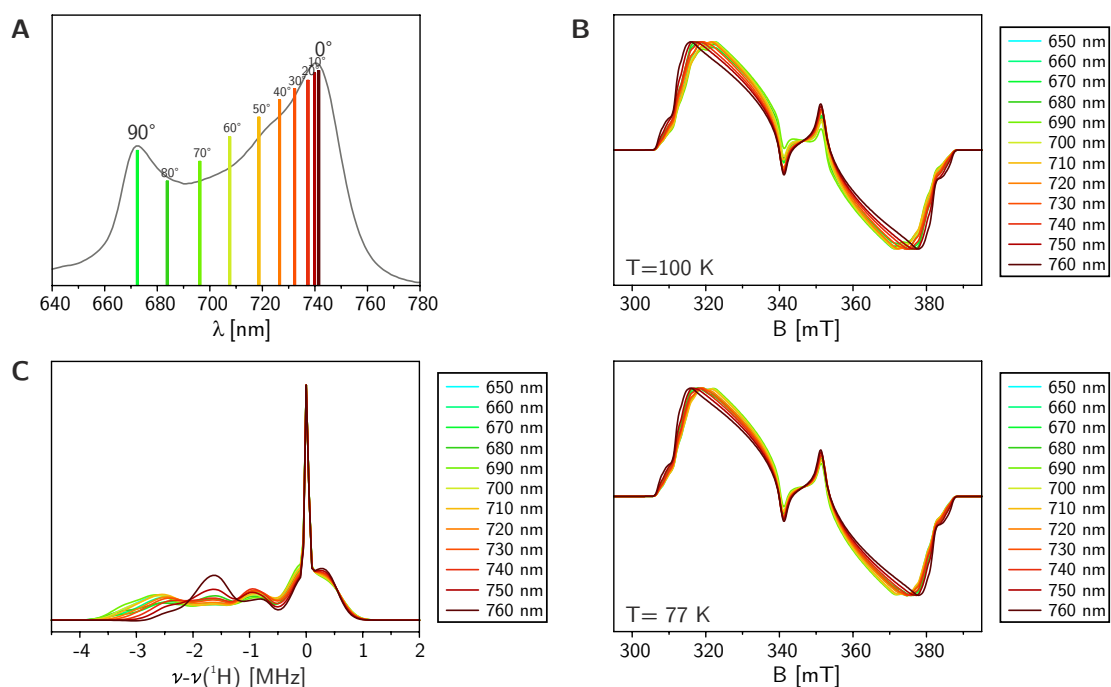


FIGURE 4.32: (A) Q band of the *l*-P2 absorption spectrum recorded at room temperature (grey) and stick spectrum indicating the positions of the absorption bands for different conformations of the porphyrin dimer. The relative positions of these absorption bands have been derived from the TDDFT calculations reported in reference [67] and the width and absolute positions have been scaled to match the experimental room-temperature UV-Vis spectrum for further analysis. The relative intensities reflect the oscillator strengths predicted by DFT. (B) Calculated EPR spectra of *l*-P2 for excitation at different wavelengths using the model described in the text for Boltzmann distributions at 100 K (top) and 77 K (bottom). (C) Calculated ENDOR spectra of *l*-P2 for excitation at different wavelengths using the model described in the text (Boltzmann distribution at 100 K).

It is well known that TDDFT often does not predict absolute energies correctly [165] and hence for the purpose of this analysis the width of the predicted distribution and the absolute positions were scaled to match the experimental room temperature UV-Vis spectrum as shown in figure 4.32 A. The UV-Vis spectrum could be reconstructed as a sum of Gaussians centred at the predicted positions, but this would not explain the presence of the spectrum corresponding to the planar dimer conformation at wavelengths from about 600 to 700 nm. As discussed above, the low temperature UV-Vis spectrum was attributed to the vibronic absorption band of the pure planar conformation, therefore instead of a Gaussian, the corresponding absorption band with its vibrational structure was used to model the contributions of different conformations at different wavelengths. The intensities for the absorption bands corresponding to the different conformations were determined based on the oscillator strengths predicted by DFT and on a weighting factor calculated from a Boltzmann population distribution at different temperatures.

The model UV-Vis spectrum thus constructed was used to predict the EPR and ENDOR spectra for excitation at different wavelengths. The calculated spectra for each conformation were summed based on the relative weights determined from the reconstructed UV-Vis spectrum at different wavelengths and the results are shown in figure 4.32 B and C. The time-resolved EPR spectra in figure 4.32 B were

calculated for two different Boltzmann distributions, at 100 and at 77 K. The calculations qualitatively reproduce the changes observed experimentally in the two different solvent systems (see figure 4.29). At the wavelengths with the largest contribution of the twisted conformation, the intensity of the central X transitions decreases and the Y transitions are shifted towards the centre. The analysis described above indicates that the observed changes are caused by the contribution of a distribution of EPR spectra that tend towards the EPR spectrum observed for the porphyrin monomer. The calculated ENDOR spectra in figure 4.32 C also predict a broadening and the appearance of an additional peak at larger hyperfine couplings, as observed experimentally (figure 4.30). The poor quantitative agreement of calculation and experiment can be attributed to the large number of approximations required to perform this analysis. Nevertheless the experimentally observed wavelength dependence could be qualitatively explained and the contribution of a distribution of different orientations to both EPR and ENDOR spectra could be confirmed.

EXCITATION WAVELENGTH DEPENDENCE IN *l*-P3 AND *l*-P4

The porphyrin trimer can assume an even larger range of conformations as the torsional angles around two butadiyne bonds can now vary independently. The room temperature UV-Vis spectrum of *l*-P3 is shown in figure 4.33 A. The Q band is shifted further to the red compared to *l*-P2 due to the increased conjugation and is also broadened. In analogy to *l*-P2, different conformations are assumed to contribute at different wavelengths, with planar conformations absorbing in the red and more twisted conformations in the blue part of the broad Q band absorption. The nature of the changes occurring upon cooling are similar to those observed for *l*-P2. The intensity of the high wavelength part of the Soret and Q band increases and the intensity at lower wavelengths decreases as shown in figure 4.33 B. An initial slow increase of the intensity at wavelengths higher than 800 nm, paralleling the behaviour observed for *l*-P2, is followed by a more pronounced and sudden increase at temperatures lower than about 138 K. The intensity of this peak relative to the broad absorption band at lower wavelengths was also found to be concentration-dependent; for lower porphyrin concentrations the well-defined peak at about 805 nm only appears at lower temperatures. A similar behaviour has been previously observed for longer porphyrin oligomers with different side-groups in

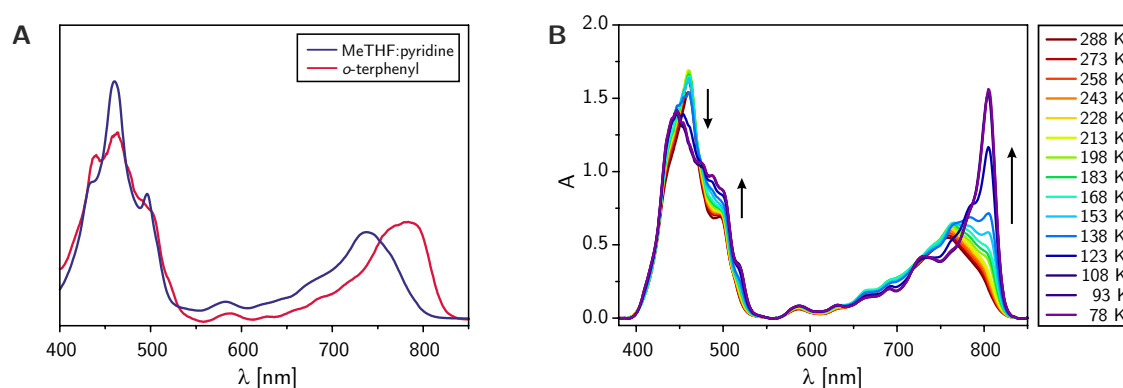


FIGURE 4.33: (A) Room temperature UV-Vis spectra of *l*-P3 in MeTHF:pyridine 10:1 compared to the spectrum recorded at 238 K in *o*-terphenyl. (B) Variable temperature UV-Vis spectra of *l*-P3 in MeTHF:pyridine 10:1. (The data shown in this figure have been recorded by Dr. Patrik Neuhaus and Mr. Martin Peeks).

the presence of aggregation and the red-shifted peak with increasing intensity at lower temperatures has been assigned to well-defined planar aggregates [166]. Aggregation is somewhat reduced if the sample is shock-frozen, but at the EPR concentrations of 100-200 μM the presence of aggregates cannot be ruled out.

The time-resolved EPR spectra recorded for *l*-P3 in MeTHF:pyridine and in *o*-terphenyl after excitation at different wavelengths in the Q band region are shown in figure 4.34. The wavelength-dependent changes are much more pronounced with respect to *l*-P2 and occur to a similar degree in both solvent systems, suggesting that a wider range of conformations is present in MeTHF as well. The changes in the EPR spectra are mainly restricted to the region of the Y transitions; in MeTHF:pyridine the intensity increases from 530 nm to higher wavelengths, reaches a maximum at about 620 nm and then decreases again, reaching a minimum at 800 nm. In *o*-terphenyl, the general behaviour is the same, but some differences are observed in the intensities at the same wavelengths. The results of analogous measurements performed for *l*-P4 in MeTHF:pyridine are shown in figure 4.35. The wavelength-dependent changes in the spectrum are again confined mainly to the Y transitions and follow similar trends to those observed for *l*-P3. The maximum intensity is reached for a wavelength of 680 nm or slightly lower and then decreases for longer wavelengths, reaching the minimum at 830 nm. At wavelengths higher than 800 nm for *l*-P3 and higher than 830 nm for *l*-P4 the intensity of the Y transitions was found to increase again, as shown in figure 4.37, no further changes are observed at even higher wavelengths before the signal intensity is reduced below the detection limit.

The changes in the time-resolved EPR spectra at wavelengths lower than 800 nm for *l*-P3 and 830 nm for *l*-P4 can be interpreted in terms of contributions of EPR spectra corresponding to twisted conformations. A conformation with two coplanar porphyrin units would give a spectrum similar to that of the porphyrin dimer, which has similar *D*- and *E*-values to the trimer and tetramer and a spin polarisation characterised by a high intensity of the Y transitions and much lower intensities of the X and Z transitions. The contribution of such a spectrum to the *l*-P3 or *l*-P4 spectrum would lead to the observed wavelength-dependent changes, i.e. increased intensity of the Y transitions in the region of

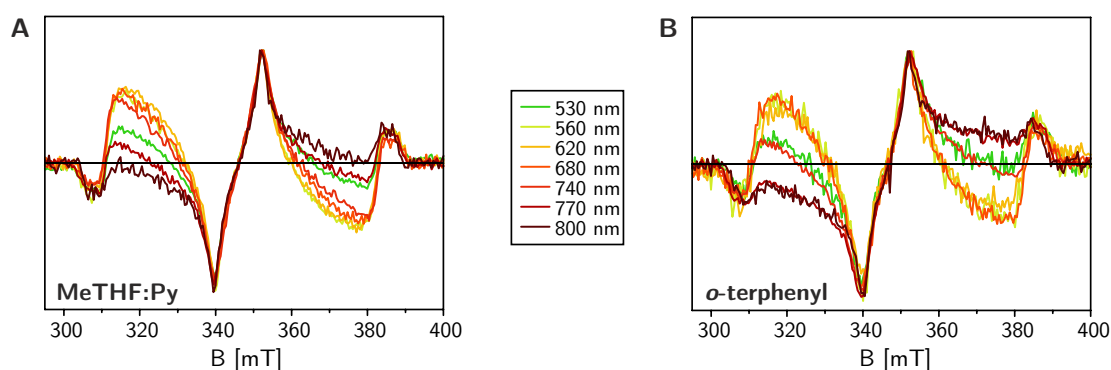


FIGURE 4.34: Time-resolved EPR spectra recorded at 20 K for *l*-P3 in MeTHF:pyridine 10:1 (A) and in *o*-terphenyl (B) using different excitation wavelengths. The spectra were recorded using light polarised parallel or perpendicular to the magnetic field and the depolarised spectra shown here were reconstructed according to $\frac{1}{3}(I_{||} + 2I_{\perp})$.

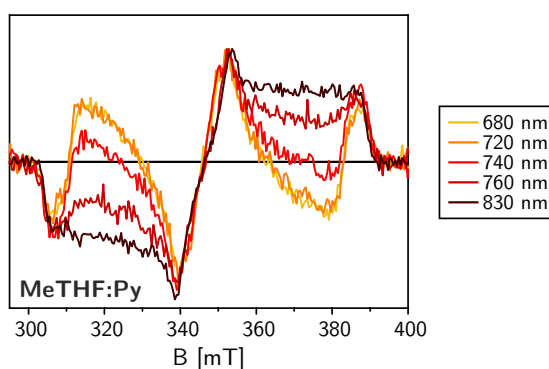


FIGURE 4.35: Time-resolved EPR spectra recorded at 20 K for *l*-P4 in MeTHF:pyridine 10:1 using different excitation wavelengths. The spectra were recorded using light polarised parallel or perpendicular to the magnetic field and the depolarised spectra shown here were reconstructed according to $\frac{1}{3}(I_{||} + 2I_{\perp})$.

the dimer absorption (approximately from 620 to 760 nm) and observation of the pure planar *l*-P3 or *l*-P4 EPR spectrum towards the red edge of the corresponding absorption spectrum. The EPR spectra recorded at 800 nm for *l*-P3 and at 830 nm for *l*-P4 were therefore assigned to the approximately planar conformation of the oligomer. The presence of a contribution of a conformation with all porphyrin units at right angles is likely, but could not be distinguished from the dimer-like contributions, since the changes would be very small, as determined from the analysis of the wavelength-dependent *l*-P2 data. Similarly, the presence of a contribution from a conformation with three coplanar porphyrin units and one unit at right angles to them in *l*-P4 cannot be determined due to the similarity of the *l*-P3 and *l*-P4 EPR spectra. All non-planar conformations will thus be referred to as twisted in the following analysis.

The hypothesis of the contribution of a dimer-like spectrum for the twisted conformations is confirmed by subtraction of the EPR spectrum obtained close to the red edge of the absorption, assigned to the planar conformation, from the spectra recorded at lower wavelengths. The subtraction spectra correspond to the EPR spectra of the twisted conformations and closely resemble the *l*-P2 EPR spectrum (figure 4.36 A and B). In order to determine the wavelength regions with the largest contribution of the twisted conformations, the spectra were reconstructed as linear combinations of the experimental spectra corresponding to the planar oligomer and the experimental dimer spectrum (see figure 4.36 C and D for *l*-P3 and *l*-P4, respectively). The contributions of the two types of conformations to the overall EPR intensity, normalised by laser power, are plotted as a function of wavelength in figure 4.36 E and F. The twisted conformations contribute mainly at wavelengths lower than 750 nm, in the region of the Q band absorption of the porphyrin dimer. The planar conformation contributes to the spectrum at all wavelengths, but reaches the highest intensity just below 800 nm for *l*-P3 and somewhere in the region from 800 to 850 nm for *l*-P4. The mismatch of the total EPR intensities as a function of wavelength with the UV-Vis absorption spectrum could be attributed to saturation effects and to differences in triplet yield between different conformations (i.e. higher triplet yields for the twisted conformations would be expected based on the observed decrease in triplet yield with the number of porphyrin units).

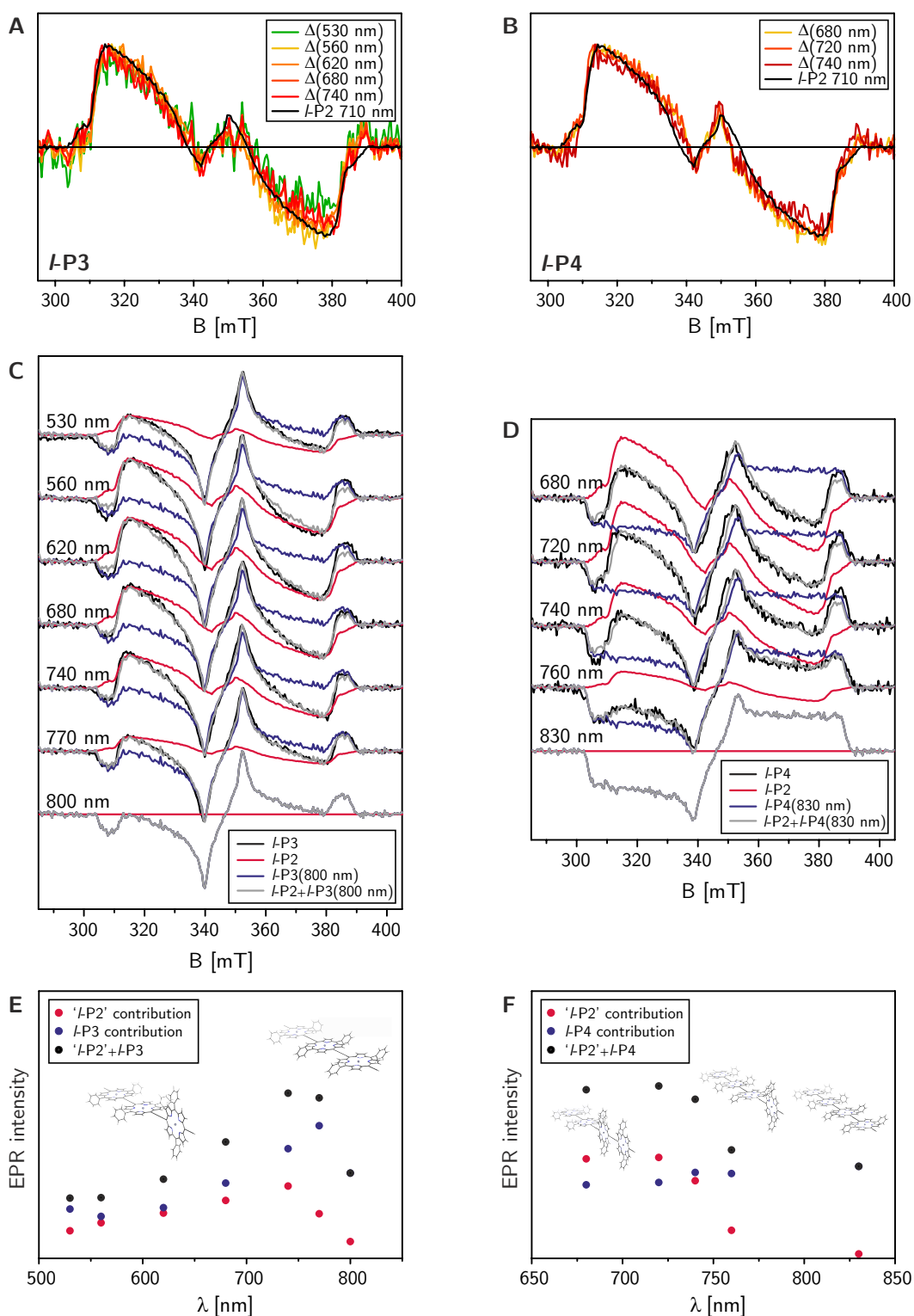


FIGURE 4.36: (A/B) EPR spectra obtained by subtraction of the normalised *l*-P3/*l*-P4 spectrum recorded at 800/830 nm from the EPR spectra recorded for excitation at lower wavelengths. The resulting subtraction spectra are compared to the *l*-P2 spectrum recorded after excitation at 710 nm. (C/D) Reconstruction of the time-resolved EPR spectra recorded for *l*-P3/*l*-P4 at different wavelengths as a linear combination of the *l*-P3/*l*-P4 spectrum recorded at 800/830 nm and the *l*-P2 spectrum. (E/F) Contributions of the *l*-P2 and planar *l*-P3/*l*-P4 conformations, resulting from the linear combinations shown in C and D, to the EPR signal intensity (divided by the laser power) as a function of wavelength.

The excitation wavelength-dependent changes in the EPR spectra of *l*-P3 and *l*-P4 can be explained in terms of the contribution of different conformations for most of the Q band absorption region. This theory does however not explain the changes occurring at higher wavelengths (figure 4.37). In the region above 800 nm for *l*-P3 and above 830 nm for *l*-P4, the intensity of the Y transitions increases again and this change could also be explained by the contribution of a dimer-like spectrum. Nevertheless, in this case, the contribution could not be due to a twisted conformation, as such a conformation would not absorb at such high wavelengths due to the decreased conjugation length. In addition to the contribution of a dimer-like spectrum, a range of different spectral shapes and widths could also lead to the observed changes. The exact nature of the contribution at high wavelengths could not be identified based on the available EPR data.

One possible explanation for the behaviour at high wavelengths would be the presence of an aggregate that absorbs at these wavelengths, a proposal in agreement with the UV-Vis data showing concentration dependent changes in this region of the absorption spectrum [166]. This hypothesis would be supported by the fact that the differences between spectra recorded by exciting at 800 nm and at 810 nm are smaller in *o*-terphenyl with respect to MeTHF, since no indications of aggregation could be detected in the UV-Vis spectra recorded in *o*-terphenyl. This would additionally explain the differences in the EPR spectra recorded at 800 nm in these two solvent systems. If aggregation is more likely in MeTHF, the aggregate could give rise to the increased Y transition intensity in the 800 nm spectrum recorded in MeTHF, while the contribution to the spectrum in *o*-terphenyl would be less pronounced. The tendency to aggregation increases with oligomer size, so that the differences are expected to be even larger for the tetramer, as is indeed observed experimentally (see figure 4.37). In order to prove this experimentally, the EPR measurements were repeated at lower concentrations, but no changes were observed in the range from 10 to 100 μM . Nevertheless, a concentration of 10 μM is still 5-10 times higher than the concentrations used for UV-Vis measurements for which signs of aggregation have already been detected. EPR measurements on samples with even lower concentrations were unfortunately not possible due to the reduced signal intensity. Similarly, increasing the concentration would lead to more significant optical saturation, also decreasing the EPR signal intensity.

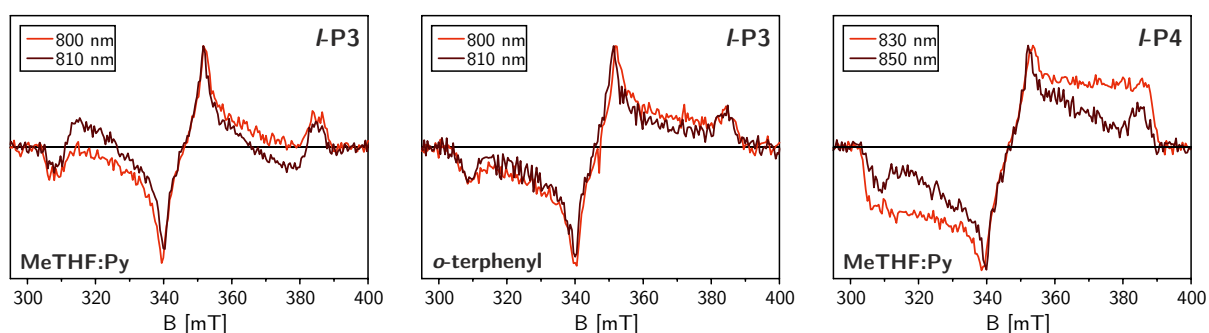


FIGURE 4.37: Time-resolved EPR spectra recorded at 20 K for *l*-P3 in MeTHF:pyridine 10:1 and in *o*-terphenyl and for *l*-P4 in MeTHF:pyridine 10:1 using different excitation wavelengths at the red edge of the absorption spectrum.

Wavelength-dependent ENDOR measurements were also attempted for *l*-P3. The magnetic field position potentially providing the most information on the presence of monomer-, dimer- and trimer-like hyperfine couplings was identified as the high field Y position (380 mT) and the ENDOR spectra recorded for *l*-P1, *l*-P2 and *l*-P3 at this field position are shown in figure 4.38 A.

The ENDOR spectra were recorded in both MeTHF:pyridine and in *o*-terphenyl with excitation at different wavelengths and are shown in 4.38 B. The worse signal-to-noise ratio for *l*-P3 is due to its decreased triplet yield. ENDOR measurements at 800 nm, where the completely planar conformation of *l*-P3 absorbs, could not be recorded with a sufficient signal-to-noise ratio in a reasonable time due to the nature of the spin polarisation in the corresponding spectrum, which leads to a signal intensity close to zero for this field position. The hyperfine peak at about -2 MHz is present in all ENDOR spectra and was assigned to the hyperfine coupling in the out-of-plane orientation of the H₁ protons, the β protons close to the triple bonds, on the central porphyrin unit (see section 4.3.3). An additional peak at -1.3 MHz appears for excitation at 760 nm for both solvent systems. The ENDOR spectrum recorded in *o*-terphenyl is characterised by broader peaks at similar positions and this broadening could be due to the wider range of conformations frozen in due to the higher freezing point. The

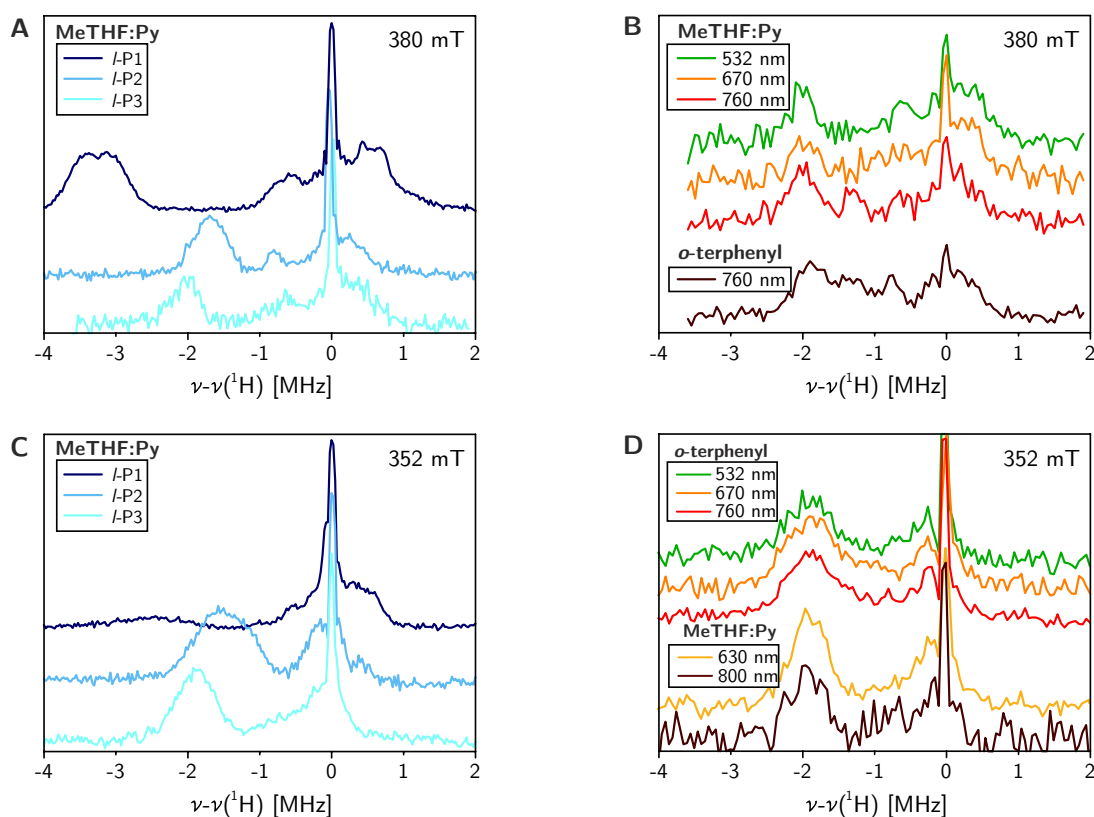


FIGURE 4.38: (A/C) Mims ENDOR spectra recorded at 380/352 mT with excitation at 532 nm for *l*-P1, *l*-P2 and *l*-P3 in MeTHF:pyridine 10:1. (B/D) ENDOR spectra recorded at 380/352 mT for *l*-P3 in MeTHF:pyridine 10:1 and in *o*-terphenyl using different excitation wavelengths. The ENDOR spectra shown in B were recorded with longer τ (200-280 ns) values compared to the ones shown in A, as this lead to higher ENDOR intensities for small hyperfine couplings; the decreased intensity of the Larmor peak with respect to the spectra in A is also due to this.

hyperfine coupling of -1.3 MHz determined from this additional peak is smaller than that corresponding to the peak in the *I*-P2 ENDOR spectrum (-1.7 MHz), but could still correspond to the hyperfine coupling for a conformation with only two coplanar porphyrin units. In that case it is somewhat surprising that this peak would not be present for excitation at lower wavelengths, corresponding to the maximum of the dimer-like contribution to the time-resolved EPR spectrum. Alternatively, since the wavelength is quite high, this signal could correspond to the planar conformation with a different spin density distribution approaching symmetrical delocalisation over the three porphyrin units. However, then the intensity and hyperfine coupling of the peak at about -2 MHz would be difficult to explain, since the intensity is too strong and the hyperfine coupling probably too large to correspond to a dimer-like triplet state. Further, the same peak at -1.3 MHz should then still be present and stronger at 810 nm, which is not observed within the signal-to-noise achievable for that field position and wavelength (data not shown).

The best signal intensities would be obtained for the X transitions and thus ENDOR measurements at the high-field X position (352 mT) were also performed and the results are shown in figure 4.38 D. No significant changes are apparent in the whole investigated wavelength range for this field position. This is not surprising if the peak at -2 MHz is assigned to the completely planar trimer, as the corresponding spectrum will always give the largest contribution to the EPR signal at this field position (see figure 4.36 C). This result renders the assignment of the peak at -1.3 MHz at the Y field position to a planar conformation very unlikely. Accordingly, assignment of this peak to a dimer-like conformation seems probable, but cannot easily be unequivocally confirmed by experiments.

In conclusion, this excitation wavelength-dependent EPR study has provided further information on the conformational distribution of the porphyrin dimer and oligomers. The contributions of different porphyrin dimer conformations at different wavelengths are well known from optical studies and time-dependent DFT calculations and the EPR results are in agreement with these results. The changes observed in both EPR and ENDOR spectra can be rationalised in terms of a very approximate model for the distributions of different conformations at different excitation wavelengths. The interpretation of the results for the porphyrin trimer and tetramer is complicated by the increased number of possible conformations and the lack of information on their contribution in different regions of the absorption spectrum. The analysis is further complicated by the possible presence of aggregates. The changes in the time-resolved EPR spectra can nevertheless be interpreted assuming two contributions: the contribution of a planar conformation with a spin polarisation determined from the EPR spectrum recorded at the maximum of the Q band absorption peak and the contribution of twisted conformations with the same spin polarisation as the porphyrin dimer.

4.3.6 PORPHYRIN OLIGOMERS BOUND TO TEMPLATES

In addition to the linear oligomers, oligomers bound to the template used for the synthesis of the six-membered ring were also investigated (see figure 4.39). The binding to a template places the

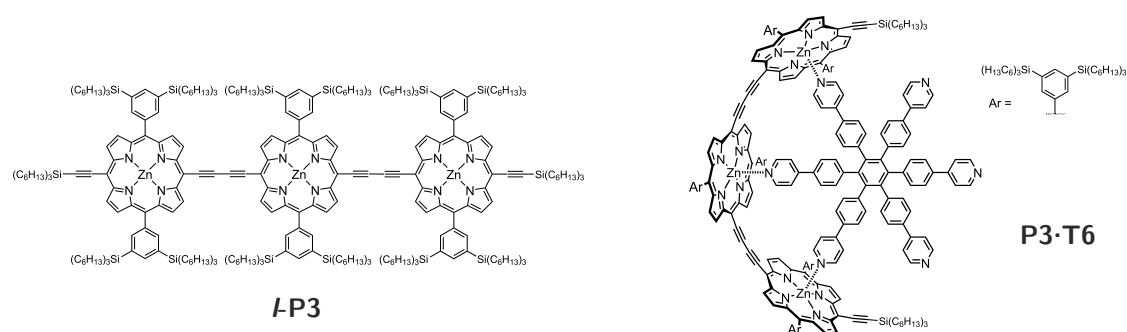


FIGURE 4.39: Molecular structure of the linear oligomer *I*-P3 and of *I*-P3 bound to the T6 template.

porphyrin units at angles of approximately 120° to each other and therefore allows the determination of the effect of the geometry on the zero-field splitting parameters and on triplet state delocalisation.

The time-resolved EPR spectra recorded for the porphyrin dimer, trimer, tetramer and hexamer in a toluene solution with an excess of T6 template are shown in figure 4.40. The binding of the porphyrin to the template was verified by UV-Vis measurements at room temperature. For the porphyrin dimer, only relatively small changes are observed upon addition of the template, while significant changes are observed for the longer oligomers. The *D*-values decrease considerably, as indicated by the reduced width of the triplet state EPR spectrum. The EPR spectrum of P3·T6 and to a certain degree also that of P4·T6 show a clearly resolved structure and spin polarisation. The spectrum is less well-defined for P6·T6, which might be due to a distribution of conformations and thus EPR spectra, causing broadening of parts of the spectrum. In all cases, a weak signal resembling the spectrum of the free oligomer is also present. Typically an excess of T6 template was used and UV-Vis data show complete binding at room temperature, but dissociation of the porphyrin oligomer from the template at low temperatures cannot be excluded.

The significant decrease in *D* would suggest increased delocalisation, according to modelling within the framework of the point-dipole approximation. It was however concluded from the analysis of the linear oligomers that much more accurate information on the triplet state delocalisation can be obtained from the hyperfine couplings. Therefore ENDOR measurements were also performed on the templated porphyrin oligomers at the high-field X position. The results for the templated porphyrin dimer and trimer are shown in figure 4.41 and are compared to the corresponding measurements on the free oligomers. The ENDOR spectra recorded for the free and bound porphyrin dimer and trimer are almost identical; similar results are obtained for the tetramer and hexamer.

The ENDOR data shown in figure 4.41 indicate that no changes in the extent of the triplet state wavefunction occur upon binding of the T6 template, hence a change in triplet state delocalisation cannot be the cause for the observed decrease of the zero-field splitting parameter *D* upon binding to the template. In order to understand the observed reduction of *D*, DFT geometry optimisations and calculations of the ZFS and hyperfine parameters were performed on the porphyrin oligomers bound to

the template. The ZFS parameters calculated at B3LYP/EPRII level are compared to the experimental results in table 4.4.

The decrease in D is well reproduced by the DFT results; experimentally the ratio of D -values for the porphyrin trimer with the bent structure on the template and for the linear structure is 0.53 and DFT predicts a ratio of 0.59. Similarly for the porphyrin tetramer the experimental ratio is 0.41 and DFT predicts a ratio of 0.52. For the porphyrin dimer the interpretation is more difficult, as only small changes are observed in the spectrum (figure 4.40). However, some discontinuities between the Y and X transition (at about 320 mT and about 375 mT, see arrows in figure 4.40) might indicate the presence of a second contribution and the spectrum can be simulated as a linear combination of the

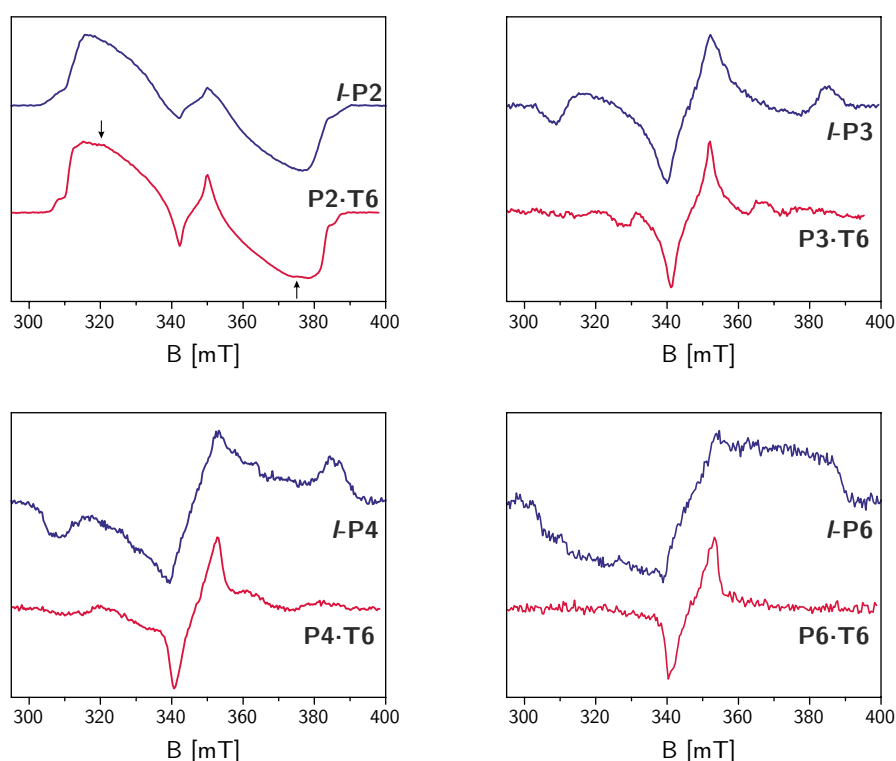


FIGURE 4.40: Time-resolved EPR spectra recorded at 20 K up to 2 μ s after the laser pulse at 532 nm for the linear oligomers l -P2 to l -P6 in toluene:pyridine 10:1 and of the oligomers bound to the T6 template in toluene.

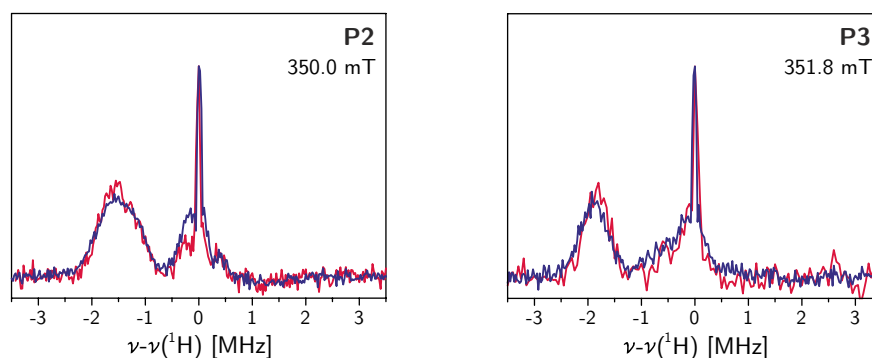


FIGURE 4.41: ENDOR spectra recorded at the high-field X position for the linear porphyrin dimer and trimer in toluene solution (blue) and bound to the T6 template (red). Excitation at 532 nm was used in both cases.

TABLE 4.4: Experimental and calculated zero-field splitting parameters for the free and bound porphyrin oligomers with two to four porphyrin units. The DFT calculations were performed at the B3LYP/EPRII level as described in section 4.2.5.

	Experiment		B3LYP/EPRII	
	D [MHz]	E [MHz]	D [MHz]	E [MHz]
<i>l</i> -P2	-1117 ± 9	-284 ± 2	-609	-72
P2·T6	-890 ± 25	-51 ± 15	-468	-51
<i>l</i> -P3	-1169 ± 7	-269 ± 2	-456	-84
P3·T6	-621 ± 6	-102 ± 2	-270	-36
<i>l</i> -P4	-1195 ± 8	-273 ± 2	-465	-60
P4·T6	-486 ± 12	-48 ± 3	-243	-6

spectrum in toluene and an additional spectrum with a decreased D -value, assigned to P2·T6, with a ratio of 0.85 : 0.15 (the corresponding ZFS values are given in table 4.4). The binding constant of the porphyrin oligomer to the template increases with the number of porphyrin units, it is thus possible that even in the presence of an excess of template, the binding is not complete for the porphyrin dimer in frozen solution.

In a recent study on the interpretation of zero-field splitting parameters in terms of electron-electron distances by Riplinger et al. [129], the dependence of the D -value on the torsion angle between two fragments of a molecule containing the unpaired electrons was investigated by studying the overlap of the localised singly occupied molecular orbitals (SOMOs). The quasi-restricted molecular orbitals from single-point calculations performed at B3LYP/EPRII level for the porphyrin monomer, dimer, trimer and tetramer are shown in figure 4.42. The corresponding orbitals of the templated oligomers are shown in figure 4.43. The SOMOs localised using the Pipek-Mezey scheme [167] are also shown and were used to separate the Coulomb and exchange contribution to the D -value. The localised orbitals are linear combinations of the delocalised orbitals and are limited to a smaller region of the molecule.

The overlap between the two SOMOs determines the magnitude of the D -value, increased overlap leads to an increase of both the Coulomb and the exchange contributions to the electron spin-spin interaction. The exchange contribution depends directly on the overlap integral and the Coulomb contribution depends on the distance between spin-carrying orbitals. A larger overlap of the SOMOs leads to more Coulomb contributions with small inter-spin distance, which correspond to larger contributions to the D -value due to the r^{-3} dependence. Comparison of the populations of the SOMOs on the different porphyrin units for the linear oligomers and the oligomers bound to a template shows that there is a larger overlap for the linear systems with respect to the bent ones, leading to larger D -values, as observed experimentally.

These results show that caution must be exerted in the interpretation of zero-field splitting D -values in terms of triplet state delocalisation in molecular-wire-type systems with extensive conjugation between the monomeric units, as changes in geometry can cause significant changes in the magnitude of D , which could be wrongly interpreted in terms of increased or decreased triplet state delocalisation.

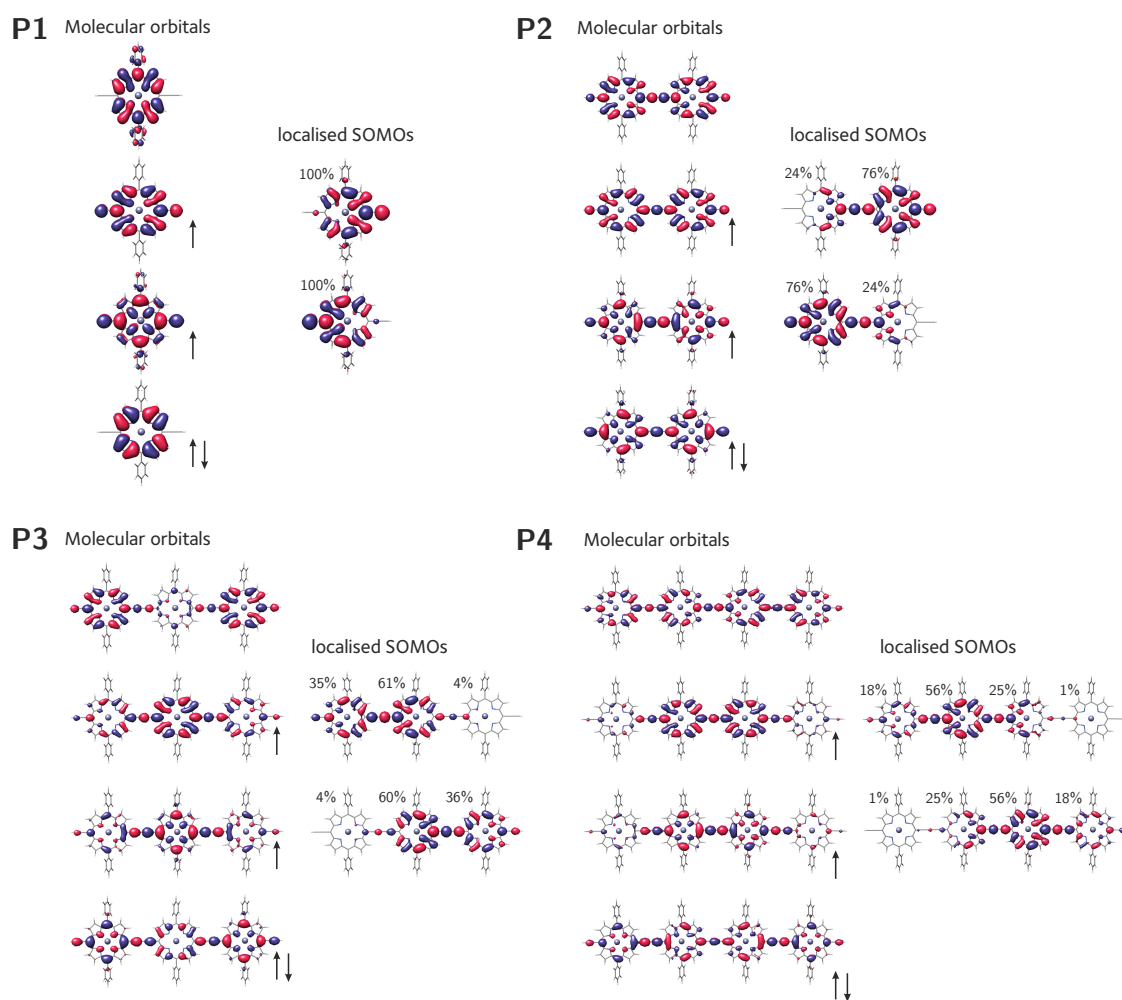


FIGURE 4.42: Plots of the frontier quasi-restricted molecular orbitals and of the localised SOMOs calculated with B3LYP/EPRII for *l*-P1, *l*-P2, *l*-P3 and *l*-P4. The populations of the SOMOs on the different porphyrin units are indicated as percentages. The arrows indicate the electrons occupying each orbital.

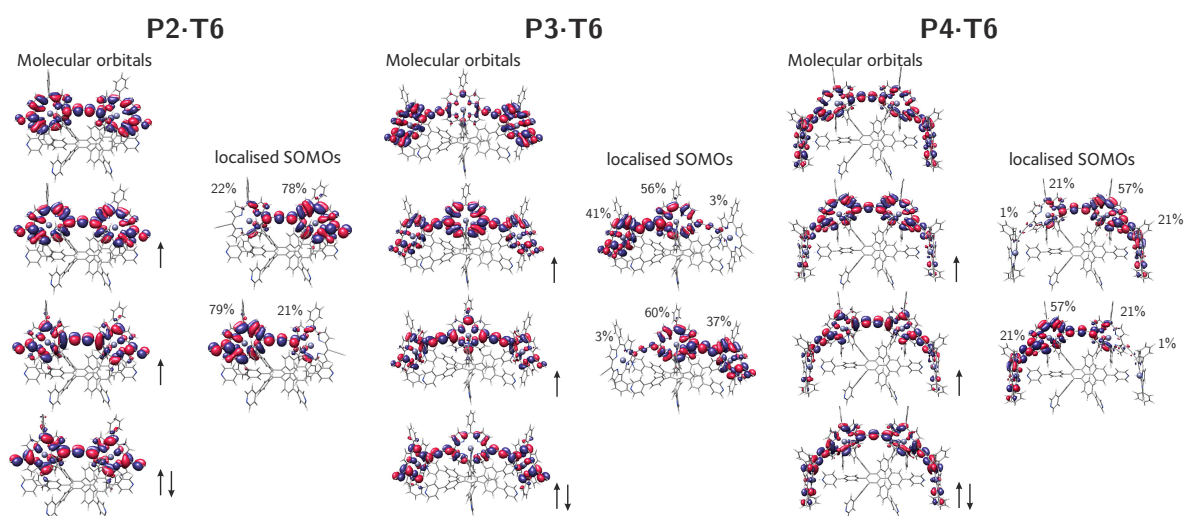


FIGURE 4.43: Plots of the frontier quasi-restricted molecular orbitals and of the localised SOMOs calculated with B3LYP/EPRII for P2-T6, P3-T6 and P4-T6. The populations of the SOMOs on the different porphyrin units are indicated as percentages. The arrows indicate the electrons occupying each orbital.

4.3.7 PORPHYRIN RINGS

In addition to the linear porphyrin oligomers, the six-membered ring has also been investigated, since the symmetry and the lack of end-group effects could influence the delocalisation of the excited triplet state. The structures of the six-membered porphyrin ring without and with rigid internal template are shown in figure 4.44.

The time-resolved EPR spectra recorded for the six-membered porphyrin ring without template (*c*-P6), the porphyrin ring with template (*c*-P6-T6) and the free base porphyrin ring are shown in figure 4.45 A. The zero-field splitting parameters and relative sublevel populations determined by simulation are reported in table 4.5. The spectra are considerably broadened and the canonical positions cannot be clearly identified based on just the time-resolved EPR data.

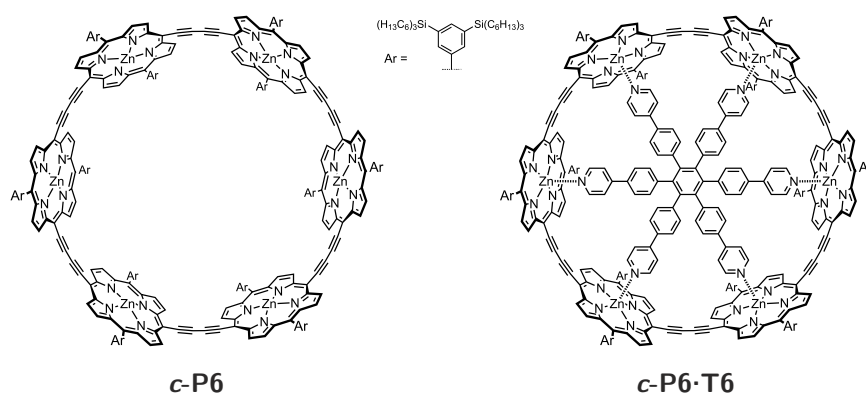


FIGURE 4.44: Molecular structures of the six-membered ring without (*c*-P6) and with T6 template (*c*-P6-T6).

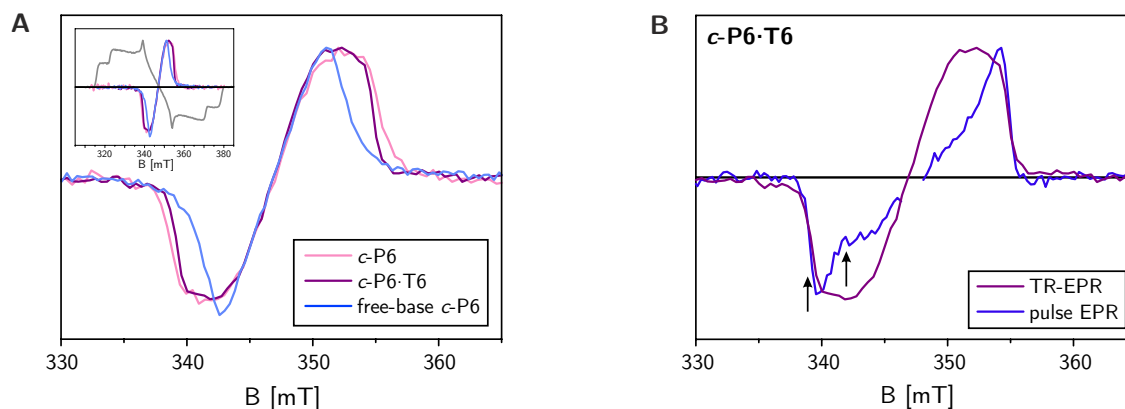


FIGURE 4.45: (A) Time-resolved EPR spectra recorded at 20 K for *c*-P6, *c*-P6-T6 and free base *c*-P6. The spectra are compared to the EPR spectrum of *l*-P1 in the inset. (B) Comparison of the time-resolved and echo-detected EPR spectra of *c*-P6-T6. The central part of the field sweep was removed due to the presence of a residual radical signal not completely removed by the pre-saturation pulse. The canonical field positions identified from the field sweep are indicated by arrows.

TABLE 4.5: Zero-field splitting parameters and relative sublevel populations determined by simulation of the time-resolved EPR data of c-P6, c-P6·T6 and free base c-P6 shown in figure 4.45 A. The relative sublevel population values are affected by errors of 0.07, 0.02 and 0.03, respectively, for the three porphyrin rings.

	$ D $ [MHz]	$ E $ [MHz]	E/D	$P_X : P_Y : P_Z$
c-P6	244 ± 16	61 ± 11	0.25	0.49 : 0.51 : 0.00
c-P6·T6	230 ± 3	52 ± 1	0.23	0.43 : 0.57 : 0.00
free base c-P6	209 ± 11	29 ± 5	0.14	0.39 : 0.61 : 0.00

Triplet state EPR spectra recorded using pulse EPR typically show increased intensities at the canonical field positions and reduced signal intensities for non-canonical orientations compared to the corresponding time-resolved cw-EPR spectra [123, 168]. These differences have been explained by the influence of modulations of the orientations of the ZFS tensor axes on the spin-spin relaxation time T_2 . T_2 is shortened for non-canonical orientations with respect to canonical orientations, as the angular dependence of the ZFS interaction is strongest and hence the modulations have the most significant effect for these off-axis orientations [123, 168]. The echo-detected field sweep of c-P6·T6 is compared to the time-resolved EPR spectrum in figure 4.45 B. The Z canonical field position can be clearly identified from the sharp increase in signal intensity. The X and Y field positions are more difficult to identify, although a discontinuity in the spectrum around 341 mT could indicate the presence of the Y canonical field position. The identification of two canonical field positions, in addition to the centre of the spectrum is sufficient to assign both D - and E -values and these values have been used to guide the simulations of the time-resolved data for c-P6·T6. Similar results, with only a small increase in D were obtained for the porphyrin ring without template. The EPR spectrum of the free base ring is characterised by an even smaller D -value and by slightly different spin polarisation. In this case no canonical field positions in addition to Z could be clearly identified from the pulse EPR data, leading to a larger uncertainty on the E -value and the relative sublevel populations.

The comparison with the spectrum of *l*-P1 shows that the D -value is significantly smaller in the rings, suggesting increased delocalisation. The results of the previous section have, however, shown that a decrease in D does not necessarily imply changes in the extent of triplet state delocalisation and hence ENDOR measurements were also performed to determine the proton hyperfine couplings.

The ENDOR spectra recorded at the high-field Z position for c-P6 and c-P6·T6 are shown in figure 4.46 A; the spectra are compared to the *l*-P1 ENDOR spectrum in the inset. The ENDOR spectra of both c-P6 and c-P6·T6 are characterised by a hyperfine peak at lower frequencies with respect to the nuclear Larmor peak and corresponding to a hyperfine coupling of about 0.6 MHz. Since the largest hyperfine couplings in the porphyrin systems investigated here are negative, the high-field Z position can be assigned to the $m_S = -1 \rightarrow m_S = 0$ transition and therefore the D -value can be concluded to be positive. Further, since the largest hyperfine couplings are observed in the direction of the phenyl substituents, the Z axis can be assigned to the out-of-plane axis of the porphyrin ring (see figure 4.47). The sign of D and the direction of the Z axis indicate an oblate spin distribution, as expected for complete delocalisation around the porphyrin ring. The hyperfine coupling for the porphyrin rings correspond to approximately one sixth of the corresponding hyperfine coupling in *l*-P1

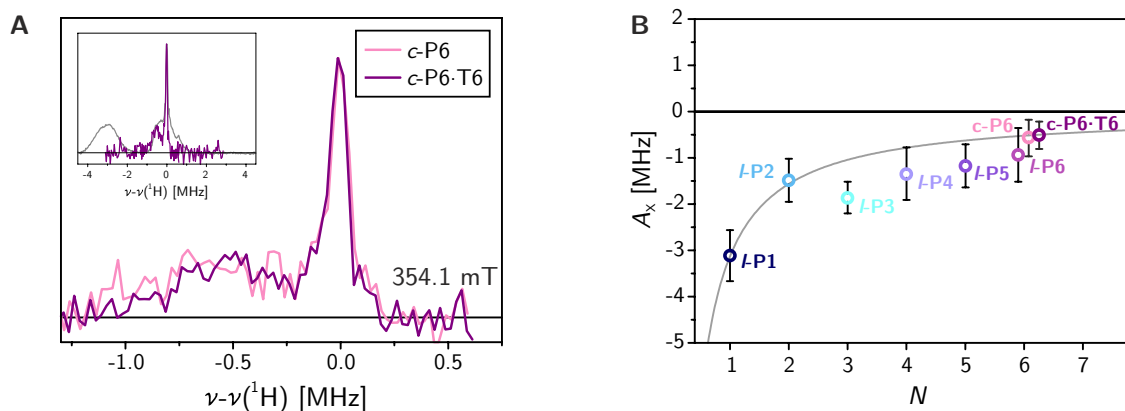


FIGURE 4.46: (A) Mims ENDOR spectra recorded at 354.1 mT for the porphyrin ring without and with template. (B) Hyperfine couplings of the H_1 protons along the ZFS axis in the porphyrin plane perpendicular to the butadiyne bonds determined by Gaussian fitting of the principal hyperfine peak in the experimental ENDOR spectra as a function of the number of porphyrin units, the error bars indicate the full width at half maximum. The grey line corresponds to the theoretical N^{-1} relationship for the hyperfine couplings in case of complete delocalisation.

(see figure 4.46 B), confirming that the triplet state is delocalised over all six porphyrin units in the porphyrin ring. The peak is shifted to slightly higher hyperfine couplings in c-P6 compared to c-P6-T6, indicating a slightly decreased extent of delocalisation in the template-free ring. The hyperfine values and ZFS tensor orientation are also in good agreement with DFT predictions for a completely delocalised triplet state.

The assignment of the orientation of the ZFS tensor with the X and Y axes in the plane of the porphyrin ring and with Z as the out-of-plane axis allows an attempt at explaining the observed spin polarisations. In all three cases, the $EEEEAA$ spin polarisation indicates that the triplet sublevels corresponding to the orientations in the ring plane are mainly populated. An analysis similar to that used to explain the spin polarisations in the linear oligomers can also be applied here. A theoretical calculation based on the assumption of population of the triplet state promoted solely by zinc spin-orbit coupling yields $P_X : P_Y : P_Z = 0.46 : 0.54 : 0.00$, with increasing population P_X for increasing deviations from circular symmetry (flattening of the porphyrin ring in one direction). The relative population ratios of the zinc porphyrin rings can again be calculated as linear combinations of the free base populations and the populations for a perfectly symmetric ring for c-P6-T6, while the populations for a ring with elliptical geometry, characterised by two internal inter-porphyrin angles of 50° and four angles of 155° , have to be used to obtain a good agreement with the experimental results for c-P6. In both cases the two ISC mechanisms, direct spin-orbit coupling promoted by mixing of the zinc d -orbitals with the porphyrin π system and vibronic spin-orbit coupling, seem to contribute to a similar extent, with ratios of $0.57 : 0.43$ for c-P6-T6 and of $0.48 : 0.52$ for c-P6. This interpretation certainly seems plausible, since the vibrations are restricted in the ring systems and therefore the contribution of the direct zinc spin-orbit coupling could be more important in these systems with respect to the more flexible linear oligomers, where the vibronic contribution was shown to carry more

weight as the size of the systems increased. The changes between the ring with and without template also seem to be in agreement with increased rigidity in the porphyrin ring bound to the template.

4.4 CONCLUSIONS

The triplet state delocalisation in linear, bent and cyclic porphyrin arrays was investigated using time-resolved EPR to characterise the ZFS interaction and ENDOR, ESEEM and HYSCORE to study the proton and nitrogen hyperfine interactions.

The results of ENDOR, ESEEM and HYSCORE measurements of proton and nitrogen hyperfine couplings indicate complete delocalisation of the triplet state over both porphyrin units in the planar dimer molecule. The orientation selectivity inherent to pulse EPR measurements on photoexcited triplet states, in combination with the results of DFT calculations, also allowed the determination of the orientation of the ZFS tensor in the molecular frame. Both these results and the corresponding results of magnetophotoselection studies have provided evidence for the reorientation of the zero-field splitting tensor from the porphyrin monomer to the dimer and for a sign change of the D -value. The transition from an oblate spin distribution in the monomer to a prolate spin distribution in the dimer leads to the shift of the axis of maximum dipolar coupling, Z , from the out-of-plane axis in the monomer to the long axis of the molecule in the dimer (figure 4.47). The occurrence of such a reorientation has previously been proposed for similar systems based on the changes in ZFS parameters [113, 124, 128], but the results shown here provide the first irrefutable experimental proof for its occurrence. The reorientation of the ZFS tensor also explains the changes in D and E as well as in spin polarisation observed in the time-resolved EPR spectra of l -P1 and l -P2. The mechanism driving the ISC in zinc porphyrins is determined by direct spin-orbit coupling due to mixing of the d -orbitals of zinc with the porphyrin π system and leads to dominant population of the out-of-plane sublevel [136, 137]. This mechanism and the reorientation of the ZFS tensor explain the spin polarisation of the l -P1 and l -P2 EPR spectra. The results of this comprehensive characterisation of the changes in triplet state properties between the porphyrin monomer and dimer have provided the necessary foundation for understanding the triplet state delocalisation in longer polymer chains.

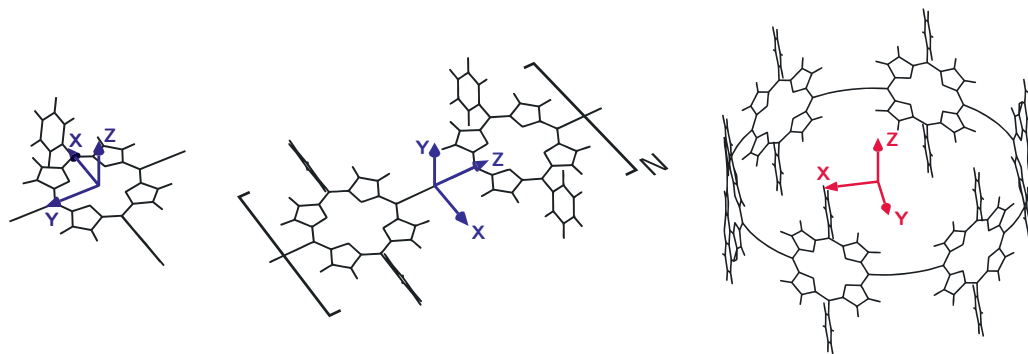


FIGURE 4.47: Orientation of the zero-field splitting tensors in the molecular frame of l -P1, l -PN and c -P6.

The results of proton ENDOR measurements on longer linear oligomers with up to six porphyrin units have been interpreted in terms of triplet states with uneven spin density distributions characterised by increased spin density on the central porphyrin units of the oligomer systems. The changes in spin polarisation of the EPR spectra of the longer porphyrin oligomers were explained by assuming the increasing importance of a competing ISC mechanism induced by molecular vibrations as the length of the oligomer increases.

Excitation wavelength-dependent time-resolved EPR and ENDOR measurements have shown that different conformations of the porphyrin oligomers contribute at different wavelengths and are characterised by different extents of the triplet state wavefunction, depending on the relative orientation of adjacent porphyrin units.

The triplet state was found to be completely delocalised in symmetric cyclic porphyrin rings with and without the rigid internal template. Evidence for increased delocalisation was obtained from the ZFS parameters and was confirmed and quantified by proton ENDOR measurements. Comparison of the experimental results with the results of DFT calculations allowed assignment of the ZFS tensor also in this system (see figure 4.47) and showed that the D -value is positive, as characteristic for an oblate spin density distribution.

Analysis of the hyperfine couplings was required to determine the extent of triplet state delocalisation. Determination of the extent of delocalisation from the ZFS D -value alone using the popular point-dipole approximation would have led to an underestimation of the true delocalisation length in the linear oligomer systems. The results of EPR and ENDOR measurements on oligomers forced into a bent conformation by binding to a template have shown that the D -value is very sensitive to the geometry of the system and does not accurately reflect the extent of triplet state delocalisation. DFT was shown to provide a useful tool for the interpretation of trends in the change of ZFS parameters with regard to triplet state delocalisation.

In contrast to previous findings [112, 113], the extent of electron delocalisation in the radical cations (at low temperatures) and in the triplet states of the multiporphyrin arrays investigated here were found to be very similar. In particular, the triplet state as well as the radical cation state was found to be delocalised over more than a single porphyrin unit. Due to the orientation selection for the triplet state and the larger proton hyperfine couplings, the delocalisation of the triplet state could be determined more accurately than the delocalisation in the radical cations, as the experimental data provided more detailed and better resolved information.

5 DIPOLAR SPECTROSCOPY

The interactions between electron and nuclear spins and the techniques used for their measurement were discussed in chapter 2. This chapter is dedicated to the measurement of weak interactions between two or more electron spins. Measurement of the dipolar interaction between electron spins can provide information on the distance between them and can thus be exploited for the determination of macromolecular structures through measurement of long-distance constraints between different paramagnetic centres at specific points of the structure. Different techniques can be applied for these measurements: short distances between 1 and 1.7 nm are typically best determined from the dipolar broadening of continuous wave EPR spectra and distances above 1.7 nm up to about 8-12 nm can be determined by the different variants of the pulse EPR technique Double Electron Electron Resonance (DEER) or synonymously Pulsed ELection DOuble Resonance (PELDOR) [169, 170].

Dipolar spectroscopy is used for the characterisation of the structure and dynamics of biological systems such as proteins, protein complexes and DNA or RNA [171, 172]. The advantages of EPR techniques over the more established X-ray crystallography and solution-state NMR techniques are the ability to study protein complexes or membrane proteins, which often do not form single crystals required for X-ray analysis and are too large to be studied efficiently by NMR. In addition to this, EPR can provide information on long-distance constraints, while NOE constraints in NMR are generally limited to short distances up to around 0.5 nm.

The paramagnetic centres used for dipolar spectroscopy are often nitroxide spin labels introduced at selected points of the protein sequence by site-directed mutagenesis. The most commonly used nitroxide spin label for proteins is (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTSL) (figure 5.1 A), which is attached to cysteine residues by a disulfide bond. Many other types of nitroxide spin labels have been developed for attachment to different sites in different biopolymers and to modulate the mobility of the spin label [173]. Trityl radicals are now also sometimes used instead of or in addition to nitroxide labels [174]. In some cases, the paramagnetic centres can be paramagnetic cofactors already present in the investigated protein structure, for example metal ions, such as copper, manganese or iron (figure 5.1 B and C), or organic radicals, such as for example tyrosyl radicals (see [175, 176] and references therein). The use of gadolinium spin labels is also becoming increasingly more common [175, 177, 178].

The EPR techniques used for distance measurements and the data analysis method required to extract the distance and in some case orientation information are described in the following sections.

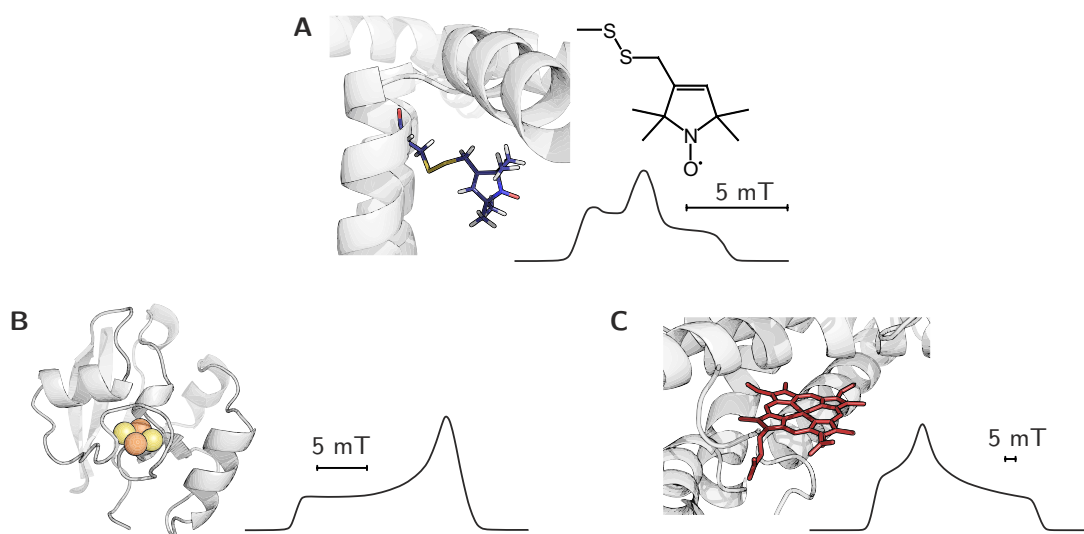


FIGURE 5.1: Paramagnetic centres intrinsically present or artificially introduced in the protein systems investigated in this thesis and their X-band EPR spectra: MTSL nitroxide spin label (A), iron-sulfur cluster (B) and low-spin ferric heme group (C).

5.1 THE DIPOLAR INTERACTION AND THE PAKE PATTERN

The electron-electron dipole and exchange interactions were described in detail in section 1.2.6. The isotropic exchange interaction requires orbital overlap and can generally be neglected if the paramagnetic centres used for structural studies have localised spins and are separated by more than 1.5 nm. The spin Hamiltonian describing a system of two weakly coupled electron spins with $S_A = S_B = \frac{1}{2}$ can be written as:

$$\hat{H}(S_A, S_B) = \frac{\beta_e}{\hbar} B_0 g_A \hat{S}_A + \frac{\beta_e}{\hbar} B_0 g_B \hat{S}_B + \hat{S}_A \mathbf{D}_{AB} \hat{S}_B \quad (5.1)$$

where, neglecting the non-secular and pseudo-secular terms, $\mathbf{D}_{AB}$ is given by

$$\begin{aligned} D_{AB} &= \frac{\mu_0}{4\pi\hbar} \frac{g_A g_B \beta_e^2}{r_{AB}^3} (1 - 3\cos^2\theta) \hat{S}_{Az} \hat{S}_{Bz} \\ &= \omega_{dd} (1 - 3\cos^2\theta) \hat{S}_{Az} \hat{S}_{Bz} \end{aligned} \quad (5.2)$$

with θ the angle between the magnetic field $\mathbf{B}_0$ and the distance vector $\mathbf{r}_{AB}$, as shown in figure 5.2 B. The point-dipole approximation is usually valid for nitroxide labels, since the two spins are sufficiently far apart to neglect the distributions of the spin densities in most cases, however, when the spin density of the paramagnetic centres is delocalised over several atoms, a distributed point-dipole model is more appropriate [129]. In the weak-coupling regime ($\Delta\omega > \omega_{dd}$, where $\Delta\omega$ is the difference in resonance frequency of the two spins), the splitting between the most prominent peaks in the Pake pattern corresponds to ω_{dd} , as shown in figure 5.2 A. In the strong-coupling regime ($\Delta\omega < \omega_{dd}$) the

pseudo-secular terms of the dipolar interaction spin Hamiltonian cannot be neglected, leading to different eigenstates and eigenvalues with respect to the weak coupling regime. In that case the splitting between the peaks in the Pake pattern increases to $\frac{3}{2}\omega_{dd}$.

The dipolar frequency $\omega_{AB} = \omega_{dd}(1 - 3\cos^2\theta)$ depends on the orientation of the two-spin systems with respect to the magnetic field and can assume a range of values from ω_{dd} , corresponding to $\theta=90^\circ$, to $2\omega_{dd}$, corresponding to $\theta=0^\circ$. In isotropic frozen solutions, all orientations of the spin system with respect to the external field contribute, giving rise to the so-called Pake pattern shown in figure 5.2.

In most cases the g - and A -anisotropies are much larger than the dipolar interaction and thus determine the appearance of the EPR spectrum. However, for short inter-spin distances, the dipolar interaction is sufficiently large to cause observable broadening of the EPR spectrum, corresponding to a convolution of the EPR spectrum of the isolated spins with the Pake pattern due to the dipolar interaction between them (shown in figure 5.2 A for two unlike spins).

Longer distances can be determined using DEER or one of its variants, where the echo intensity is modulated by the dipolar frequency ω_{dd} (see section 5.3). A Fourier transform of the modulated DEER trace yields a Pake pattern similar to that shown in figure 5.2 C.

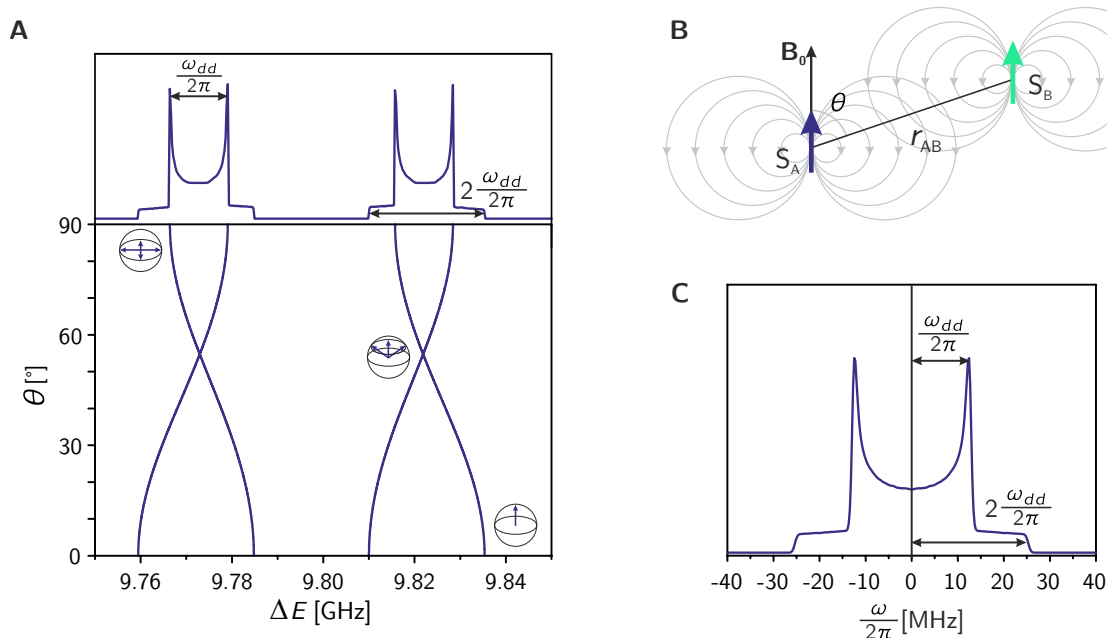


FIGURE 5.2: (A) EPR transition frequencies for a two-spin system described by the Hamiltonian given in equations 5.1 and 5.2 in the weak coupling regime ($\Delta\omega > \omega_{dd}$) for different relative orientations of the magnetic field and the distance vector r_{AB} depicted in B ($r_{AB} = 1.6$ nm, $g_A = 2.005$, $g_B = 1.995$, $B_0 = 350$ mT). The weighted sum over all contributions gives the Pake patterns shown on top of the graph. (B) Schematic diagram of a two electron spin system and definition of the angle θ between the magnetic field B_0 and the distance vector r_{AB} connecting the two spins. (C) Dipolar spectrum for two spins at a distance $r_{AB} = 1.6$ nm obtained by a weighted sum of the differences between the two pairs of EPR transition frequencies shown in A over all values of θ (weak-coupling regime Pake pattern). The turning points correspond to $\omega_\perp$ and $\omega_\parallel$ and are equal to ω_{dd} and $2\omega_{dd}$ in the absence of exchange interaction.

In the presence of a single fixed distance, the positions of the turning points in the dipolar spectrum can be directly related to the inter-spin distance r_{AB} due to the r_{AB}^{-3} dependence of ω_{dd} . In the presence of distance distributions or multiple distances, a different type of analysis is required as will be described in the following sections. In some cases, not all the orientations of a spin system are excited in pulse dipolar spectroscopy, so that only incomplete Pake patterns can be detected and an analysis explicitly considering orientation selection is required for the extraction of the average distance and distance distribution.

5.2 CONTINUOUS WAVE EPR

Continuous wave EPR can provide information on the distance between paramagnetic centres if the dipolar interaction is sufficiently large to cause a broadening of the cw-EPR powder lineshape. In the case of nitroxide spin labels the upper limit for the inter-spin distance that can be reliably determined by cw-EPR corresponds to 1.5-1.7 nm [169]. The typical width of a nitroxide EPR spectrum is around 200 MHz and a distance of 1.5-1.7 nm would correspond to a dipolar coupling of 10-15 MHz, causing a visible broadening. Frozen solution cw-EPR can provide qualitative information on the presence of distances shorter than about 2.0 nm ($\frac{1}{2\pi}\omega_{dd} = 6.5$ MHz) by comparison of the spectrum recorded in the presence of multiple spin labels with the spectrum recorded for the corresponding singly-labelled system.

The extraction of accurate quantitative distance information is more difficult and a range of different methods have been proposed [169, 171]. Second moment analysis and convolution methods are described below. Another method frequently used is Fourier deconvolution [179] based on the determination of the dipolar broadening function by deconvolution in Fourier space. Other, less commonly used methods also exist and are described, for example, in reference [171]. All of these methods are however affected by significant errors, since extremely high-quality data are required for accurate analysis, as the effects on the EPR spectrum are typically very small. Incomplete spin labelling can also lead to significant errors in the analysis, since the spectrum is then a combination of the broadened and unbroadened spectrum. In addition to this, the contributions of dipolar and exchange interaction cannot be distinguished, leading to errors in the determined distances if the spectra are also affected by exchange interaction (normally only important at distances below about 1.5 nm [129]).

5.2.1 SECOND MOMENT ANALYSIS

The difference between the second moments, M_2 , of the EPR spectra of a doubly labelled (d) and the corresponding singly-labelled (s) system can be shown to be proportional to r_{AB}^{-6} [180, 181]:

$$\begin{aligned} \mu_2[T^2] &= \frac{3}{5} \frac{g^2 \mu_B^2 \mu_0^2}{(4\pi)^2} S(S+1) \frac{1}{r_{AB}^6} \\ \mu_2 &= M_2^{(d)} - M_2^{(s)} \end{aligned} \quad (5.3)$$

where the second moments of the singly- and doubly-labelled spectra are calculated according to equation 3.3. The average inter-spin distance r_{AB} can thus be calculated as:

$$r[m] = \left(\frac{3}{5} \frac{g^2 \mu_B^2 \mu_0^2}{(4\pi)^2} S(S+1) \right)^{\frac{1}{6}} \mu_2^{-\frac{1}{6}} \quad (5.4)$$

The derivation of these expressions is based on the assumption of strong coupling between the two electron spins ($\Delta\omega < \omega_{dd}$), in the presence of weak coupling a correction factor of $\frac{3}{2}^{-\frac{1}{3}}$ has to be considered for the distance.

The second moment of an EPR spectrum is very sensitive to changes in the outer wings of the spectrum. Consequently, errors in the baseline correction can lead to significant errors in the extracted inter-spin distance. The extent of the error on the extracted distance can be evaluated based on the standard deviations of the two second moments, evaluated from a series of baseline corrections with different polynomial functions and different baseline fitting limits, as:

$$\Delta r[m] = \frac{1}{6} \left(\frac{3}{5} \frac{g^2 \mu_B^2 \mu_0^2}{(4\pi)^2} S(S+1) \right)^{\frac{1}{6}} \mu_2^{-\frac{7}{6}} \sqrt{\left(\sigma_{M_2}^{(d)} \right)^2 + \left(\sigma_{M_2}^{(s)} \right)^2} \quad (5.5)$$

5.2.2 CONVOLUTION

The broadening of the cw-EPR spectrum due to the dipolar interaction arises from the convolution of the spectrum corresponding to the isolated spins with a dipolar broadening function, consisting of the superposition of the Pake patterns for all of the distances included in the distance distribution for the coupled spins. The distance distribution can for example be modelled by a Gaussian or a sum of Gaussians with positions and widths determined through least-square fitting [169, 182]. The experimental doubly-labelled spectrum is compared to the spectrum calculated as the convolution of the singly-labelled spectrum with the dipolar broadening function corresponding to a given distance distribution.

The dipolar broadening function can be constructed based on weak- or strong-coupling regime Pake patterns, depending on the type of system. In the case of nitroxides, the strong-coupling regime usually has to be considered for distances below 0.8 nm, while the weak-coupling regime should be used for distances above about 1.5 nm [183]. Hence in most cases an intermediate-coupling regime would give the most accurate description of the two-spin system. Consideration of the wrong coupling regime leads to a maximum error of $\frac{3}{2}^{\frac{1}{3}}$, corresponding to about 0.2 nm for a distance of 1.5 nm (ca. 15%).

5.3 PULSE DIPOLAR SPECTROSCOPY

The distance between two coupled electron spins can be determined by pulse EPR experiments designed to measure the dipolar frequencies ω_{dd} . These experiments include three-pulse DEER, four-pulse DEER, the single frequency experiment '2+1', SIFTER (Single Frequency Techniques for Refocusing electron-electron couplings) and DQC (Double Quantum Coherence) [3, 184, 185]. The pulse sequences for the three- and four-pulse DEER experiments used in this thesis are shown in figure 5.3 and these techniques will be described in detail in section 5.3.1.

Three- and four-pulse DEER are based on the use of two different microwave frequencies to selectively excite different coupled spins. The spins excited by the observer or detection pulse sequence, generating the electron spin echo, are referred to as A spins and the spins excited only by the pump pulse are called B spins. The pump pulse causes an inversion of the B spins, resulting in a change in the local field at the position of the A spins, which influences the echo intensity at the end of the detection sequence. As the position of the pump pulse is varied, the echo intensity is modulated by the frequency of dipolar coupling between the A and B spins.

The analysis and interpretation of the time-domain DEER data, involving the extraction of a distribution of distances between the coupled spins and its interpretation in terms of macromolecular structure, is not trivial and has been developed and continuously improved over the last decade. The most

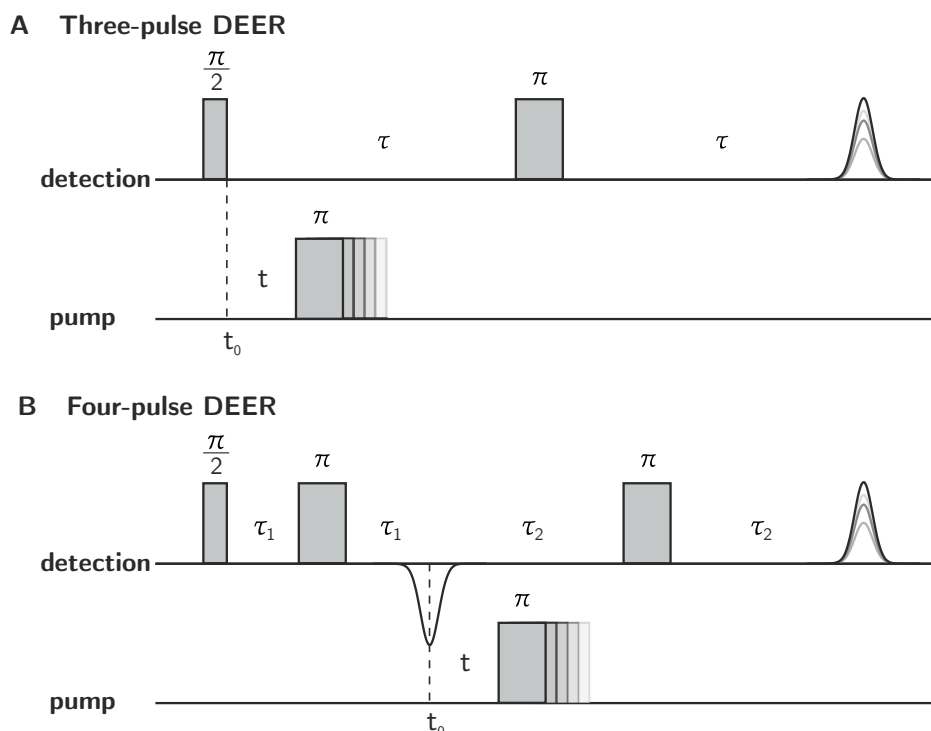


FIGURE 5.3: Pulse sequences for three-pulse (A) and four-pulse (B) DEER. The position of the inversion pulse at the pump frequency is incremented and the change in echo intensity is recorded. The dashed line indicates the zero-time of the experiment.

widely used analysis software is the freely available DeerAnalysis [186]. The principles behind the analysis of DEER data will be described in section 5.3.2.

5.3.1 THREE-PULSE AND FOUR-PULSE DEER

The three-pulse DEER or PELDOR experiment was first developed by Milov et al. [187, 188] and consists of a two-pulse echo sequence at the detection frequency and a pump π pulse at the pump frequency (figure 5.3 A and figure 5.4). The initial $\frac{\pi}{2}$ pulse selectively creates electron coherence of the A (detection) spins. The pump pulse applied at time t flips the B (pump) spins and hence transfers coherence between the two single-quantum transitions of the A spins. In terms of the vector model, the change in local field at the A spins causes a change in their precession frequency, resulting in re-focusing of the magnetisation off the y axis and hence a reduction in echo intensity (see figure 5.4). The position of the pump pulse is varied within the interval τ between the two detection pulses and

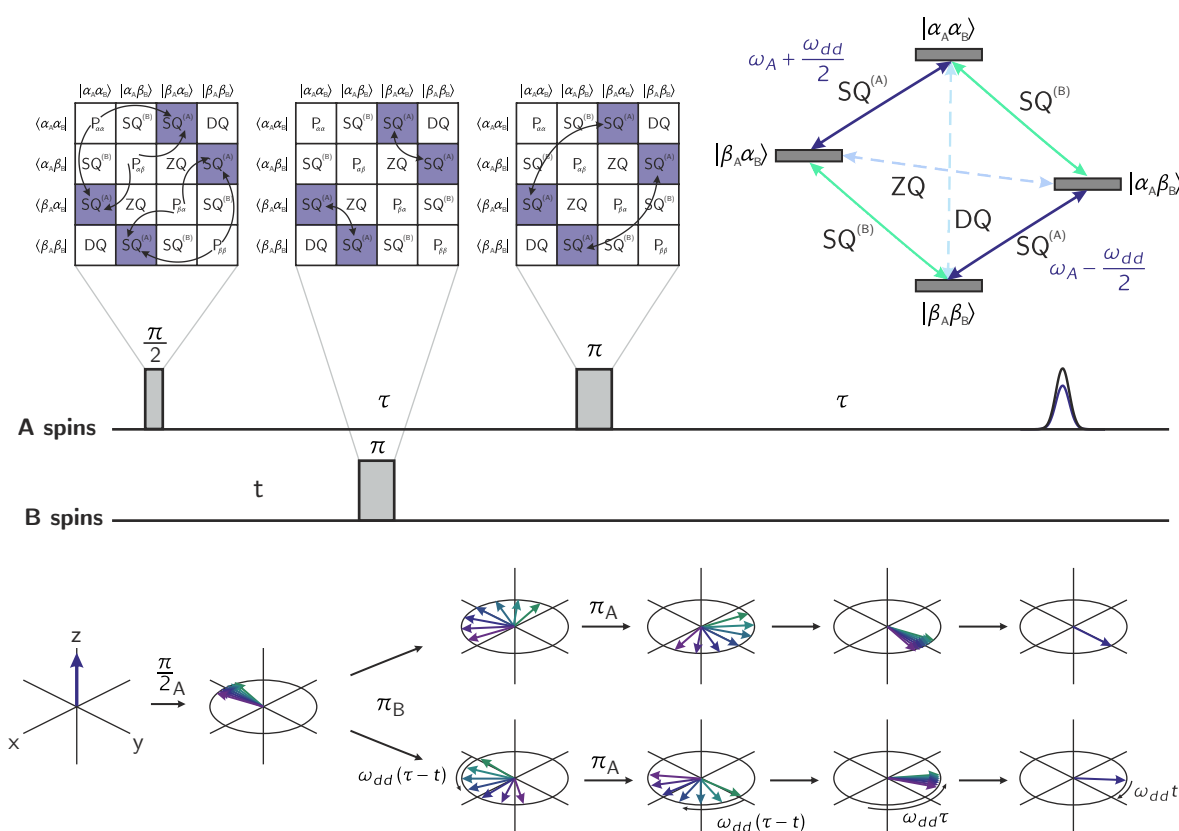


FIGURE 5.4: Three-pulse DEER sequence explained in terms of coherence transfer and vector model. The four-level system of two coupled electron spins with $S = \frac{1}{2}$ with the coherences connecting different states are shown on the top right. The transition frequencies for the A spins are determined by the spin Hamiltonian $\hat{H}(S_A)$ and the dipolar interaction ω_{dd} . The three-pulse DEER sequence is shown in the centre and the effect of the pulses on the coherences in terms of density matrix elements is shown on top. Vector model diagrams representing the evolution of the magnetisation of A spins during the three-pulse DEER sequence are shown on the bottom, in the absence of B spins in the top part of the graph and initially coupled to B spins in the β state on the bottom. The vector diagrams are shown in the rotating frame with a frequency of $\omega_A - \frac{1}{2}\omega_{dd}$.

the echo intensity is recorded as a function of pump pulse position resulting in a modulation with a frequency equal to ω_{dd} . The length of the pulse sequence is fixed and the maximum length is limited by the phase memory time. Typically a phase cycle is applied to the initial $\frac{\pi}{2}$ pulse to adjust for receiver offset.

The change in echo amplitude as a function of time in the three-pulse DEER experiment can be derived using the product operator or the density matrix formalism. Assuming ideal pulses and selective excitation, the modulation formula for the echo amplitude can be determined as:

$$f_{3p}(t) = \cos(\omega_{dd}t) \quad (5.6)$$

This modulated contribution arises from only a fraction of the coupled spins in the sample; the A spins coupled to B spins that are not excited by the pump pulse give rise to an additional unmodulated contribution. This is taken into account with the parameter λ , the inversion efficiency of the pump pulse, and the overall echo amplitude thus varies with time as:

$$F_{3p}(t) = 1 - \lambda[1 - \cos(\omega_{dd}t)] \quad (5.7)$$

The three-pulse DEER sequence has the disadvantage that the first part of the DEER trace is often distorted due to the overlap of the first detection and the pump pulse at short times. This dead-time prevents an accurate determination of the zero-time and leads to suppression of broader features of the dipolar spectrum obtained by Fourier transform of the time-domain data [189]. This problem is solved in the four-pulse DEER experiment (figure 5.3 B) proposed by Pannier et al. [190]. This experiment uses a two-pulse sequence followed by a refocusing π pulse at the detection frequency. The pump pulse position varies within the interval $(\tau_1 + \tau_2)$ between the two π pulses of the detection sequence. The zero-time in this case corresponds to the position of the primary echo generated by the first two detection pulses. The generation of a DEER signal in the four-pulse experiment can be explained in the same way as shown for the three-pulse experiment in figure 5.4. If the pump pulse coincides with the position of the primary echo, the precession frequency is the same during dephasing and refocusing, leading to complete refocusing of the magnetisation at τ_2 after the last π pulse. Variation of the pump pulse position at times t after the primary echo leads to modulation of the magnetisation and thus the echo intensity at the frequency ω_{dd} .

The modulation of the echo amplitude in the four-pulse DEER experiment, again taking both the modulated and unmodulated contributions into account, is given by:

$$F_{4p}(t) = 1 - \lambda[1 - \cos(\omega_{dd}t)] \quad (5.8)$$

where $t = 0$ at the time of the primary echo.

The expressions given in equations 5.7 and 5.8 only account for the dipolar interaction between two spins in the same nano-object and not for the dipolar interactions between distant electron spins on

different nano-objects. The overall DEER trace is given by the product of these two contributions as:

$$V(t) = F(t)B(t) \quad (5.9)$$

where $F(t)$ is called form factor and $B(t)$ is the background factor, described by an exponentially decaying function of the form

$$B(t) = \exp\left(-kt^{\frac{d}{3}}\right) \quad (5.10)$$

where k is a factor proportional to the spin concentration and d is the dimensionality of the background [191]. The dimensionality of the background is usually close to three for nano-objects homogeneously distributed in three dimensions, such as proteins or organic biradicals in solution. In case of preferential ordering in one dimension, such as for membrane-bound systems, the dimensionality d becomes close to two. In addition to the dimensionality of the distribution, d is also affected by the under-representation of short distances due to excluded volume and hence can deviate from the predicted values of two and three. In general, the inter-nano-object distances exceed the intra-nano-object distances significantly and the form and background factor can be separated. The form factor is then used after background correction for further analysis (see next section). In order to avoid distance artefacts due to incorrect background corrections, the best approach is to record an experimental background on a singly-labelled equivalent of the investigated system.

The DEER experiments are based on the selective excitation of the A spins and B spins by the microwave pulses at the detection (ω_{det}) and pump (ω_{pump}) frequencies, respectively. Selective excitation is possible even if A spins and B spins have the same EPR spectrum, as long as the spectral width exceeds the excitation bandwidth of the microwave pulses. In the case of nitroxides, the A and B spins correspond to nitroxides in different hyperfine states, typically $m_I = +1$ and $m_I = 0$, respectively. The spectral width of the nitroxide spectrum is compared to the excitation profiles of detection and pump pulses in figure 5.5. The excitation profile of the detection pulse sequence in four-pulse DEER is

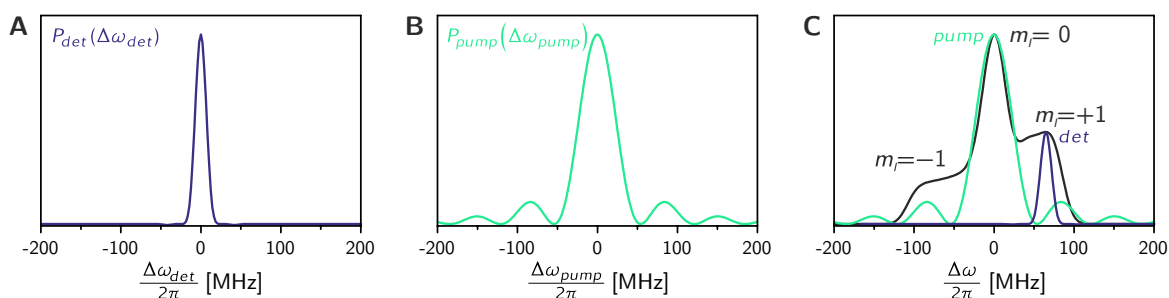


FIGURE 5.5: (A) Excitation profile of a four-pulse DEER detection sequence with 32 ns pulses calculated using equation 5.11. (B) Excitation profile of a $t_\pi = 16$ ns pump pulse calculated using equation 5.12. (C) Excitation profiles of the detection and pump pulses compared to an X-band nitroxide spectrum in the frequency domain. The pump frequency is typically centred on the $m_I = 0$ transition, while the detection frequency is set on the $m_I = +1$ transition, shifted by $\Delta\nu = 65$ MHz. The nitroxide spectrum was simulated with g -values of 2.0090, 2.0060 and 2.0024 and an axial ^{14}N hyperfine tensor with eigenvalues $A = [18, 18, 96]$ MHz.

defined as [190]

$$P_{det}(\Delta\omega_{det}) = \left(\frac{\omega_1}{\omega_{eff}} \right)^5 \sin^5 \left(\omega_{eff} t \frac{\pi}{2} \right) \quad (5.11)$$

with $\Delta\omega_{det} = \omega_{det} - \omega$, $\omega_{eff} = \sqrt{\Delta\omega_{det}^2 + \omega_1^2}$ and ω_1 as the detection pulse B_1 field strength. The excitation profile of the pump pulse is defined as [190]

$$P_{pump}(\Delta\omega_{pump}) = \left(\frac{\omega_1}{\omega_{eff}} \right)^2 \sin^2 \left(\omega_{eff} t \frac{\pi}{2} \right) \quad (5.12)$$

where $\Delta\omega_{pump}$, ω_{eff} and ω_1 are defined as above, but with respect to the pump pulse frequency and strength. The excitation profiles of detection and pump pulses are shown in figure 5.5 A and B and are compared to a nitroxide EPR spectrum at X-band in figure 5.5 C. In DEER experiments on nitroxides at X-band, the pump pulse is generally placed at the centre of the EPR spectrum ($m_I=0$), where a short enough pump pulse (12-16 ns) can excite essentially all orientations with respect to the magnetic field. The detection pulses are usually placed on the $m_I = +1$ transition, exploiting the slightly larger signal intensity of this transition with respect to $m_I = -1$, which is determined by the anisotropy of the g - and A -tensors.

A frequency overlap of the detection and pump pulses should be avoided as excitation of A spins by the pump pulse can lead to the occurrence of ESEEM modulations in the DEER trace, which could be falsely interpreted as inter-spin distances. The presence of proton ESEEM modulations can lead to artefacts in the distance distribution at 1.5 nm, while deuterium ESEEM modulations produce artefacts at a distance of about 2.8 nm [191]. The effects of ESEEM modulations on the recorded DEER traces can be reduced by averaging over different τ_1 values in the four-pulse DEER experiment. The initial τ_1 value is optimised for maximum signal intensity and the increment $d\tau$ is chosen to cover a full period of the nuclear modulation. This commonly leads to summation over eight traces with values of $\tau_1 = 176$ ns and $d\tau = 8$ ns for ^1H and of $\tau_1 = 400$ ns and $d\tau = 56$ ns for ^2H [192].

The determination of a particular distance and distance distribution is only reliable if the recorded DEER trace is sufficiently long to characterise this distance. The determination of different properties of the distance distribution require different lengths of the experimental DEER trace; normally a length corresponding to half an oscillation is sufficient for the determination of the mean distance, a length corresponding to at least one and a half oscillations is required to determine the width of the distribution and more than three oscillations are required to accurately determine the shape of a distance distribution [191]. A DEER trace with a length of 2 μs can thus be used to reliably determine mean distances up to $\langle r \rangle_{max,mean} = 5$ nm, widths of distance distributions up to $\langle r \rangle_{max,width} = 4$ nm and shapes of distance distributions up to $\langle r \rangle_{max,shape} = 3$ nm [191]. The maximum length required for the determination of the characteristics of distance distributions centred at a value r_{exp} can be determined based on the following equation:

$$t_{max} = 2\mu\text{s} \left(\frac{r_{exp}}{r_{max,i}} \right)^3 \quad (5.13)$$

where i stands for mean, width or shape. The length of the DEER trace that can be recorded is determined by the phase memory time. Since the DEER experiment is a constant-time experiment, the chosen length also determines the signal-to-noise ratio of the experimental data. The phase memory time can often be prolonged by decreasing the temperature down to the rigid-lattice limit, but the accompanying increase in spin-lattice relaxation time requires a lower shot repetition time. The optimum temperature determined by balancing the effect of these two relaxation times on the signal-to-noise ratio was found to correspond to ca. 50 K for nitroxides [192]. As discussed in section 1.4.2, the phase memory time in protonated solvents is typically determined by nuclear spin diffusion and can thus be significantly extended by the use of deuterated instead of protonated solvents. Even more significant improvements in data quality can be achieved by deuteration of both the spin labels and the protein as well as the solvent [170].

The four-pulse DEER experiment is more severely affected by the limitation of the phase memory time with respect to the three-pulse DEER experiment due to additional relaxation during the initial part of the pulse sequence with length $2\tau_1$. The advantages of four-pulse DEER in determining the initial part of the DEER trace more reliably and of three-pulse DEER in providing higher sensitivity for the acquisition of long DEER traces can be combined using the DEER-Stitch method proposed by Lovett et al. [193]. This method is based on recording a short four-pulse DEER trace and a longer three-pulse DEER trace and combining the two to reconstruct an overall DEER trace.

Distances from about 1.7 nm to an upper limit determined by the length of the DEER trace can be reliably detected using the DEER experiments. Short distances corresponding to a dipolar coupling of the same magnitude as the excitation bandwidth are suppressed [192, 194]. If the excitation bandwidth is comparable to the dipole-dipole interaction, the static spin Hamiltonian has to be taken into account during the pulses, leading to a dependence of the modulation depth on the dipolar frequency ω_{dd} [169, 186]. The variation of the modulation depth under these conditions was found to approximately follow a Gaussian function [169]:

$$\lambda = \lambda_0 \exp\left(-\frac{\omega_{dd}^2}{\sigma_{exc}^2}\right) \quad (5.14)$$

where σ_{exc} is the effective excitation bandwidth of the DEER pulse sequence. Short distances can be extracted by taking the suppression into account in the analysis of the experimental data [169, 186].

5.3.2 ANALYSIS OF DEER DATA

The interpretation of experimental DEER data in terms of distance constraints and macromolecular structure can be achieved using different approaches:

- extraction of distance distributions from the experimental DEER traces and deduction of the 3D structure of the nano-object based on a series of inter-spin distance distributions;
- calculation of DEER traces based on a proposed model structure and refinement of the model structure based on the comparison of calculation and experiment.

The first approach is inherently model-free and thus widely applicable, while the second approach requires the availability of a single or multiple model structures of the macromolecular system. As will be explained below, extraction of a distance distribution from the DEER trace requires solution of an ill-posed problem. The uncertainty related to this procedure can be avoided by following the second approach. The second approach does also allow for more flexibility in terms of explicitly including multi-spin or orientational effects in the simulation of the DEER traces.

A general DEER data analysis procedure based on the first approach is implemented in the freely available software DeerAnalysis [186]. The different steps in the data analysis procedure are shown in figure 5.6 [186, 191]. The first step consists of the determination of the background factor $B(t)$ by fitting of equation 5.10 to the final part of the DEER trace, where the modulations are damped and the decay is governed by the background factor (figure 5.6). Both the factor k and the dimensionality d can be fitted to the experimental data. If an experimental background recorded on a singly labelled sample is available, this can be used for the background correction. The division of the experimental data by the background function $B(t)$ yields the form factor $F(t)$ shown in figure 5.6. The modulation depth, Δ , is determined from the difference between the intensity at zero-time and the intensity at long times of the form factor, after the modulations have decayed. For further analysis, the reduced form factor $f(t)$, containing just the modulated contribution to the DEER trace, is calculated as:

$$f(t) = \frac{F(t) - (1 - \Delta)}{\Delta} \quad (5.15)$$

An approximate estimate of the average inter-spin distance can already be obtained from the reduced form factor $f(t)$, by considering the time $t_{\frac{1}{2}}$ at which the signal has decayed to 0.5. In the case of two nitroxide spin labels, the average distance $\langle r \rangle$ can be calculated as [191]:

$$\langle r \rangle [nm] \approx 5 \left(\frac{t_{\frac{1}{2}} [\mu s]}{0.48} \right)^{\frac{1}{3}} \quad (5.16)$$

This approximation is strictly only valid for unimodal distributions and is related to the fact that the initial part of the reduced form factor is determined almost exclusively by the position of the distance peak and not by its width and shape [191].

The DEER spectrum shown in figure 5.6 is obtained by Fourier transform of the reduced form factor. In the presence of a well-defined pair distance, the dipolar frequency can be determined from the position of the peaks of the Pake pattern as described in section 5.1. In the presence of broader distance distributions or multimodal distance distributions, the dipolar spectrum corresponds to the weighted average of the Pake patterns corresponding to the different distances present, leading to poorly resolved features. The determination of the inter-spin distance distribution in that case requires a different method.

A series of different approaches have been proposed for the model-free extraction of a distance distribution from the experimental DEER data [195–198]. The distance distribution $P(r)$ and the reduced

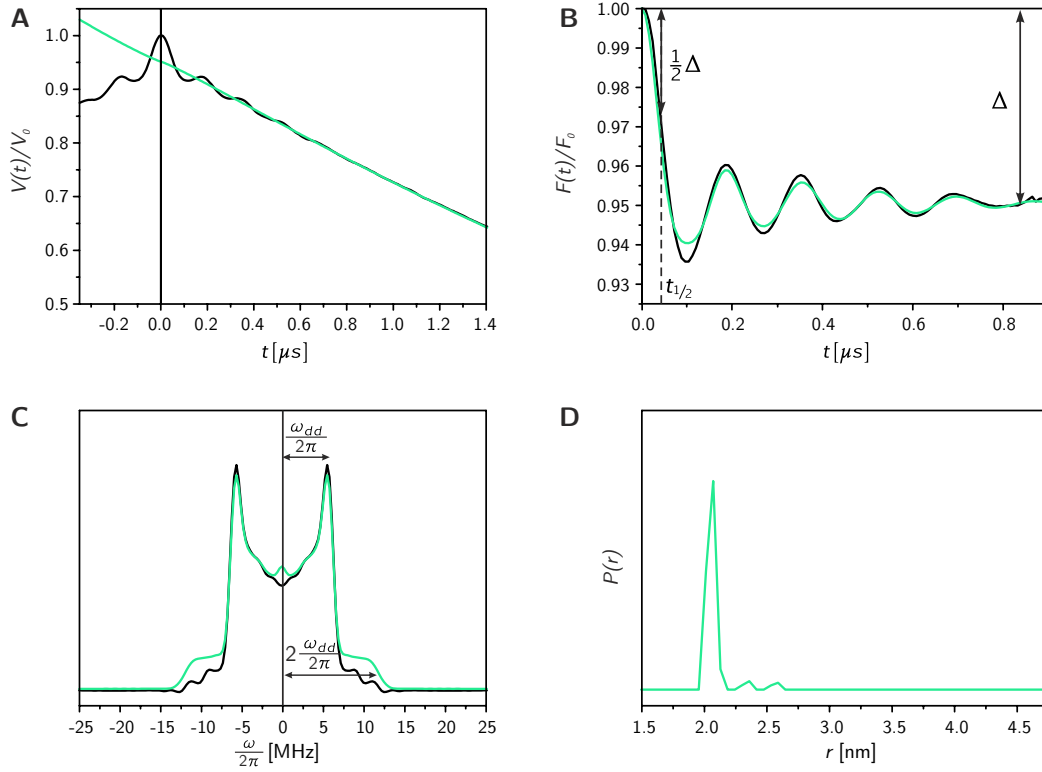


FIGURE 5.6: DEER data analysis. (A) Experimental DEER trace $V(t)/V_0$ recorded for a Bruker standard biradical sample at 50 K and fitted background $B(t)$. (B) Experimental and simulated form factor $F(t)/F_0$ obtained after background correction. Differences between experiment and simulation are most likely due to orientation selection effects (C) Experimental and simulated DEER spectrum obtained by Fourier transform of the reduced form factor. (D) Distance distribution extracted by Tikhonov regularisation applied to the data shown in B and C with a regularisation parameter α of 0.1. The analysis was performed with the program DeerAnalysis [186].

form factor $f(t)$ are related by the following function:

$$f(t) = \int_{r_{min}}^{r_{max}} K(r, t) P(r) dr \quad (5.17)$$

where $K(r, t)$ is the kernel function defined as:

$$K(r, t) = \int_0^1 \cos\left[\left(1 - 3\cos^2\theta\right)\omega_{dd}t\right] d\cos\theta \quad (5.18)$$

and corresponds to the modulation due to the dipolar interaction averaged over all orientations of the inter-spin vector with respect to the magnetic field. If the suppression of short distances by limited excitation bandwidth needs to be taken into account as well, the kernel function is modified to also contain the exponential term from equation 5.14. The inversion of this equation to determine the distance distribution from the reduced form factor is an ill-posed problem, i.e. the solution is highly sensitive to changes in the initial data, such as for example experimental noise and differences in background correction. The mathematical approach that was chosen for the solution of this problem

in the context of the analysis of DEER data is called Tikhonov regularisation [196, 198]. The concept behind Tikhonov regularisation is stabilisation of the solution of the inverse of equation 5.17 by imposition of two types of constraints: the distance distribution $P(r)$ is constrained by imposing a smoothness criterion, since conformational flexibility in most of the investigated systems leads to smooth distance distributions, and is also constrained to positive values ($P(r) \geq 0$), since negative probabilities have no physical meaning. An error function $G_\alpha(P)$ is defined and minimised:

$$G_\alpha(P) = \|KP(r) - f(t)\|^2 + \alpha \left\| \frac{d^2 P(r)}{dr^2} \right\|^2 = \rho + \alpha\eta \quad (5.19)$$

The first term in equation 5.19 represents the square norm of the deviation between the calculated reduced form factor given by $KP(r)$ and the experimental reduced form factor $f(t)$ and its minimisation improves the agreement between simulated and experimental data. The second term is the square norm of the second derivative of the distance distribution (representing the roughness) and imposes the smoothness constraint on the solution $P(r)$. The factor α is called regularisation parameter and controls the solution by balancing resolution and smoothness of the distance distribution. A small value of the regularisation parameter α leads to a distance distribution with many narrow peaks due to fitting of the noise in the experimental form factor as distances. A large value of α leads to broadening of the distance distribution and excessive damping of the modulations in the time domain. The optimal value for the regularisation parameter is usually determined based on the L-curve criterion [186, 196, 198]. The L-curve is a plot of $\log \eta$ against $\log \rho$ for different values of α and generally forms an approximate L-shape. The first vertical part corresponds to under-smoothing of the distance distribution, while the second horizontal part corresponds to over-smoothing. The best solution is obtained by minimisation of both η , representing the smoothness, and ρ , determining the quality of the fit, and can be identified from the left corner of the L-curve. Tikhonov regularisation gives good results in most cases, but can cause problems if a distance distribution is characterised by a combination of narrow and broader peaks [186, 196].

Since in this approach the errors on the distance distribution are not a linear function of the noise in the input data, the error evaluation of the results obtained by Tikhonov regularisation is not trivial. So far the best approach for error estimation consists of repeating the extraction of the distance distribution for different noise levels and different background corrections and to determine the distance distribution giving the best fit to the experimental results and the standard deviation. This approach is currently implemented in DeerAnalysis [186, 191] and can be used for example to identify artefact peaks arising from errors in background correction or noise level.

Another possibility for the solution of the ill-posed problem in DEER is the maximum entropy method proposed by Freed et al. [199]. This approach combines Tikhonov regularisation with the maximisation of the entropy function associated with the distance distribution $P(r)$, implicitly excluding negative values and increasing the resistance to noise artefacts. The problem of the solution of an ill-posed problem can be circumvented altogether, if a model distance distribution is available; then a least-square fitting of $K(r, t)P_{model}(r)$ to $f(t)$ can be performed. Model distance distributions can for example be generated based on a series of model macromolecular structures, for example different

crystal structures, structures generated by molecular dynamics (MD) or structures of protein-protein or protein-cofactor complexes generated using docking procedures.

5.3.3 CONFORMATIONAL DISTRIBUTIONS AND FLEXIBILITY OF SPIN LABELS

In order to be able to relate distances determined based on experimental DEER measurements to a macromolecular structure, it is important to take the size and spatial distribution of the paramagnetic centre used for the DEER measurement into account. Metals or metal clusters used as intrinsic spin labels are often in a well-defined and known position in the macromolecular structure and their spatial distribution is related to the flexibility of the surrounding structure, providing only information on the investigated system. Spin labels artificially attached to a nano-object at specific positions on the other hand can assume a wide range of conformations, leading to a distribution of inter-spin distances that need to be taken into account for the correct interpretation of the distance distribution in terms of protein structure [191, 200]. In dynamical studies, the flexibility of the spin label and of the nano-object itself are difficult to separate unless the flexibility of the spin label can be modelled allowing the extraction of information on just the flexibility of the nano-object.

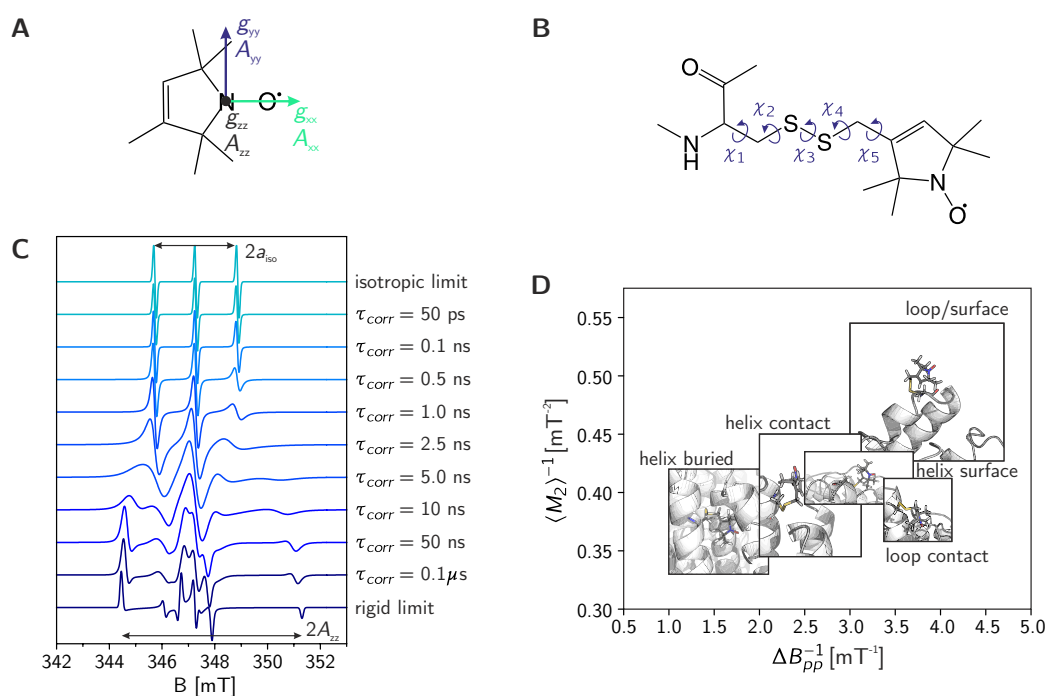


FIGURE 5.7: (A) g - and A -tensor orientations for a nitroxide. (B) Molecular structure of MTSL with highlighted torsional angles determining the possible conformations of the MTSL side chain. (C) X-band nitroxide EPR spectra in the isotropic limit, in the fast and slow motion regime with different correlation times τ_{corr} and in the rigid limit. The spectra were simulated using EasySpin's *garlic*, *chili* and *pepper* functions [23, 95] for a g -tensor with the principal values 2.0090, 2.0060 and 2.0024 and a hyperfine tensor with eigenvalues $A = [18, 18, 96]$ MHz. (D) Topological regions in a plot of $\langle M_2 \rangle^{-1}$ against ΔB_{pp}^{-1} determining the mobility of spin labels in different regions of an α -helical protein [201].

The continuous wave EPR spectra recorded at room temperature can provide information on the mobility of the protein backbone and the spin label, since the spectral lineshape is sensitive to motions occurring on the EPR timescale determined by the spectral anisotropy. In the description of nitroxide spin labels attached to proteins, three different correlation times can be defined [201, 202]: the correlation time related to the overall tumbling of the protein, the correlation time related to the protein backbone motion and the correlation time related to the internal motion of the spin label (transitions between different rotameric states). A distinction of these three contributions is quite difficult and requires a multi-frequency approach allowing isolation of different timescales [203, 204]. At X-band frequencies, the overall tumbling of the protein can often be neglected, as the correlation time exceeds the EPR sensitivity for large protein systems (>200 kDa) [201]. The sensitivity of the EPR lineshape to backbone and spin label motions is due to the anisotropies characterising the nitroxide g - and A -tensors. The orientations of the two tensors with respect to the molecular frame are shown in figure 5.7 A. The g -tensor is orthorhombic with principal values of about 2.0090, 2.0060 and 2.0024 and the A -tensor is axial with principal values of about 18, 18 and 96 MHz. The motion of a free spin label in solution at room temperature averages out all the anisotropies, leading to an isotropic limit spectrum as shown in figure 5.7 C. In frozen solution, the spectrum is the average over all possible orientations of the molecule with respect to the magnetic field and hence contains all of the anisotropic contributions; the corresponding rigid limit spectrum is also shown in figure 5.7 C. If the spin label is attached to a protein, its motion is restricted with respect to the free label in solution and hence the anisotropies are not averaged out completely. The spectral changes occurring in the X-band nitroxide EPR spectrum as a function of correlation time are depicted in figure 5.7 C. A quantitative interpretation of nitroxide spectra recorded for a specific protein system and labelling position requires simulations based on dynamic models specifically developed for the description of nitroxide spin labels attached to proteins such as for example the Microscopic Order with Macroscopic Disorder model (MOMD) or the Slowly Relaxing Local Structure model (SRLS) first proposed for this application by Freed et al. [205–208].

The relative mobility of a spin label can also be qualitatively evaluated by considering two simple spectral parameters [201, 202, 209]:

- the peak-to-peak linewidth of the central $m_I = 0$ peak of the nitroxide spectrum ΔB_{pp} ;
- the overall width of the nitroxide spectrum, defined by the second moment $\langle M_2 \rangle$.

Both parameters are a measure of the extent of averaging of the anisotropic interactions. In the presence of multiple superimposed components, the central linewidth is dominated by the most mobile contribution, while the second moment is dominated by the most immobile component, as can be appreciated from figure 5.7 C. The spectral lineshapes of spin labels attached at several positions in model protein systems with known structures were analysed and it was shown that the pairs of ΔB_{pp}^{-1} and $\langle M_2 \rangle^{-1}$ values are related to spin labels in similar topological regions of the protein cluster within certain regions of a plot of $\langle M_2 \rangle^{-1}$ against ΔB_{pp}^{-1} [201, 202, 210, 211] (see figure 5.7 D). The regions were found to be applicable for a series of mainly α -helical protein systems (T4L lysozyme

[202, 210], annexin [211], bacteriorhodopsin [201]). Spin labels attached in buried sites are characterised by restricted motion and fall into the bottom left corner of the plot, spin labels close to the surface and in regions without defined secondary structure are characterised by high mobility and fall into the top right corner of the plot. Different regions with intermediate spin label mobilities can also be distinguished.

The conformational distribution of macromolecular systems in frozen solutions is assumed to reflect the dynamics at the glass transition temperature of the solvent matrix. The information on relative spin label mobility obtained by room temperature continuous wave EPR measurements can thus be useful in the interpretation of the widths of distance distributions relating to different pairs of spin labels. More precise information is however required for the accurate interpretation of DEER results, in particular if the motion of the spin label and of the protein backbone need to be distinguished. The distribution of conformational states of a nitroxide spin label can be taken into account for DEER data interpretation using a series of different methods. The glass transition temperature of the solvation shell of proteins in aqueous solution was estimated to occur at about 170 K [212], hence this temperature can be used to identify thermally accessible conformations if the relative energies are known.

A simple approach for considering the conformational distribution of a spin label is the tether-in-a-cone model, which assumes the electron spin to be localised on a cone centred at the backbone C_α atom [213]. Other more sophisticated methods involve the explicit consideration of the conformational space of the spin label by computation of rotamer distributions. One approach is based on the calculation of a rotamer library for selected spin labels and weighting based on the potential energy of the different rotamers in a specific protein environment and is implemented in the Matlab program MMM [214]. A second approach is based on a conformational search based on random rotations around the torsional angles without weighting and is implemented in the Pymol plug-in MtsslWizard [215]. The two approaches generally lead to similar results, however the second approach can sometimes provide a better agreement with experimental data in cases of tight binding, when the spin label is forced into unfavourable conformations [215]. In this thesis, the rotamer library approach of MMM has been used for the analysis of protein DEER results and hence only this approach will be described in more detail below.

The MTSL spin label previously introduced is connected to the protein backbone by five rotatable single bonds (figure 5.7 B), hence a large number of conformations, characterised by different values of the five torsional angles χ_i , is theoretically possible. The electron spin is assumed to be localised at the midpoint of the N-O bond, and can thus be situated at a range of different positions with respect to the protein backbone.

The rotamer library approach implemented in MMM is based on a pre-computed library of rotameric states of a specific nitroxide spin label with relative energies defined for a standard protein environment. Additional energy terms are included based on the experimentally investigated protein and the chosen labelling position. The distributions of dihedral angles for the free MTSL spin label were investigated by molecular dynamics simulations performed at a temperature of 175 K. The potential

energy profiles for rotation around the five angles in MTSL were found to be characterised by two or three minima [214]. The complete conformational space of the MTSL spin label could be reasonably well defined by about 200 generated rotamers, based on combinations of these torsional angles.

The populations of different rotameric states are defined using Boltzmann statistics based on the sum of an internal energy pre-computed for the free spin label and an external energy depending on the specific spin label environment [214]. The evaluation of the internal energy was based on assignment of conformations sampled in MD trajectories of the free spin label to specific rotameric states and calculation of the energy from the frequency of occurrence of these states using the equation of a Boltzmann distribution [214]. The external energy is calculated for each pre-computed rotameric state attached at a specific site of the investigated protein by modelling a rigid protein environment and evaluating the energy through the Lennard-Jones interaction potential for each pair of non-hydrogen atoms on the spin label and on the protein (within a defined cutoff radius). The flexibility of the protein environment is accounted for by a reduction of the van der Waals radii [214]. The uncertainty related to this approach has been quantified based on studies of protein systems with a known structure. The error on the mean position of a single MTSL spin label was estimated to about 0.22 nm and the uncertainty on the mean distance determined using such an approach was estimated to about 0.3 nm [200].

5.3.4 MULTI-SPIN EFFECTS

The standard DEER experiments and the data analysis procedures described in sections 5.3.1 and 5.3.2 were designed for an isolated pair of coupled spins in each nano-object. Complications arise in the presence of more than two coupled spins within a nano-object and multi-spin correlations have to be considered for a reliable structure determination.

The signal of a multi-spin system of a detection spin A coupled to multiple pump spins (B, C, etc.) can be expressed as a product of two-spin contributions if the experiment is such that the couplings between the different pump spins can be neglected [216]. In a multi-spin system, the detection spin A is coupled to multiple pump spins within the same nano-object and the pump pulse of the DEER experiment, causing a spin-flip for the pump spins, probes the coupling to the pump spins. Since the pump pulse is usually sufficiently short to be able to neglect the coupling between B spins for its duration and since only the single-quantum coherences of the A spin evolve and are observed in the DEER experiment, it is possible to express the form factor of the DEER experiment in an N spin system as [216–218]:

$$\frac{F(t)}{F_0} = \frac{1}{N} \sum_{A=1}^N \prod_{B \neq A}^N [1 - \lambda_B (1 - \cos \omega_{AB} t)] \quad (5.20)$$

The summation accounts for the fact that each of the N spins can act as the detection spin. If the inversion efficiency λ_B is assumed to be the same for all of the pump spins, equation 5.20 can be

written out as [218]:

$$\begin{aligned}
 \frac{F(t)}{F_0} = & (1 - \lambda)^{N-1} + \frac{1}{N} \sum_{A=1}^N \left[(1 - \lambda)^{N-2} \lambda \sum_{B \neq A} \cos \omega_{AB} t \right] \\
 & + \frac{1}{N} \sum_{A=1}^N \left[(1 - \lambda)^{N-3} \lambda^2 \frac{1}{2} \sum_{A \neq B} \sum_{C \neq A, B} \cos \omega_{AB} t \cos \omega_{AC} t \right] \\
 & + \frac{1}{N} \sum_{A=1}^N \left[(1 - \lambda)^{N-4} \lambda^3 \frac{1}{3!} \sum_{A \neq B} \sum_{C \neq A, B} \sum_{D \neq A, B, C} \cos \omega_{AB} t \cos \omega_{AC} t \cos \omega_{AD} t \right] \\
 & + \dots
 \end{aligned} \tag{5.21}$$

In addition to the unmodulated contribution and the two-spin contribution modulated at a single dipolar frequency, which is also present for a two-spin system, N -spin contributions depending on $N - 1$ dipolar frequencies appear. The multi-spin contributions contain products of cosine functions, which can be rewritten in terms of sums of cosines of linear combinations of the dipolar frequencies. For example the product of cosines in the three-spin contribution can be rewritten as:

$$\cos \omega_{AB} t \cos \omega_{AC} t = \frac{1}{2} [\cos [(\omega_{AB} + \omega_{AC}) t] + \cos [(\omega_{AB} - \omega_{AC}) t]] \tag{5.22}$$

This shows that the DEER time trace of a N -spin system is modulated not only by the dipolar frequencies corresponding to the $\frac{1}{2}N(N-1)$ inter-spin distances, but also by their sums and differences in the three-spin case and by other linear combinations of the dipolar frequencies for more than three spins. The additional modulations at higher and lower frequencies with respect to the dipolar frequencies lead to artefacts in the distance distributions at shorter and at longer distances, if the multi-spin effects are not taken into account in the analysis.

Different methods for evaluating the number of coupled spins within a nano-object and for analysing multi-spin effects were proposed. The dependence of the modulation depth on N as [188, 217–219]:

$$\Delta = 1 - (1 - \lambda)^{N-1} \tag{5.23}$$

can be used for the determination of the number of coupled spins by inversion of equation 5.23:

$$N = \frac{\ln(1 - \Delta)}{\ln(1 - \lambda)} + 1 \tag{5.24}$$

The overall modulation depth Δ can be determined from the DEER time trace at long times after background correction. Knowledge of the inversion efficiency λ is also required for the determination of N . The inversion efficiency is specific to a spectrometer setting and depends on the measurement conditions and therefore requires calibration using model compounds with a known number of spins ($N = 2-4$) [217, 219]. This method of spin counting can be affected by many different types of errors. One limitation is the error on the modulation depth Δ , which depends on the background correction and the signal-to-noise ratio. In the case of a high signal-to-noise ratio, the error on Δ has been estimated as 0.02, leading to an error on N of about 5% for up to four spins [217]. A second source

of error is the λ value determined through calibration; the calibration will lead to determination of a λ value and its standard deviation. Further errors in the calibration can occur if the properties of the model systems and the actual investigated system, such as the A_{zz} value for the two nitroxides and the relaxation times, differ significantly [191, 219]. Since the change in modulation depth decreases for an increasing number of spins N , the precision of this method decreases significantly for systems with more than four spins and hence for large multi-spin systems only lower limits can be determined reliably [191, 217].

Inspection of equation 5.21 shows that the different N -spin contributions to the overall form factor are weighted by terms depending on the inversion efficiency λ . As N increases, the weighting factor depends on increasingly higher powers of λ and the corresponding contributions to the DEER trace become less significant. The dependence of the different contributions on λ can be exploited to extract the two-spin contribution by measuring the dependence of the modulation depth Δ on the flip angle of the pump pulse [218]. The dependence of the inversion efficiency on the pump pulse flip angle β is given by

$$\lambda = \lambda_{max} \frac{(1 - \cos \beta)}{2} \quad (5.25)$$

where λ_{max} corresponds to the inversion efficiency achieved with a pulse with flip angle $\beta = \pi$. In order to distinguish the effects of the variation of the flip angle from effects due to changes in excitation bandwidth and hence orientation selection, the flip angle of the pump pulse should be varied by changing the microwave power at constant pulse length [218]. The variation of the modulation depth Δ as a function of the inversion efficiency calculated according to equation 5.25 follows a polynomial with an order depending on the number of coupled spins N and can thus be used to distinguish systems with a different number of coupled spins. In three-spin systems the two- and three-spin contributions can be reconstructed from the experimental data and the coefficients of the polynomial fit [218]. This analysis method requires knowledge of the spin labelling efficiency and only provides reliable results for systems with a limited number of coupled spins. The method has proven to be efficient in distinguishing two- and three-spin systems in model compounds [218], but no reliable quantitative results could be obtained when applied to a biological multi-spin system [220].

The effect of neglecting multi-spin effects in the analysis of DEER data recorded for systems with more than two coupled spins on each nano-object was investigated by calculations based on equation 5.21. Multi-spin systems were modelled as regular polygons and spin distributions were defined based on the tether-on-a-cone model as shown in figure 5.8. The calculations were performed by randomly selecting points on the spherical caps for each vertex and using the corresponding distance to calculate the DEER time trace for each orientation of the magnetic field with respect to the spin system based on equation 5.21 with N set to 3, 4 or 5. The different contributions were calculated separately and combined for different values of the inversion efficiency λ . The final DEER trace was obtained after 1000 Monte Carlo iterations. The calculated DEER traces are shown in figure 5.8 and show clear differences in both modulation depth and frequencies for different inversion efficiencies. The effect of neglecting the multi-spin nature of the spin system was evaluated by extracting the distance distributions from the calculated traces using DeerAnalysis and by comparing the results

with the distance distributions used in the calculations. In the three-spin system, increasing inversion efficiencies lead to the appearance of an additional well-resolved peak at shorter distances and of artefacts at longer distances. In addition to this, a broadening of the distance distribution is also observed. In the four- and five-spin systems, the peak at shorter distances increases with respect to the peak at longer distances and again additional peaks or broadening appear at shorter distances and artefacts start to appear at longer distances. The results of the calculations in figure 5.8 show that neglect of multi-spin effects can lead to misinterpretation of the experimental results. Typical inversion efficiencies at X-band for a 12 ns pump pulse correspond to approximately $0.2 \leq \lambda \leq 0.6$ [221] and this inversion efficiency leads to clearly visible deviations of the extracted from the real distance distribution. Similar results are also obtained by artificially adding noise to the calculated traces and repeating the data analysis.

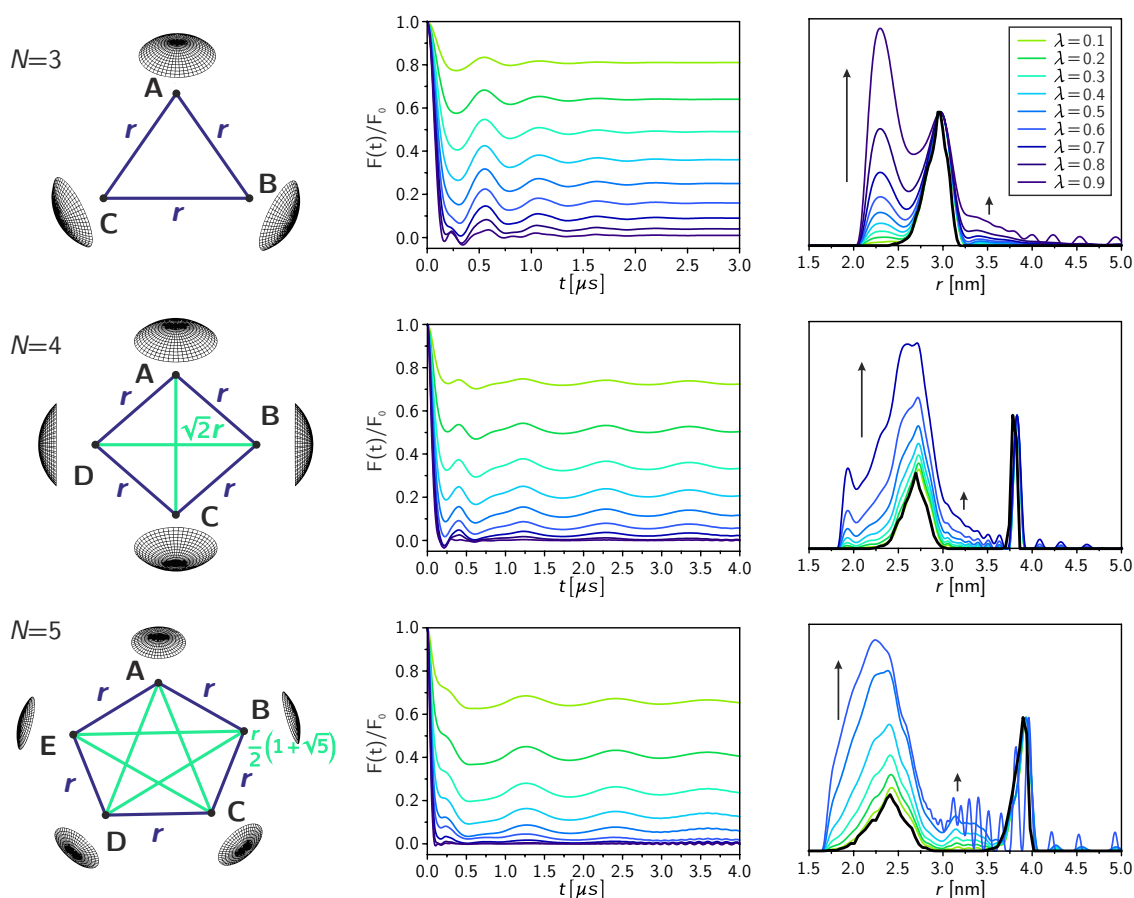


FIGURE 5.8: Illustration of the multi-spin effects in DEER on model systems with $N=3$, $N=4$ and $N=5$ with spins arranged in regular polygons. Spin distributions were modelled with a tether-on-a-cone model as illustrated on the left using a Monte Carlo approach. The DEER traces calculated from equation 5.21 for different values of λ are shown in the centre and the distance distribution extracted using DeerAnalysis (from $8 \mu\text{s}$ DEER traces) are compared to the real distance distribution (black) on the right. The distance distributions are normalised at the position of the maximum for the real distance distribution and the arrows indicate the direction of changes due to multi-spin effects for increasing inversion efficiencies. Severely distorted distance distributions for high values of λ are not shown.

A method for the suppression of multi-spin artefacts was proposed by von Hagens et al. [222] and is based on power scaling of the experimental form factor before extraction of the distance distribution. The authors found that scaling of the logarithm of the experimental form factor for an N -spin system with the factor ζ_N leads to the logarithm of the form factor of the two-spin contribution:

$$\zeta_N \ln[F_N(t)] \approx \ln[F_2(t)] \quad (5.26)$$

with optimum ζ_N equal to

$$\zeta_N = \frac{1}{N-1} \quad (5.27)$$

This method was evaluated theoretically for multi-spin systems with up to five spins and experimentally for model systems with three spins and was found to work well for symmetric and relatively rigid systems. The power scaling is implemented in DeerAnalysis. Another approach to data analysis for multi-spin systems consists of fitting a model to the experimental DEER data using simulation routines including the higher-order contribution of the DEER form factor [223].

5.3.5 ORIENTATION SELECTION

The spectra of spin systems characterised by large anisotropies, such as metal ions like copper or iron, triplet states or spin systems investigated at high magnetic fields, cannot be completely excited by the microwave pulses of the DEER pulse sequence [176]. The pulses only excite a limited range of orientations of the molecule with respect to the magnetic field, determined by the magnetic field and by the pulse lengths. Orientation selection in DEER experiments can lead to incomplete Pake patterns in the frequency domain, i.e. parts of the dipolar spectrum are underrepresented or completely absent. Orientation selection effects can also occur for nitroxides at X-band if the orientation and the inter-spin vector are correlated, as is the case for example in rigid biradicals [224] or for rigid spin labels attached to DNA or RNA [225]. In these cases the distance distribution cannot be accurately determined without explicitly considering the effects of orientation selection.

The first theoretical description of orientation-selective DEER was presented by Larsen and Singel [189] and later different simulation routines for orientation-selective DEER were developed [224–229]. In the derivation of equations 5.7 and 5.8 complete excitation of the detection spin was assumed. In order to account for the effects of orientation selection, the excitation fractions of both detection and pump spins need to be considered. The general form factor can thus be written as [176]:

$$F(t) = \frac{\sum_{i \in \theta, \phi} f_A^i \left[1 - \lambda_B^i \left(1 - \cos(\omega_{AB}^i t) \right) \right] + f_B^i \left[1 - \lambda_A^i \left(1 - \cos(\omega_{AB}^i t) \right) \right]}{\sum_{i \in \theta, \phi} (f_A^i + f_B^i)} \quad (5.28)$$

where the summation corresponds to the average over all selected orientations of the magnetic field with respect to the molecular frame. f_A^i and f_B^i are the echo intensities from the spins A and B at the detection frequency ω_{det} and the sum of two terms accounts for the fact that either one can be the

detection or the pump spin. λ_A^i and λ_B^i are the efficiencies for spin flips of the A and B spins at the pump frequency ω_{pump} .

A program for the calculation of orientation-selective DEER (OriDEERSim) was previously developed in the group [227] and is outlined in figure 5.9. The coordinate system used by the program is also shown on top in figure 5.9. The reference frame is defined by the $\mathbf{g}$ -tensor frame of the detection spin A; the position and orientation of the pump spin B is defined with respect to this reference frame by the distance r_{AB} , the vector $\mathbf{n}_{AB}(\xi, \psi)$ and three Euler angles α , β and γ describing the relative orientations of the two $\mathbf{g}$ -tensor frames. The detection and pump spins can either be defined as point spins or with distributions of spin densities over several points. In the latter case, multiple distance vectors are defined and the sum over all ij pairs of spin densities is considered with weights determined by the spin densities ρ_A^j and ρ_B^k .

The first step in the computation is the generation of a grid of i orientations of the magnetic field $\mathbf{B}_0^i(\theta, \phi)$ on a unit sphere in the reference frame defined above. The resonance frequencies and intensities I_{det}^i and I_{pump}^i of the detection and pump spin EPR spectra are calculated for each of these orientations based on a spin Hamiltonian containing the main interactions for the two paramagnetic centres. For each position of the magnetic field vector in the reference frame the excitation fraction of the detection spin is computed. The contribution of each orientation to the detection spin echo is calculated as

$$f_{det}^i = \int_0^\infty I_{det}^i(\omega) P_{det}(\omega_{det} - \omega) d\omega \quad (5.29)$$

where P_{det} is the excitation profile of the detection sequence defined in equation 5.11. The orientations of the magnetic field vector that do not excite a significant fraction of the detection spins are neglected in the subsequent analysis. The excitation fraction of the pump spin is then computed for the remaining orientations of $\mathbf{B}_0$ as

$$\lambda_{pump}^i = \int_0^\infty I_{pump}^i(\omega) P_{pump}(\omega_{pump} - \omega) d\omega \quad (5.30)$$

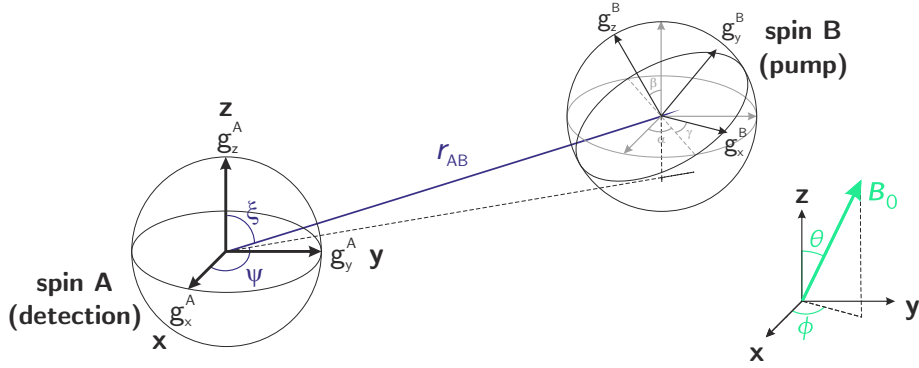
where P_{pump} is the excitation profile of the pump pulse defined in equation 5.12. The overall DEER signal for a single orientation $\mathbf{B}_0^i(\theta, \phi)$ is given by

$$F^i(t) = f_{det}^i \left[1 - \lambda_{pump}^i (1 - \cos(\omega_{AB}^i t)) \right] \quad (5.31)$$

with a dipolar frequency ω_{AB}^i defined as

$$\omega_{AB}^i = \frac{\mu_0 \beta_e^2}{4\pi \hbar} g_A^i g_B^i \sum_j \sum_k \rho_A^j \rho_B^k \frac{1 - 3 \cos^2 \theta_{Aj, Bk}^i}{r_{Aj, Bk}^3} \quad (5.32)$$

The OriDEERSim program computes the frequency spectrum instead of the time trace. This is done by binning of the dipolar frequencies calculated through equation 5.32 and computation of the final



- 1 Construct vectors $\mathbf{n}_{jk}$ and distances r_{jk} between detection and pump spin densities in the molecular frame.
- 2 Generate a grid of n vectors $\mathbf{B}_0^i(\theta, \phi)$ over half the unit sphere defined by polar (θ) and azimuthal (ϕ) angles and calculate the solid angle weights w^i .
- 3 Calculate the set of orientations $\mathbf{B}_0^i(\theta, \phi)$ excited by the detection pulses at the frequency ω_{det}

$$f_{det}^i = \int_0^\infty I_{det}^i(\omega) P_{det}(\omega_{det} - \omega) d\omega$$

where P_{det} is the pulse excitation profile and I_{det}^i is the detection spin spectrum at the orientation $\mathbf{B}_0^i(\theta, \phi)$.

- 4 Remove all non-resonant $\mathbf{B}_0^i(\theta, \phi)$ vectors.
- 5 Generate the pump pulse profile P_{pump} as a function of $\Delta\omega_{pump} = \omega_{pump} - \omega$ and store

$$P_{pump}(\Delta\omega_{pump}) = \left(\frac{\omega_1}{\omega_{eff}} \right)^2 \sin^2(\omega_{eff} t_{\pi/2})$$

- 6 Calculate the pump spin spectrum I_{pump}^i which is centred around ω_{pump} for the orientation $\mathbf{B}_0^i(\theta, \phi)$.
- 7 Calculate the modulation depth, i.e. the fraction of pump spin signal at $\mathbf{B}_0^i(\theta, \phi)$ that is flipped by the pump π pulse, as:

$$\lambda_{pump}^i = \int_0^\infty I_{pump}^i(\omega) P_{pump}(\omega_{pump} - \omega) d\omega$$

- 8 Calculate the dipolar frequency corresponding to the orientation $\mathbf{B}_0^i(\theta, \phi)$ as:

$$\omega_{dd}^i = \frac{\mu_0 \beta_e^2}{4\pi\hbar} g_A^i g_B^i \sum_j \sum_k \rho_A^j \rho_B^k \frac{1 - 3 \cos^2 \theta_{Aj, Bk}^i}{r_{Aj, Bk}^3}$$

- 9 Calculate the intensity at the frequency ω for the orientation $\mathbf{B}_0^i(\theta, \phi)$ as:

$$F^i(\omega) = \frac{\lambda_{pump}^i}{2} (\delta(\omega_{dd}^i + \omega) + \delta(-\omega_{dd}^i + \omega)) + (1 - \lambda_{pump}^i) \delta(\omega)$$

- 10 Calculate the final intensity at the frequency ω as:

$$F(\omega) = F(\omega) + (w^i f_{det}^i) F^i(\omega)$$

Loop over all i $\mathbf{B}_0^i(\theta, \phi)$ vectors for which detection spins are excited.

Loop over all jk spin densities of the two spin systems

FIGURE 5.9: Definition of the coordinate system used for the definition of a two-spin system for orientation-selective DEER calculations and outline describing the calculation of an orientation-selective DEER trace using the OriDEERSim program [227].

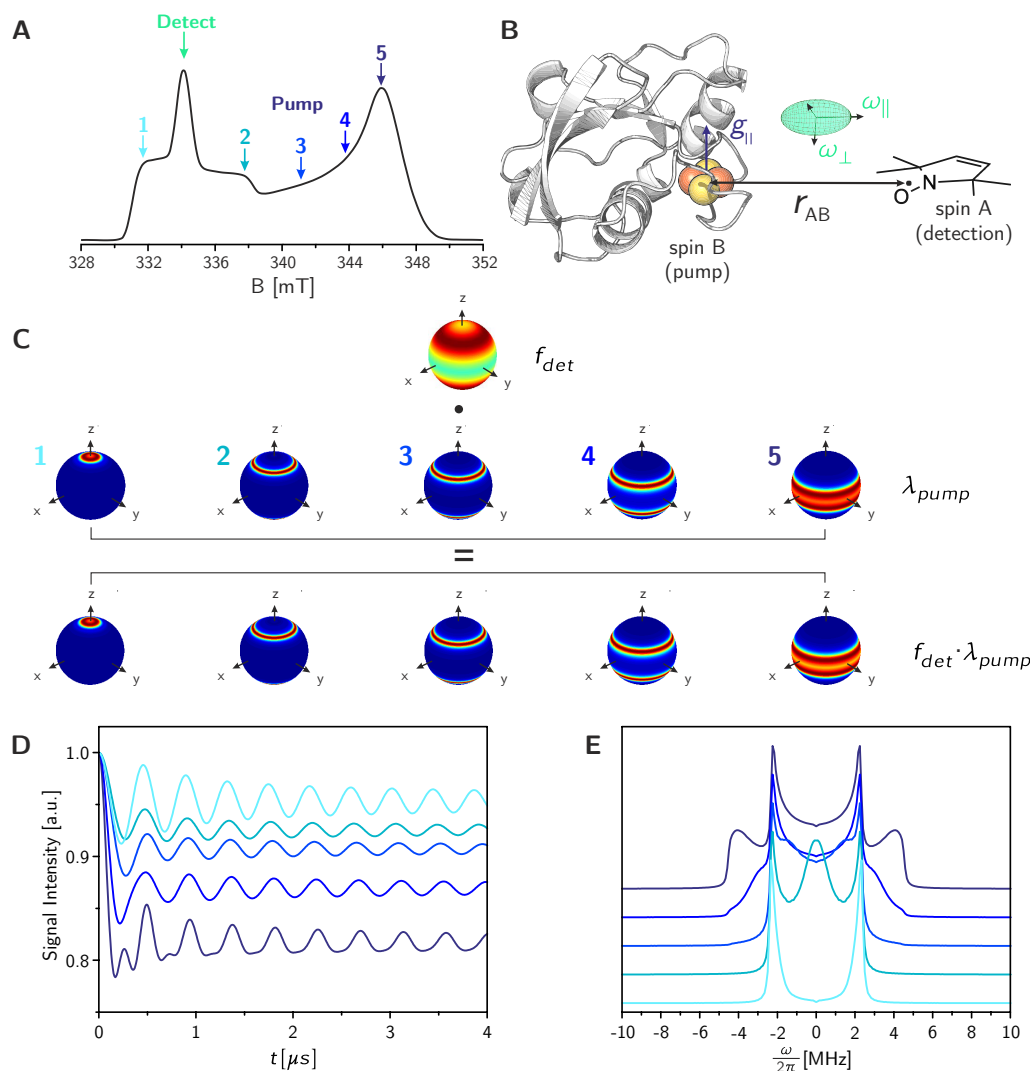


FIGURE 5.10: X-band orientation-selective DEER between an iron-sulfur cluster (pump) and a nitroxide spin label (detect). (A) X-band EPR spectrum with arrows indicating the pump and detection positions. (B) Relative orientation of the iron-sulfur cluster and the nitroxide label. (C) Orientations selected by detection ($t_{\pi} = 20$ ns) and pump ($t_{\pi} = 20$ ns) pulses for the nitroxide and iron-sulfur cluster, respectively. The intensity of excitation of different orientations is colour-coded with intensely excited orientations in red and not excited orientations in blue. (D) DEER time traces for the different pump positions indicated in A simulated for a distance of 3 nm between the centre of the iron-sulfur cluster and the midpoint of the N-O bond and an angle of $\xi = 90^{\circ}$ between $g_{\parallel}$ and the inter-spin vector. (E) Orientation-selective dipolar spectra corresponding to the DEER time traces shown in D. The maximum intensity has been normalised to 1 for better comparison of the Pake patterns. The calculations were performed for the following spin Hamiltonian parameters: nitroxide principal g -values of 2.0090, 2.0060 and 2.0024 and principal hyperfine values of 18, 18 and 96 MHz, iron-sulfur cluster with an axial g -tensor with $g_{\perp} = 1.933$ and $g_{\parallel} = 2.023$. The spin density of the nitroxide was considered to be localised on the midpoint of the N-O bond, while a distribution of spin density over the two Fe and six S atoms was considered for the iron-sulfur cluster (spin density distribution and g -tensor orientation from reference [230]).

intensity at each frequency according to:

$$F^n(\omega) = \frac{\lambda_{pump}^i}{2} \left(\delta(\omega_{AB}^i + \omega) + \delta(-\omega_{AB}^i + \omega) \right) + (1 - f_{pump}^i) \delta(\omega) \quad (5.33)$$

and

$$F(\omega) = F(\omega) + (w^i f_{det}^i) F^i(\omega) \quad (5.34)$$

The DEER time trace is then determined by inverse Fourier transform.

The results of an example calculation for orientation-selective DEER between a nitroxide spin label and an iron-sulfur cluster are shown in figure 5.10. The nitroxide spin label is chosen as the detection spin, while the iron-sulfur cluster is the pump spin. Detection is always performed on the maximum of the nitroxide spectrum, while the pump position changes across the EPR spectrum of the iron-sulfur cluster, varying the selected orientations. The orientation selection spheres show the orientations excited at the detection and the different pump positions and the overall contribution of each orientation of the magnetic field vector with respect to the g -tensor frame of the detection spin. The DEER traces calculated for the different pump positions show clear differences in both modulation depth and modulation frequencies. In the corresponding dipolar spectra the external peaks of the Pake pattern corresponding to $\omega_{||}$ are attenuated or missing for all of the pump positions except the one selecting the $g_{\perp}$ region of the iron-sulfur spectrum (pump position 5 in figure 5.10). The reason for this can be clearly seen from the orientation of the dipolar coupling tensor with respect to the g -tensor frame of the iron-sulfur cluster shown in figure 5.10 B. The frequency $\omega_{||}$ can only be detected if the orientations lying in the direction of the inter-spin vector are selected, corresponding to $g_{\perp}$ for the iron-sulfur cluster. The strongest peaks of the dipolar spectra, corresponding to $\omega_{\perp}$ in the complete Pake pattern, are also shifted with respect to each other for different pump pulse positions. This illustrates that an analysis of the orientation-selective DEER results without explicit consideration of the effects of orientation selection could lead to significant errors in the determination of an inter-spin distance.

Orientation-selective DEER traces contain not only information on the inter-spin distance, but also on the relative orientation of the two paramagnetic centres. The possibility of extracting this additional information can reduce the number of distance constraints needed for the determination of a macromolecular structure.

6 STRUCTURE DETERMINATION OF THE MULTIMERIC TATA CHANNEL

The work described in this chapter was part of a collaboration with the research groups of Prof. Ben C. Berks and Prof. Jason R. Schnell. Their groups investigated the structure of the twin-arginine translocase A channel complex in micelles using electron microscopy and solution state NMR. Since only short-distance constraints were determined by NMR, two different model structures in agreement with the detected intermolecular NOEs could be proposed. The ability of EPR to measure longer distances was exploited to differentiate between the two model structures.

The studied protein complex is in a homo-oligomeric state with a number of monomers depending on the experimental conditions. An approximate estimate of the number of protein molecules in a complex is crucial for the biological relevance of the proposed structure, since complexes with four monomeric units are believed to represent the inactive state, while larger complexes assemble as a response to a signalling protein and are involved in biological function.

Continuous wave EPR and three- and four-pulse DEER were combined to distinguish the two possible multimeric assemblies and to validate the structure proposed based on the NMR constraints for this type of assembly. A quantification of the number of units in the oligomer was also attempted using several different approaches.

6.1 TWIN-ARGININE TRANSLOCATION PATHWAY

The twin arginine translocation (Tat) system mediates the transport of folded proteins across the bacterial membrane or the chloroplast thylakoid membrane [231]. The transport of fully folded proteins poses different requirements to the translocase involved with respect to the more common Sec translocation pathway for unstructured proteins. The transmembrane channel of the Tat pathway has to be able to accommodate different types of substrates, while still preventing diffusion of small molecules or ions between cell compartments. The transport mechanism has been proposed to be based on the formation of channels of variable diameter determined by the size of the substrate to be transported [232].

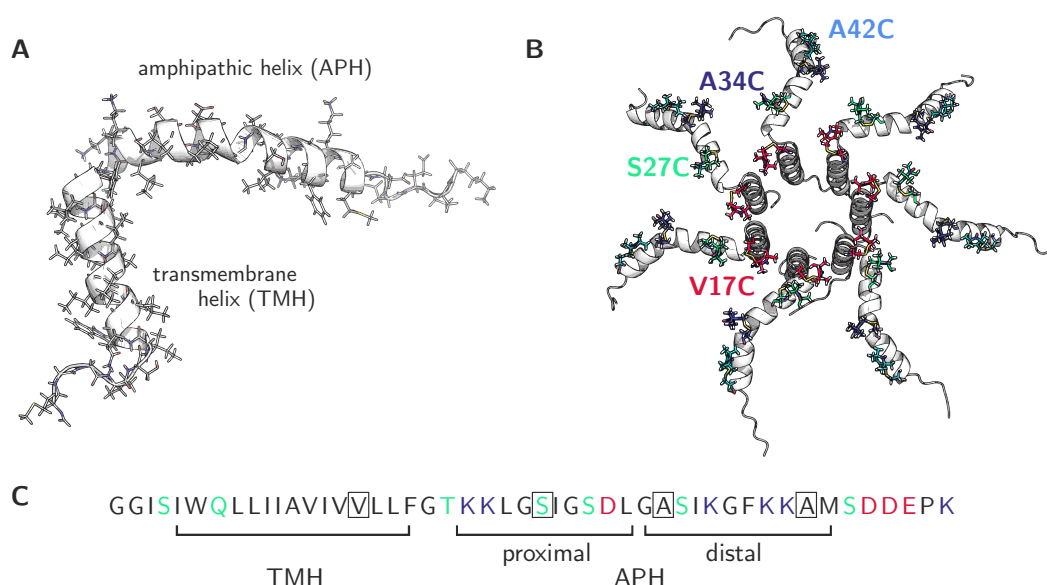


FIGURE 6.1: Structure of a single TatA subunit (A), model structure of a seven-membered TatA protein complex proposed based on the NOE constraints (B) and the aminoacid sequence of the truncated TatA subunit of *E. coli* (C). The nitroxide spin label rotamer with the highest population predicted by MMM [214] is shown for each labelling position in B. The aminoacids in C are colour coded based on their properties: hydrophobic (black), polar (green), negatively charged (red) and positively charged (blue). The positions selected for mutation and spin labelling are highlighted.

In general the Tat system consists of three membrane proteins: TatA, TatB and TatC. TatA is the component forming the protein-translocating channels of variable size, which are thought to conduct the proteins through the membrane. TatB and TatC form a multimeric protein complex involved in protein recognition, substrate binding and regulation of the substrate transport. The particular features required for this transport pathway have been proposed to be related to a significant conformational change of the TatA component [233].

The single TatA unit consists of two α -helices arranged in an approximate L-shape: a short hydrophobic transmembrane helix (TMH) and a longer amphipathic helix (APH) [234] (figure 6.1 A). The length of the transmembrane helix is not sufficient to fully span the membrane bilayer, possibly causing a distortion of the bilayer. The amphipathic helix is characterised by a kink at residues Leu32-Gly33, dividing it into a proximal and a distal segment. Several selective mutation studies have shown that the amphipathic helix is crucial for the function of the TatA protein complex [233, 235].

Since knowledge of the structure of the TatA protein complex is the basis for an understanding of the translocation mechanism, several studies aimed at characterising the structure both in natural and in artificial environments have been conducted [232, 233, 236, 237]. *In vivo*, the assembly of the TatA protein complex requires activation by the TatBC complex, but TatA in the oligomeric state has also been observed after solubilisation in detergents, for example nona-polyoxyethylene dodecyl ether ($C_{12}E_9$) or dodecylphosphocholine (DPC). The type and concentration of the solvent was found to modulate the size of the TatA oligomers. High DPC detergent concentrations (detergent-to-protein ratio (DPR) of about 250) allow isolation of the TatA monomer, while lower concentrations (DPR of

about 30) lead to the formation of complexes. The complexes formed in DPC detergent are smaller with respect to the complexes formed in $C_{12}E_9$. Electron microscopy studies on TatA complexes in $C_{12}E_9$ led to the proposal of a ring-shaped architecture partly closed on one end of the central channel (see figure 6.2 A and reference [232]). The channel wall was found to have a diameter of 2.5-3 nm, approximately one to two helices wide, independent of the size of the oligomer. The inner diameter was found to be highly variable, supporting the proposal of a dynamic translocation mechanism involving changes in the oligomeric state of the channel complex to allow close packing around the substrate. Several model topologies of the TatA subunit in the oligomeric state have been proposed based on these results and can be separated into models where the amphipathic helix lies along the membrane and models where the amphipathic helix is part of the channel spanning the membrane bilayer [233, 238, 239].

The work presented in this thesis is part of a more extensive investigation of the structure of the TatA complex in DPC micelles by solution state NMR, analytical ultracentrifugation, water accessibility measurements and molecular dynamics simulations. Analytical ultracentrifugation suggested the presence of an average of nine TatA units in the oligomeric TatA state at a DPC detergent-to-protein

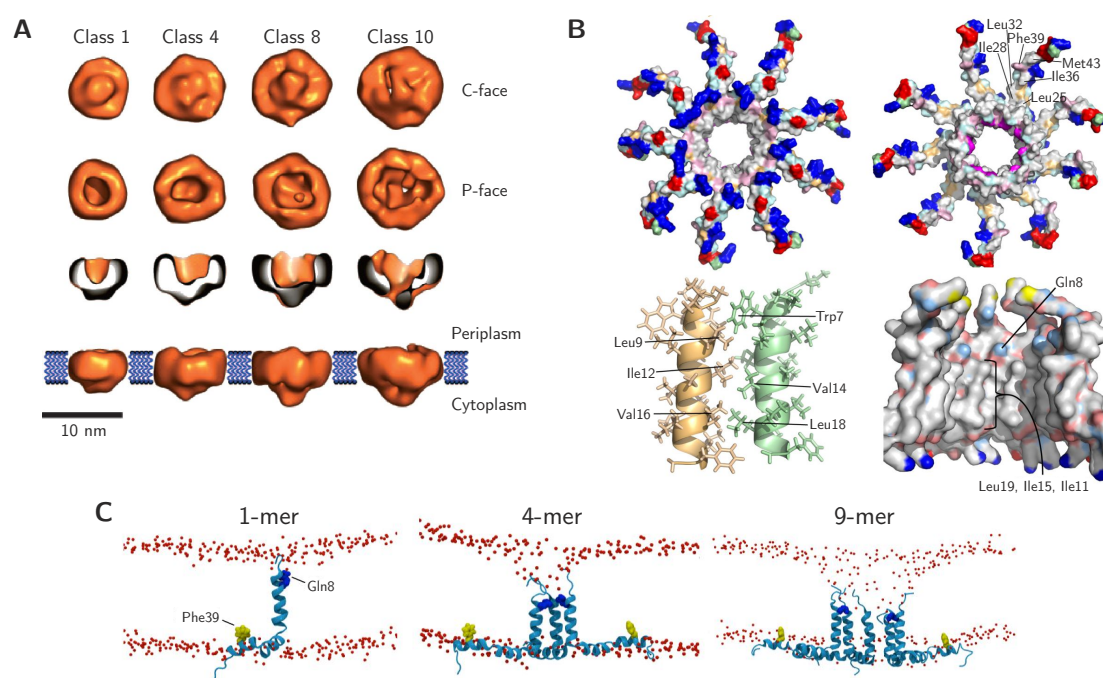


FIGURE 6.2: (A) The 3D architecture of TatA complexes determined by random conical tilt electron microscopy [232]. Four classes of complexes with variable sizes are shown as viewed from the cytoplasmic (C-face), periplasmic (P-face) side of the membrane and as a section through the membrane. [Adapted from [232], copyright 2005 National Academy of Sciences, USA]. (B) Structure of the oligomeric TatA complex as proposed based on rigid-body docking guided by intermolecular NOEs [233]. Surface plots as viewed from the cytoplasmic and periplasmic side of the membrane are shown on the top; the transmembrane helix packing interface and a surface plot of the pore interior are shown on the bottom. [Adapted from [233], copyright 2013 National Academy of Sciences, USA]. (C) Coarse-grained molecular dynamics simulations of monomeric (1-mer), tetrameric (4-mer) and nonameric (9-mer) TatA structures in an *E. coli* lipid membrane [233]. The protein is displayed as a cartoon and the phosphate particles of the lipid headgroups are shown as red spheres. [Adapted from [233], copyright 2013 National Academy of Sciences, USA].

ratio of about 30. The water accessibility measurements have shown that in the complex the transmembrane helix is isolated from the aqueous solution and that the amphipathic helix is also partly protected from solvent exposure, except for the region of the kink between the proximal and distal segments. The solution NMR results of the oligomeric TatA complex showed small changes in chemical shifts with respect to the monomer for the amphipathic helix and larger changes for the transmembrane helix, attributed to the effect of intermolecular packing. In particular, the chemical shift of Gln8 changed significantly between monomeric and oligomeric TatA and this change was attributed to exposure of this residue to a more polar environment in the oligomeric state, suggesting that it is located on the inside of the protein channel. Intermolecular NOEs were determined between residues on the transmembrane helices up to Phe20, an additional NOE between residues Val17 (TMH) and Leu25 (APH) suggested a similar relative orientation of the transmembrane and amphipathic helix in the monomeric and multimeric states. The intermolecular NOEs were combined with rigid-body docking to determine a model structure of the protein complex, which is depicted in figure 6.1 B and figure 6.2 B and C. Since no intermolecular NOEs could be detected between residues on the amphipathic helices, two models could potentially be proposed, one with the amphipathic helices pointing outwards and one with the amphipathic helices pointing inwards. The EPR results of this chapter in combination with the results from the other techniques, led to the proposed structure with the amphipathic helices pointing outwards with respect to the centre of the complex [233].

Molecular dynamics studies have shown that this model forms stable channel complexes with up to 25 TatA units, and hence could be a relevant model for the oligomeric TatA state in its native environment. The results of molecular dynamics simulations in a lipid bilayer have shown that a single TatA unit assumes a conformation that allows both the transmembrane helix and the proximal segment of the amphipathic helix to be inside the bilayer without causing distortions. This conformational state is characterised by a transmembrane helix tilted with respect to the membrane normal and by a more pronounced kink between the proximal and distal part of the amphipathic helix (figure 6.2 C). The conformation assumed by the TatA subunits in larger complexes is close to the solution state NMR structure shown in figure 6.1 A. In the complexes, only the transmembrane helix is inside the membrane, leading to more significant distortions and thinning of the bilayer for increasing oligomer size (figure 6.2 C).

The previously proposed mechanism for Tat translocation [231] has been refined in light of these results and is illustrated in figure 6.3 [233]. The translocation is initiated by the binding of the substrate to the TatBC complex. The recognition and binding occurs through a signal peptide, which in the case of the Tat pathway is characterised by a conserved twin-arginine motif interacting with TatC [231]. Prior to activation, the TatA component exists in a monomeric state (with a conformation suggested by the molecular dynamics simulations) or as a protomer with an average of four units. After formation of the TatBC-substrate complex, the association of TatA with TatBC is triggered and polymerisation of TatA is induced. The polymerisation is proposed to occur with a conformational change similar to that predicted by the molecular dynamics simulations and thus induces a thinning of the membrane in preparation of translocation. The oligomeric state of the channel complex changes dynamically until the diameter is sufficiently large to allow translocation of the substrate through

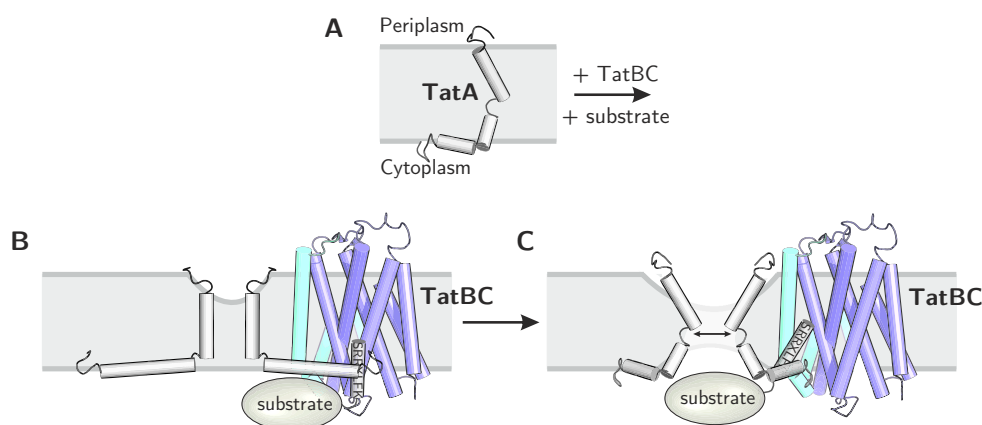


FIGURE 6.3: Model representation of the Tat translocation pathway as a section through the membrane [233]. (A) The inactive monomeric form of TatA is proposed to be in a conformation with the transmembrane and a significant part of the amphipathic helices within the membrane. (B) Substrate binding to the TatBC protein complex and to TatA induces oligomerisation and a conformational change that forces a thinning of the membrane in preparation for translocation. The substrate is bound to TatBC through a signal peptide characterised by two arginines (R) close to the end of the sequence. (C) The TatA complex opens and ruptures the membrane as the substrate begins to move through the membrane.

the disrupted membrane. After translocation the TatA complex is disassembled into monomers or protomers.

The TatA complex in ($C_{12}E_9$) has previously been investigated using continuous wave EPR by White et al. [237]. The study focused on characterising the mobility of spin labels attached at different positions on the transmembrane helix and on determining the distances between them by dipolar convolution. Their results showed increased mobilities of spin labels attached at the upper and lower end of the transmembrane helix with respect to spin labels attached in the central part and identified a close contact between residues Ile12 and Val14. The frozen solution cw-EPR measurements showed similar inter-spin distances in the range of 1.40 to 1.55 nm for all of the residues from Leu9 to Leu18 (on the transmembrane helix).

In this investigation, spin labelling at different amino acid positions along the transmembrane and amphipathic helices, combined with X-band cw-EPR and DEER measurements, was used to discriminate between the two structures of the proposed NMR model complexes, in particular concerning the orientation of the amphipathic helices, for which no intermolecular NOEs could be detected by NMR. Quantification of the number of subunits in the oligomeric state of TatA in DPC was also attempted.

6.2 EXPERIMENTAL METHODS

6.2.1 SAMPLE PREPARATION

The TatA protein of *E. coli* truncated at residue 49 was expressed and purified according to the procedure described in reference [233]. TatA protein was labelled with MTSL at positions 17, 27, 34 and 42

by Dr. Fernanda Rodriguez in Prof. Ben C. Berks' group as described in reference [237]. The mutated residue V17C is located on the transmembrane helix, while the other three residues, S27C, A34C and A42C, are located at different points along the amphipathic helix (see figure 6.1 B). EPR measurements were performed on samples in a 1:1 mixture of deuterated and protonated water containing 0.15% of DPC (dodecylphosphocholine) detergent (corresponding to a detergent to protein ratio of approximately 30, promoting complex formation). Prior to freezing, 20% of deuterated glycerol were added as glass-forming agent. NMR test measurements on solutions containing glycerol confirmed that the presence of glycerol does not cause any conformational changes.

All measurements were performed on samples with a detergent-to-protein ratio of 30, at which the TatA protein exists as an oligomeric complex. Samples with different relative concentrations of wild type (WT) to spin labelled (SL) variant were prepared by mixing solutions of the two proteins. Samples with wild-type to spin labelled (WT:SL) ratios of 0:1, 4:1, 8:1 and 16:1 were prepared. Previous studies have shown that subunit exchange is fast and that equilibration is complete after approximately 1 minute [237]. The protein concentration in the spin labelled sample (0:1 WT:SL) was 100 μM and for all other samples the overall protein concentration was 500 μM , with varying ratios of wild-type and spin labelled protein.

6.2.2 CONTINUOUS WAVE EPR

Continuous wave X-band EPR measurements were performed on a Bruker Biospin EMX spectrometer equipped with a high sensitivity Bruker probehead and an Oxford Instruments helium-flow cryostat. Room temperature measurements were performed on 8 μL solutions in capillary tubes at a microwave frequency of 9.87 GHz and with a modulation amplitude of 0.1 mT. Spin counting was performed using the Bruker Xepr software; a test measurement with a TEMPO sample at a concentration of 100 μM under the same conditions estimated the error to about 10%. The background was recorded for an empty tube and scaled to fit the external regions of the experimental spectra before subtraction. The $m_I = -1$ part of the nitroxide spectrum in some cases might still be affected by background artefacts. Low temperature EPR spectra were recorded at 170 K at a microwave frequency of 9.40 GHz and with a modulation amplitude of 0.1 mT.

6.2.3 PULSE EPR MEASUREMENTS

DEER measurements were performed at 50 K on an X-band Bruker E680 spectrometer with a Bruker 3 mm split-ring resonator (ER 4118X_MS3) equipped with a liquid helium cryostat and a variable temperature unit from Oxford Instruments.

Three-pulse DEER was performed with the sequence $\frac{\pi}{2} - \tau - \pi - \tau - \tau - echo$ at ν_A and a pump pulse at ν_B moved during τ of the observer sequence; about 400 data points were collected with 8 ns increments. Four-pulse DEER experiments were performed with the sequence $\frac{\pi}{2} - \tau_1 - \pi - \tau_1 - \tau_2 - \pi - \tau_2 - echo$ at the observer frequency ω_A , and a single π pulse at the pump frequency ω_B is moved within the section

$\tau_1 - \tau_2$ of the observer sequence in 8 ns steps. A two-step phase cycle was employed for the first observer pulse in both cases. Deuterium nuclear modulations were averaged by increasing τ_1 from 400 to 792 ns by 56 ns steps in the four-pulse sequence and by increasing τ in eight 56 ns steps in the three-pulse sequence. The pulse lengths were 32 ns for the observer and 16 ns for the pump pulses. The pump frequency was set to the maximum of the nitroxide spectrum and the observer frequency was set 65 MHz higher. Data were accumulated for 24-48 h depending on spin concentration.

The four- and three-pulse DEER data were combined using the DEER-Stitch procedure described by Lovett et al. [193]. For the final analysis, an average trace was calculated from ten DEER-Stitch traces obtained with different overlap regions.

6.3 RESULTS AND DISCUSSION

6.3.1 SPIN LABEL MOBILITY

TatA complexes labelled at four different positions, one on the transmembrane helix (V17C) and three at different points of the amphipathic helix (S27C, A34C and A42C), were investigated in aqueous solution with a concentration of detergent inducing formation of small oligomeric complexes (DPR of 30). The room temperature continuous wave EPR spectra are shown in figure 6.4 A. The EPR spectra are characteristic of the slow motion regime and confirm binding of the MTSL spin labels to the protein. The sharper signals superimposed on the $m_I = -1$ feature (see arrow in figure 6.4) could be indicative of the presence of free MTSL. However, according to simulations, the free spin label only amounts to about 2% of the total EPR signal.

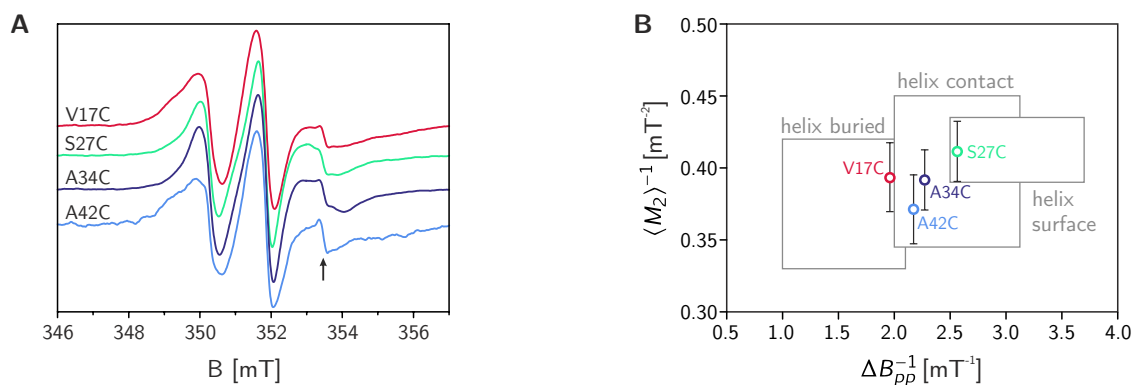


FIGURE 6.4: (A) Room temperature continuous wave EPR spectra of TatA spin labelled at different positions in water with a DPC-to-protein ratio of 30 and 20% glycerol. The 0:1 wild-type:spin labelled protein samples were used for this measurement. Experimental details can be found in section 6.2.2. The arrow indicates a potential contribution of free MTSL to the EPR spectrum. (B) Correlation of the mobility parameters $\langle M_2 \rangle^{-1}$ and ΔB_{pp}^{-1} determined from the spectra shown in A. The different topological regions in the correlation plot are highlighted (see section 5.3.3) [201].

The mobility of spin labels attached to different regions of a protein can be estimated based on the peak-to-peak linewidth of the central line of the nitroxide spectrum, ΔB_{pp} , or on the second moment of the nitroxide spectrum, $\langle M_2 \rangle$, as discussed in section 5.3.3. The experimental values of these two parameters for the four spin label positions in the TatA complex are compared to the topological regions characteristic of different spin label environments in α -helical proteins in figure 6.4 B [201]. The spin label at position V17C is characterised by low mobility, as expected for this spin label on the upper end of the transmembrane helix, close to the hinge region between the transmembrane and amphipathic helices and to the direct contact region between different TatA subunits. The spin label at position S27C is characterised by the highest mobility. Its position on the first part of the amphipathic helix would indicate high solvent exposure in the proposed model structure (figure 6.1 B), in agreement with the assignment to a helix surface region. The second part of the amphipathic helix with spin labels at positions A34C and A42C is proposed to be partially inserted into the detergent micelle and this would be in agreement with the decreased mobility as compared to the spin label at position S27C.

Simulations of the nitroxide EPR spectra resulted in predicted correlation times ranging from three to four nanoseconds, with higher values for V17C and A42C as compared to S27C and A34C, in agreement with the semi-quantitative analysis reported above.

Spin counting experiments provided information on the spin labelling efficiency, which was found to be generally low and to correspond to about 47% for V17C, 34% for S27C, 49% for A34C and 23% for A42C.

6.3.2 LOW TEMPERATURE EPR

The TatA complex was previously investigated by frozen solution continuous wave EPR in a different detergent forming larger oligomeric complexes. In that case, dipolar broadening could be detected for spin labels introduced at different positions in the transmembrane helix. The proposed model structure of the TatA complex in DPC detergent predicts inter-spin distances of only about 1.7 nm, too short to be reliably detected by DEER, but within the range of cw-EPR.

X-band continuous wave EPR spectra were recorded on oligomeric TatA samples with different ratios of spin labelled subunits at 170 K. The spectra recorded for spin labelled TatA samples and for samples with a ratio of 16:1 wild-type (WT) to spin labelled (SL) TatA are shown in figure 6.6. Short inter-spin distances lead to dipolar broadening of the cw-EPR spectrum, consequently a difference between the spectra recorded on the spin labelled sample and on the sample containing only a small number of spin labelled proteins within a TatA complex should be detected if the distance between the spin labels is smaller than about 1.7 nm [169]. The V17C variant, with the spin label on the transmembrane helix, shows a broadening of the nitroxide spectrum at a higher spin labelling stoichiometry, while the spectra for the S27C, A34C and A42C variants, with spin labels on the amphipathic helix, are almost independent of spin label stoichiometry (see figure 6.6). This first qualitative result implies a closer average proximity of the transmembrane helices with respect to the amphipathic helices, supporting

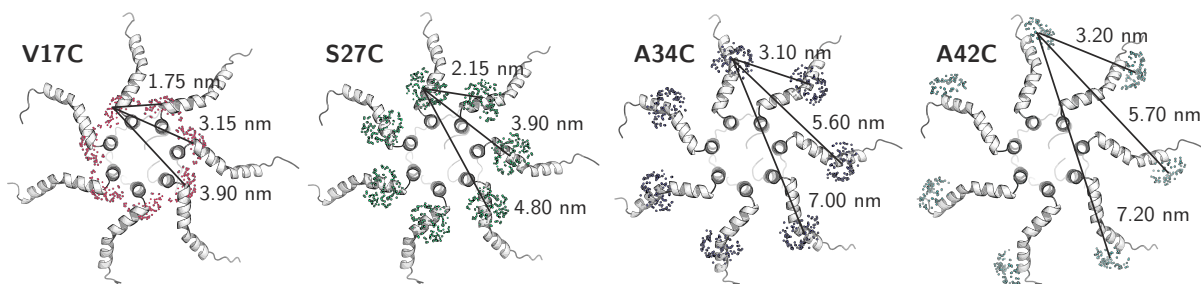


FIGURE 6.5: Model structure of the seven-membered oligomeric TatA complex with highlighted NO midpoint positions corresponding to the rotamer distributions at each spin label position calculated using MMM [214]. The average distances between the different pairs of spin labels in the complex are indicated for each of the variants.

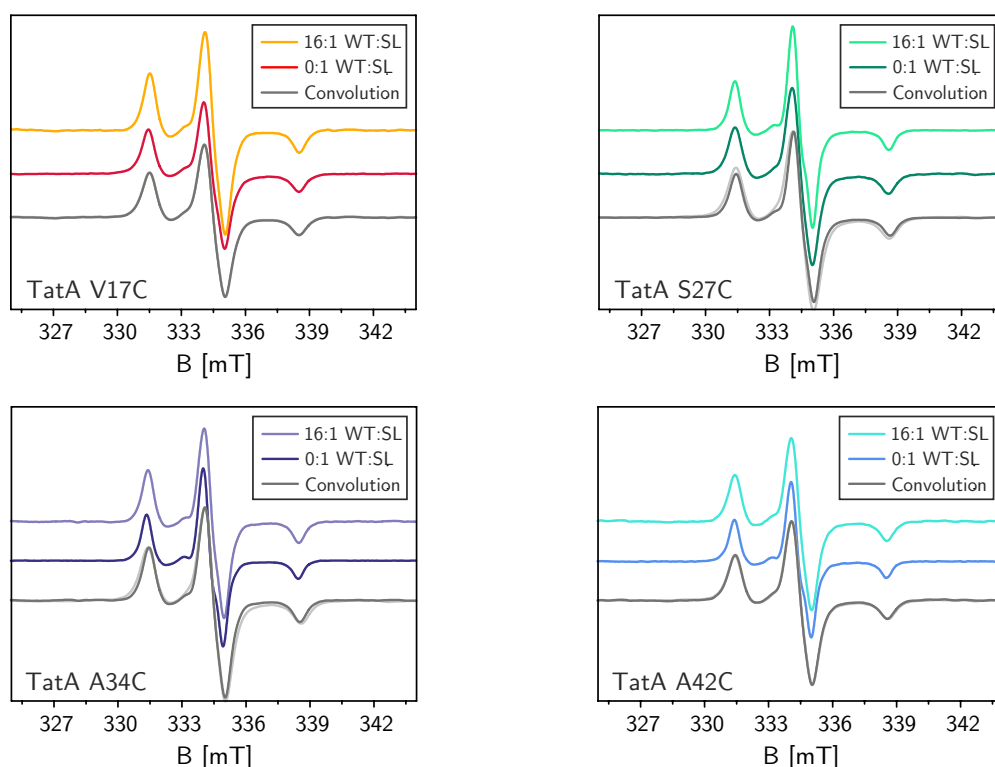


FIGURE 6.6: Cw-EPR spectra recorded at 170 K on oligomeric TatA samples for different relative concentrations of wild-type (WT) and spin labelled (SL) protein for TatA labelled in positions 17, 27, 34 and 42. The convolution of the 16:1 wild-type to spin labelled EPR spectrum with the distance distribution obtained from the model of the seven-membered oligomer is shown in grey, the results obtained using the model of the nine-membered oligomer are shown in light grey in the background. Experimental details can be found in section 6.2.2.

a model structure with the amphipathic helices pointing outwards with respect to the centre of the complex (figure 6.5).

A quantitative analysis of the results using second moment analysis (see section 5.2.1) predicts a shorter nearest-neighbour distance for the V17C variant as compared to the other variants, but

the extracted distances are affected by a large error (0.3-1.0 nm). Convolution analysis (see section 5.2.2) predicts a broad distance distribution covering the whole range from 0.5 to 4 nm for the V17C variant and was not successful for the others.

In order to verify the agreement of the low temperature EPR data with the model structure proposed based on the NOE constraints, distance distributions were predicted based on rotamer distributions computed using the MMM program [214], as explained further on in section 6.3.5 (see also figure 6.5). These distance distributions were then used to calculate the dipolar broadened spectrum by convolution of the 16:1 WT:SL spectrum with Pake patterns calculated based on the predicted distance distributions. The resulting spectra calculated for a seven-membered oligomeric complex are shown in grey in figure 6.6 and are in good agreement with the experimental results, predicting a clearly visible broadening for V17C and only smaller changes for the other variants. Spectra resulting from the convolution based on the nine-membered oligomeric model complex give identical results for V17C and A42C, but show a slightly larger broadening for S27C and A34C (see light grey spectra in figure 6.6), as the amphipathic helices move closer together.

The continuous wave EPR data thus are in agreement with the proposed model and clearly confirms a complex structure with the amphipathic helices pointing outwards and with the transmembrane helices forming the inside of the protein translocation channel.

6.3.3 DEER MEASUREMENTS

The continuous wave EPR measurements provided information on short distances between spin labels. Information on distances longer than about 1.7 nm can be determined using the DEER experiment. The largest distances that can be reliably determined using DEER depend on the length of the DEER trace that can be recorded (see section 5.3.1). The distance distributions predicted for the four spin label positions in the TatA complexes range from relatively short nearest-neighbour distances to distances longer than 6 nm across the channel complex, hence long DEER traces would be desirable. The maximum length of a DEER trace is, however, determined by the phase memory time of the sample. The phase memory time of the TatA complex in DPC micelles was increased from about 0.4 μ s to 1.8 μ s by using deuterated water and adding 20% deuterated glycerol to the sample, but could not be easily extended further. Three- and four-pulse DEER measurements were performed for TatA at 50 K and the experimental DEER traces were combined using the DEER-Stitch method [193] and are shown in figure 6.7 for a wild type to spin labelled protein ratio of 4:1. The combination of three- and four-pulse DEER allows measurements of longer DEER traces than standard four-pulse DEER, which in turn enables the determination of longer inter-spin distances.

The DEER traces are characterised by an initial drop in intensity, but no pronounced dipolar modulations are observed beyond this. A similar behaviour has often been observed for systems in detergent micelles [240]. The flexibility of the spin label and protein backbone in this protein systems probably leads to a distribution of distances that causes damping of the dipolar modulations.

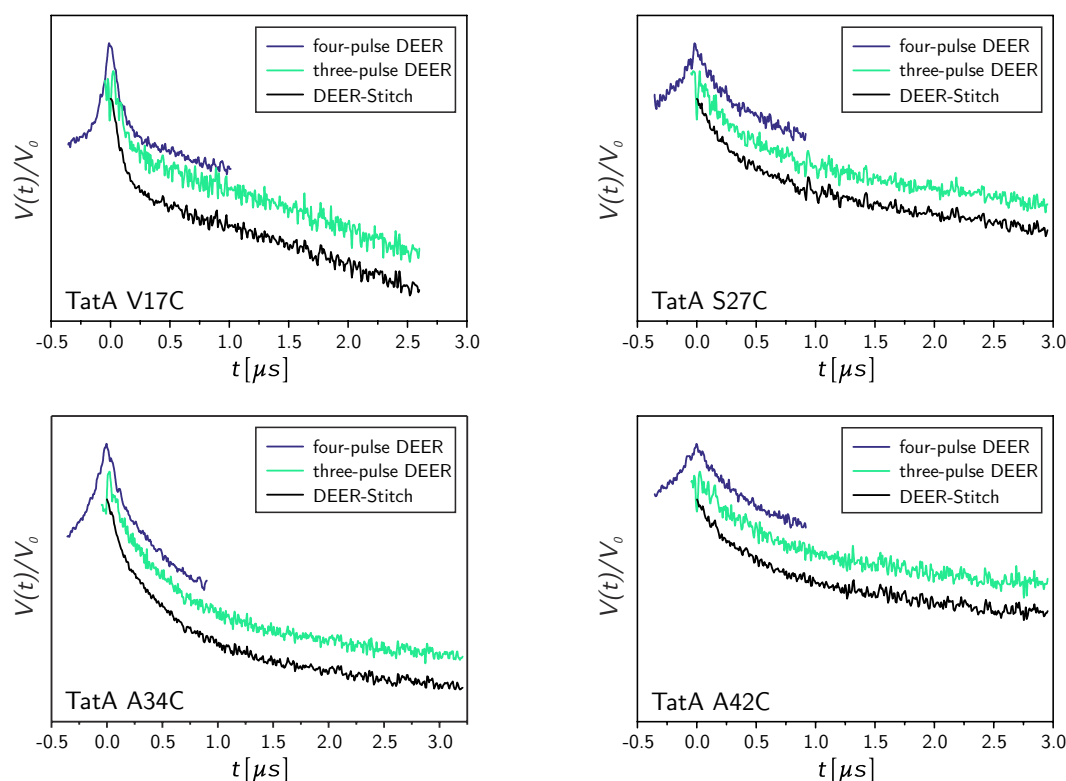


FIGURE 6.7: Three- and four-pulse DEER traces recorded at 50 K for the TatA complex spin labelled at positions V17C, S27C, A34C and A42C. The ratio of wild-type to spin labelled TatA is 4:1 for the data shown here. The overall trace obtained by combining three- and four-pulse data is also shown in black. The DEER traces are offset for better visualisation. Experimental details on the DEER experiments and on the DEER-Stitch procedure can be found in section 6.2.3.

The initial drop of the signal can be used to obtain a first qualitative estimate of the distances present. The average distance can be estimated from the half-time, i.e. the time it takes for the reduced form factor to decrease to half its initial value (see equation 5.16). The average inter-spin distances thus estimated for the different spin labelled TatA variants are 2.7 nm for V17C, 3.7 nm for S27C, 3.8 nm for A34C and 3.9 nm for A42C, where the distances estimated for A34C and A42C are affected by larger errors due to the likely presence of longer distances, which render an accurate background correction difficult.

The background factor of the DEER time traces is determined by the distribution of the spin labelled nano-objects in the sample. As discussed in Chapter 5, for a homogeneous distribution of nano-objects in solution, the background factor is an exponential function (dimensionality $d=3$ in equation 5.10) or a stretched exponential in case of preferential distribution in specific directions, such as for example in lipid bilayers. A study by Bode et al. [240] has shown that this is generally not the case for samples in detergent micelles, where spatial restriction leads to largely inhomogeneous distributions of the nano-objects. The background decay arising from intermolecular interactions was found to be determined by the aggregation number of the detergent, i.e. the number of detergent molecules per micelle. The changes observed in the DEER traces recorded for spin labels at different

positions of the TatA complex provide evidence for the detection of actual inter-spin distances in TatA complexes rather than of size-restriction effects.

The knowledge of the background factor is nevertheless important for further interpretation of the DEER data and hence measurements were performed at a dilution of 16:1 wild-type to spin labelled protein. For this ratio of wild-type to spin labelled protein, calculations of statistical distributions predict that, assuming a seven-membered complex, for a spin labelling efficiency of 100%, only about 4% of the complexes contain two spin labelled subunits, with even lower percentages for the actual labelling efficiencies determined for the TatA variants investigated here. Hence a DEER measurement performed for this degree of dilution should provide a good estimate of the background decay. Background fits with a two-dimensional background factor, different fractal background factors (non-integer d in equation 5.10) and 3^{rd} -order polynomials gave similarly good agreement with the experimental trace and led to considerable improvement with respect to the standard three-dimensional background. Analysis of the DEER traces recorded with reduced dilution factors with these different experimental background corrections did not considerably affect the distance distributions resulting from Tikhonov regularisation in DeerAnalysis [186]. Two-dimensional background corrections were used for the following analysis, unless otherwise specified.

6.3.4 MULTI-SPIN EFFECTS

The presence of more than two spin labels in each TatA complex can lead to multi-spin effects which affect both modulation depth and modulation frequencies [217, 218, 222] (see section 5.3.4). Measurements were performed for different ratios of wild-type (WT) and spin labelled (SL) protein to analyse these multi-spin effects and to avoid misinterpretation of the experimental results. Based on the spin concentration measured by cw-EPR at room temperature, it was estimated that, assuming a seven-membered complex, about 72-75% of the complexes should contain three or more spin labels for the V17C and A34C variants in the 0:1 WT:SL sample, but only 2-3% of the complexes in the 4:1 WT:SL sample and only about 0.5% of the complexes in the 8:1 WT:SL sample. This is reflected in the different modulation depths λ obtained for the samples with different WT:SL protein ratios as shown in figure 6.8.

The size of the TatA oligomers in DPC micelles has been estimated by analytical ultra-centrifugation and cross-linking studies to lie between about seven and ten TatA subunits per channel complex [233]. Analysis of the modulation depths of the DEER traces could potentially provide additional information on the size of the complexes, since the modulation depth of a DEER trace depends on the number of coupled spins as shown in section 5.3.4 (see equation 5.23). Jeschke et al. [218] and Bode et al. [217] showed that in rigid, well-defined model systems of three and four coupled spins this number can be extracted from the experimental data. In the presence of more than four coupled spins, typically only a lower limit can be determined, as the differences in modulation depth decrease with an increasing number of spins.

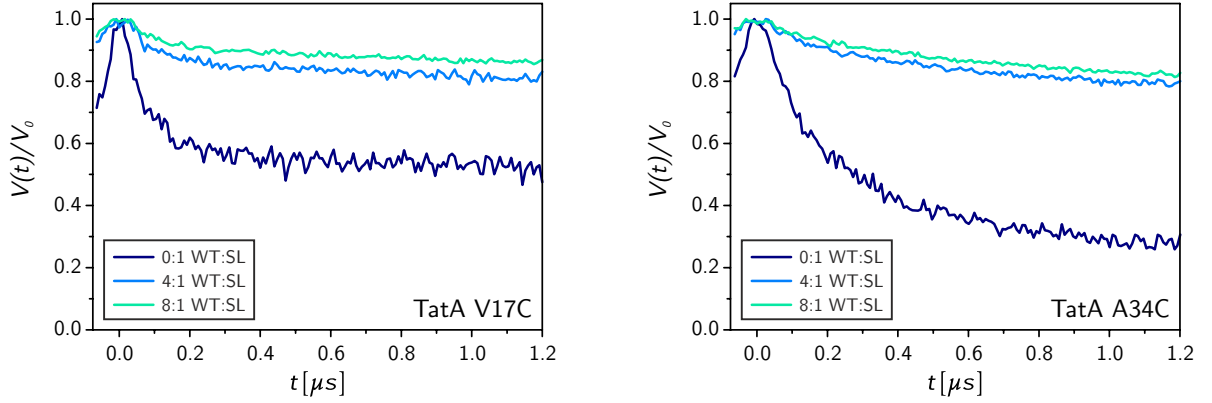


FIGURE 6.8: Four-pulse DEER traces recorded at 50 K for the TatA complex spin labelled at positions V17C and A34C for different ratios of wild-type (WT) to spin labelled (SL) TatA protein.

The accurate evaluation of the modulation depth of a DEER trace requires high signal-to-noise ratios, the precise knowledge of the spin labelling efficiency and the correct background factor. The experimental data recorded for the TatA complex do not satisfy these conditions, but an approximate quantitative analysis was nevertheless attempted, also exploiting the information on modulation depths for different ratios of wild-type to spin labelled protein. The evaluation of relative modulation depths should be less affected by the errors in background correction.

The number of spins in a TatA complex is not only determined by the size of the oligomer, but also by the statistical distribution of labelled and unlabelled TatA subunits. The probability distributions for n -membered complexes with k spin labels can be determined as:

$$P_k = \frac{n}{k(n-k)} p^k (1-p)^{n-k} \quad \text{with} \quad p = \frac{\text{spin labelled protein}}{\text{total protein}} \quad (6.1)$$

where p is the degree of labelling and the concentration of spin labelled protein was determined by taking the protein concentration and spin labelling efficiency into account. The resulting probability distributions for the spin labelling efficiencies of the V17C and A34C variants of TatA are depicted in figures 6.9 A and 6.10 A, respectively. The statistical distributions for three different sizes of the oligomeric complex are shown: the four-membered complex, proposed as the inactive protomer, and the seven- and nine-membered complexes, lying within the range of oligomer sizes predicted to be present under the experimental conditions.

The modulation depth, Δ , predicted for a complex with k spin labels is [218]:

$$\Delta_k = 1 - (1 - \lambda)^{k-1} \quad (6.2)$$

where λ is the inversion efficiency of the pump pulse, dependent on the specific spectrometer configuration. For the experimental setup used for the measurements on the TatA complexes, λ was estimated to 0.20 ± 0.05 . The overall modulation depth expected for statistically labelled complexes

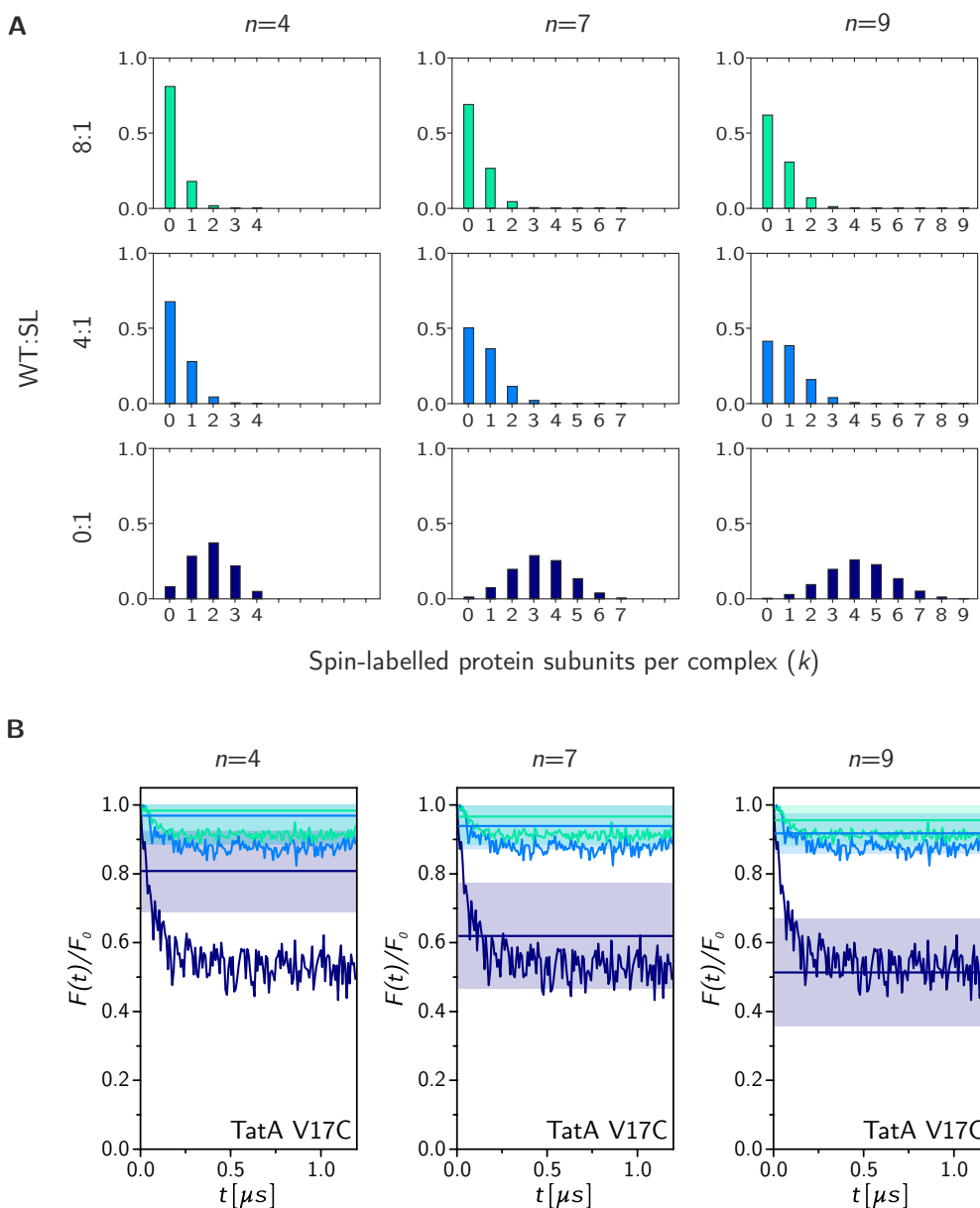


FIGURE 6.9: (A) Statistical distributions of n -membered TatA complexes with k spin labelled subunits for different wild-type to spin labelled protein stoichiometries calculated based on equation 6.1 for a spin labelling efficiency of 0.47. (B) Experimental, background-corrected four-pulse DEER form factors for different ratios of wild-type to spin labelled TatA A17C compared to modulation depths predicted based on the statistical distributions in A and a pump pulse inversion efficiency of 0.20 ± 0.05 for different oligomeric sizes of the TatA complex.

can be calculated as the weighted sum of n -membered complexes with 1 to n spin labels [240]:

$$\Delta_{total} = \frac{\sum_{k=1}^n P_k \Delta_k}{\sum_{k=1}^n P_k} \quad (6.3)$$

The modulation depths calculated for the statistical distributions discussed above are compared to the experimental form factors of the V17C and A34C DEER traces in figures 6.9 B and 6.10 B, respectively. The error on the calculated modulation depth is indicated by the shaded regions and was

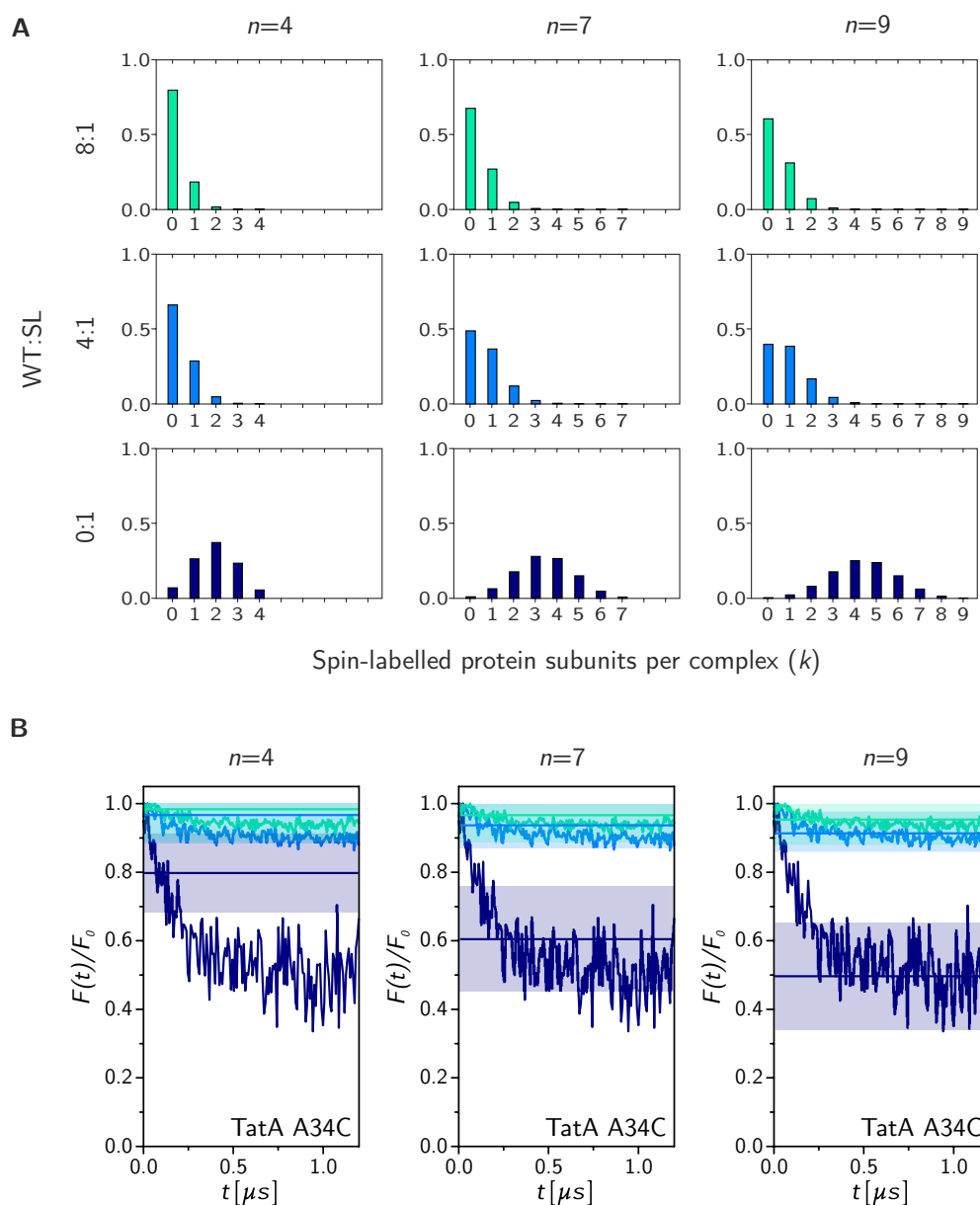


FIGURE 6.10: (A) Statistical distributions of n -membered TatA complexes with k spin labelled subunits for different wild-type to spin labelled protein stoichiometries calculated based on equation 6.1 for a spin labelling efficiency of 0.49. (B) Experimental, background-corrected four-pulse DEER form factors for different ratios of wild-type to spin labelled TatA A34C compared to modulation depths predicted based on the statistical distributions in A and a pump pulse inversion efficiency of 0.20 ± 0.05 for different oligomeric sizes of the TatA complex.

estimated through error propagation based on the errors on the inversion efficiency λ and the spin labelling efficiency. The error on the experimental modulation depth was estimated as the standard deviation of the results for different background corrections and amounts to about ± 0.05 .

The modulation depths predicted for the four-membered complex are much smaller than those determined experimentally for both TatA variants. The experimental modulation depths for both TatA variants fall within the error regions calculated for seven- and nine-membered complexes, a slightly

better agreement is obtained for the nine-membered complex. Considering the errors on the calculation and on the determination of the experimental modulation depth, the only conclusion that can be drawn is that the oligomeric size lies somewhere between seven and probably ten TatA subunits. The presence of a distribution of complexes with different sizes can also not be excluded. The results of this analysis thus only confirm previous conclusions based on other methods [233], but do not provide any further information on the size of the TatA complexes due to the low signal-to-noise, the uncertainty in background correction and the intrinsic difficulty in determining the number of spins in large systems [217, 220].

In addition to causing changes in the modulation depth, the presence of more than two coupled spins leads to the contribution of sum and difference frequencies as well as the dipolar frequencies, which lead to artefacts in the distance distributions determined by Tikhonov regularisation, as was discussed in section 5.3.4. The presence of such multi-spin artefacts was verified by comparing the distance distributions obtained by Tikhonov regularisation in DeerAnalysis for different ratios of wild-type and spin labelled TatA. The distance distributions obtained for the four-pulse DEER traces depicted in figure 6.8 are shown in figure 6.11. The error on the distance distributions was estimated with the validation tool implemented in DeerAnalysis [186] by changing the noise level and the start time for the background correction and is shown as shading in the graphs. The length of the DEER traces limits the accurate determination of the mean distance to about 4.2 nm (based on equation 5.13).

The experimental distance distributions are affected by a considerable error, but nevertheless the same trends can be identified for both TatA variants. The shortest distance determined from Tikhonov regularisation increases as the dilution of spin labelled TatA with wild-type protein increases. This is in agreement with the conclusions from model calculations in section 5.3.4, which predicted artefacts and generally higher weighting of short distances in the presence of more than two coupled spins.

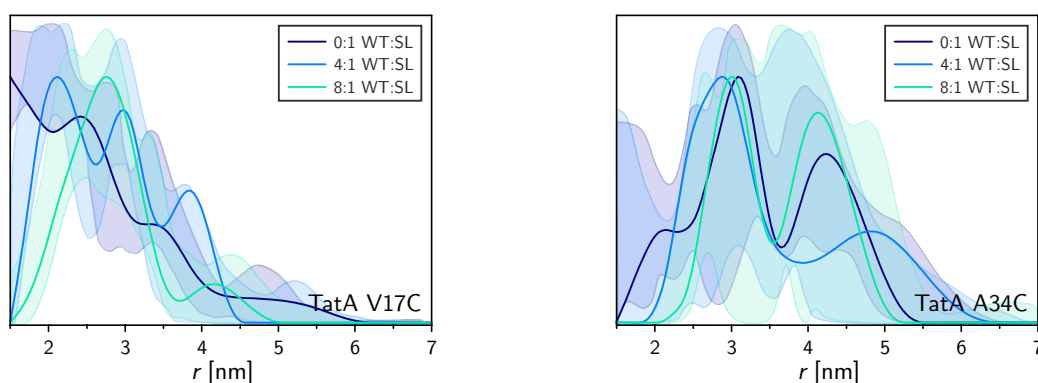


FIGURE 6.11: Distance distributions obtained by Tikhonov regularisation based on the four-pulse DEER traces recorded for the TatA complex spin labelled at positions V17C and A34C for different ratios of wild-type (WT) to spin labelled (SL) TatA protein. The DEER traces are shown in figure 6.8. The experimental DEER traces were background-corrected with a two-dimensional background and the distance distributions were extracted by Tikhonov regularisation with a regularisation parameter of $\alpha=1000$. The error on the distance distributions was estimated by evaluating the effects of noise and different background start times with the validation tool in DeerAnalysis [186].

Evaluation of the presence and reduction of multi-spin artefacts in the distance distribution are possible using power scaling of the experimental form factor as proposed in reference [222]. This method is implemented in DeerAnalysis as 'ghost suppression' and was applied for the extraction of the distance distribution from the 0:1 WT:SL traces. The power scaling with a factor based on 3-4 spins, corresponding to the most probable complexes based on the statistical distributions, led to somewhat decreased intensities at short distances, confirming the presence of artefacts. In the further analysis only the 4:1 WT:SL traces were considered, where the multi-spin effects should be negligible (only 2-6% of the complexes should contain more than two spins) and the modulation depth is still reasonable, providing an acceptable signal-to-noise ratio of the DEER data.

Theoretically it should be possible to estimate the number of spins in a complex with rotational symmetry from the number of different distances and their positions relative to the shortest, nearest-neighbour distance. The different inter-spin distances between spin labels in n -sided polygons are shown in figure 6.12. In the presence of significant distributions of rotamers, the broadening of the different peaks renders absolute assignments impossible. The distance distributions for seven to ten spins are very similar and only differ at very long distances, hence long DEER traces would be required to accurately extract the shape of the distance distribution up to the longest distance present. In the present study, the error on the distance distributions extracted by Tikhonov regularisation is too large to allow interpretation of the shapes of the distance distributions.

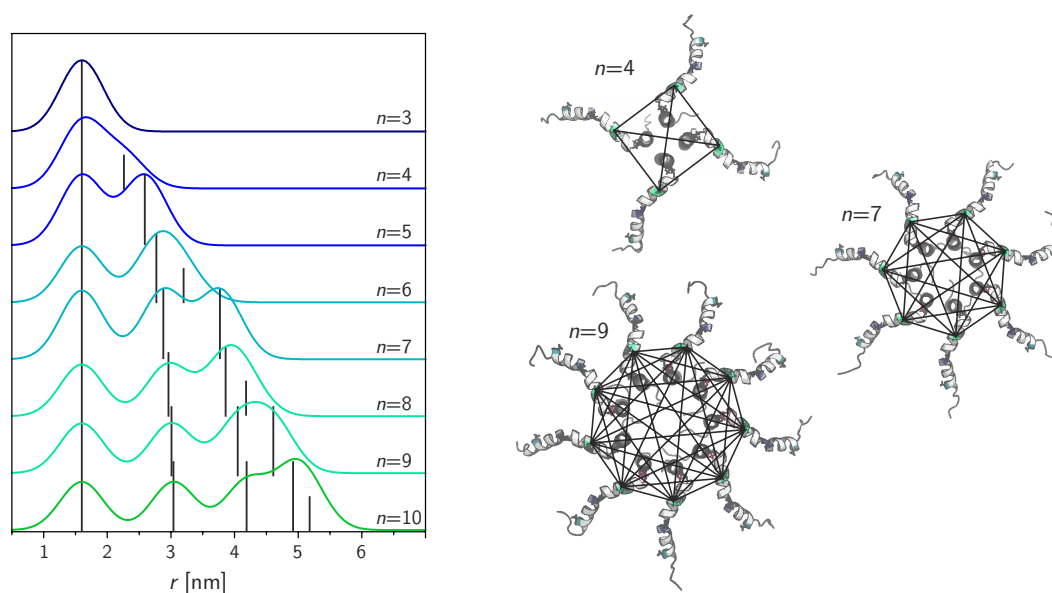


FIGURE 6.12: Distance distributions for spin label arrangements in regular polygons with n corners. The nearest-neighbour distances are taken as 1.6 nm and Gaussians with a FWHM of 0.8 nm were modelled to take distributions of NO positions into account. Models of TatA complexes with four, seven and nine subunits and the different possible inter-spin distances are also shown.

6.3.5 MODELLING AND INTERPRETATION OF THE RESULTS

The results of the data analysis described in the previous section suggest that a model-based simulation approach might be preferable for the interpretation of the DEER results on the TatA complex. Since a structural model proposed based on the NMR data already exists, this has been used as the basis for the modelling and interpretation of the experimental results.

The rotamer distributions were calculated for each spin label using the MMM program [214] on the model structure for the complex [214] and the resulting NO midpoint positions are depicted in figure 6.13. The distance distributions predicted by MMM at a simulation temperature of 175 K [214] were compared to predictions of the MtsslWizard software [215]. The distance distributions predicted by Mtssl-Wizard were generally broader, while the distributions predicted by MMM showed a series of narrow peaks. The overall extent of the predicted distance distributions was however almost identical. If a simulation temperature of 298 K was chosen in MMM, the predicted distance distribution was much closer to the results of MtsslWizard [215]. The distance distributions for the TatA complex were computed with a Monte Carlo approach based on the NO midpoint positions predicted by MMM and by randomly adding a deviation of up to ± 1.0 nm to each predicted inter-spin distance. This approach is believed to best reflect the flexibility of both the nitroxide spin labels and of the protein backbone. The resulting distance distributions for each spin label position are shown in figure 6.13.

The DEER reduced form factors were calculated based on equation 5.8 with the same Monte Carlo approach used for determination of the distance distributions. The dipolar frequency was calculated for each of the predicted distances and the reduced form factor was constructed as an average of 10^5

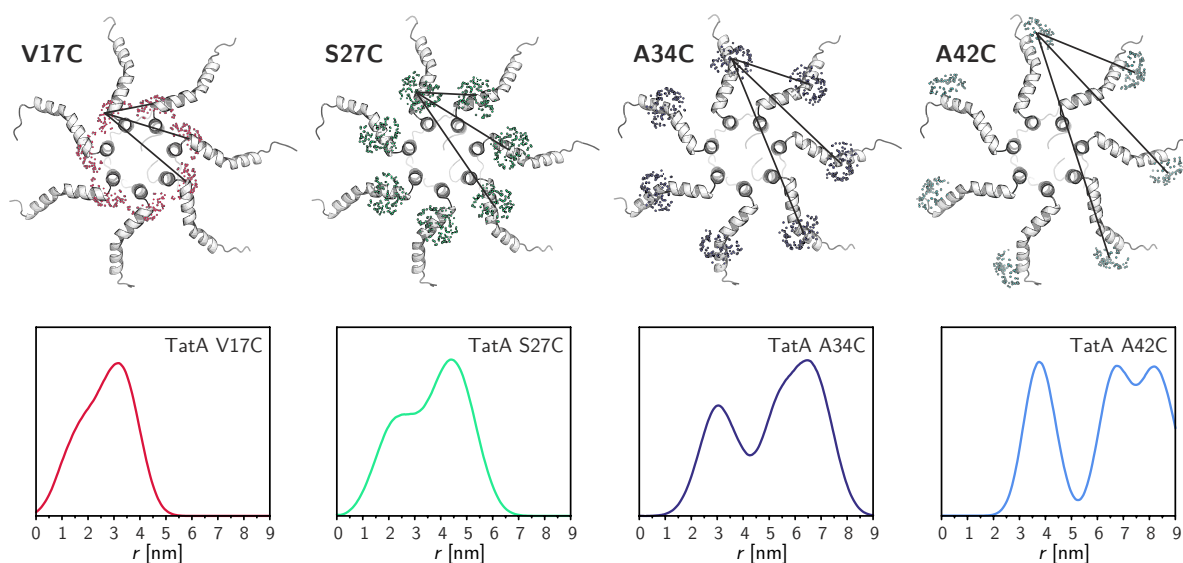


FIGURE 6.13: Model structure of the seven-membered oligomeric TatA complex with highlighted NO midpoint positions corresponding to the rotamer distributions at each spin label position calculated using MMM [214]. The distance distributions predicted based on these NO positions as described in the text are shown below the corresponding model. The distance distributions were calculated considering all of the possible pairs of spin labels in the complex.

Monte Carlo iterations. Similar computations were repeated with multi-spin effects taken explicitly into account by adding the higher-order contributions to the form factor according to equation 5.21. In the simulations for the spin label on the transmembrane helix (V17C), the excitation bandwidth of the pulses was taken into account to model the drop in sensitivity of DEER for short distances, less than about 1.5 nm, according to the procedure described in reference [169]. The simulation programs were based on MMM subroutines [214].

The final DEER traces were calculated by fitting each experimental DEER trace with a function comprising the calculated reduced form factors and a background function according to

$$\frac{V(t)}{V_0} = [1 - \lambda(1 - f(t))] \exp^{-kt^{\frac{d}{3}}} \quad (6.4)$$

where λ , k and d were optimized using a least mean square fitting algorithm. The DEER traces computed for the four spin labelling positions based on the four-, seven- and nine-membered model TatA structures constructed based on the NOE constraints are shown in figure 6.14. The simulations were compared to the 4:1 WT:SL DEER traces and multi-spin effects were not taken into account in the simulations shown, as the multi-spin contributions at this degree of dilution are expected to be negligible. The difference between the simulations with and without inclusion of higher-order contributions falls within the signal-to-noise of the experimental data.

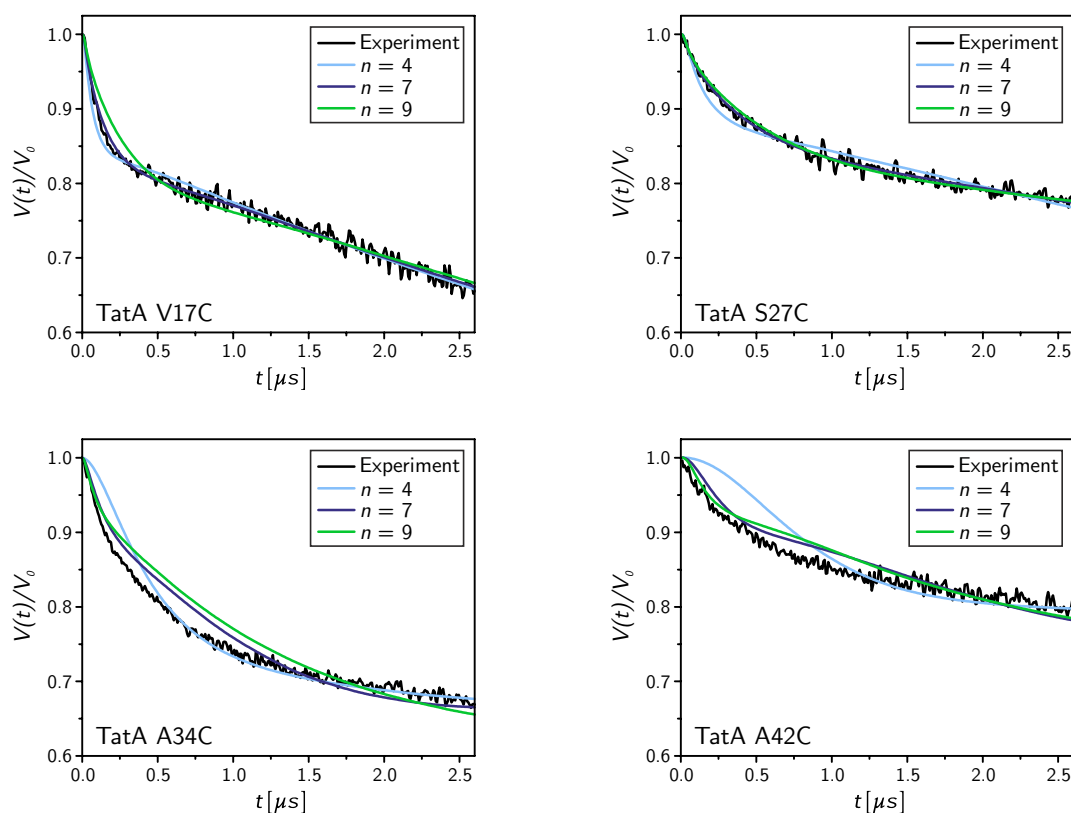


FIGURE 6.14: Comparison of the experimental 4:1 WT:SL DEER traces for the TatA V17C, S27C, A34C and A42C variants with traces simulated based on four-, seven- and nine-membered model complex structures with the method described in the text.

A good overall agreement between simulation and experiment is obtained for the TatA complexes labelled at positions V17C and S27C, on the transmembrane helix and on the proximal segment of the amphipathic helix, while larger deviations are observed for the spin label positions A34C and A42C, on the distal segment of the amphipathic helix. In all cases, larger deviations are observed for the simulations based on the structure of the four-membered TatA complex. In particular, the agreement with the initial drop, determined by the shortest distance present, is significantly worse. The observed DEER traces are thus not due to a tetrameric protomer and should reflect the structure of the active channel complex. The differences between simulations based on a seven- or nine-membered complex are generally quite small due to the similar mean inter-spin distances in both oligomers and their broad distance distributions. However, marginally better agreement is obtained for the seven-membered complex. In reality, a distribution of different sized complexes is probably present and contributes to the DEER signal.

The V17C and S27C labelling positions are close to the region of the TatA complex where NOE constraints could be determined and used to guide the generation of a model structure. The good agreement between the calculated and experimental DEER data confirms the NMR model structure with the amphipathic helices pointing outwards with respect to the centre of the TatA complex. The larger deviations between calculation and experiment for the A34C and A42C labelling positions, located at the beginning and towards the end of the distal segment of the amphipathic helix, could be attributed to a deviation from the proposed structure. In the NMR model structure, the structure of the amphipathic helix was considered to be the same as in the NMR structure of the monomeric unit [233].

Further modelling was attempted to identify the nature of the deviations from the proposed model structure. Since the TatA complex is rotationally symmetric, the distance distributions are only determined by the radius of the circle defining the average NO midpoint position. Calculations of the DEER traces as described above were repeated for different symmetric displacements of the MMM rotamer

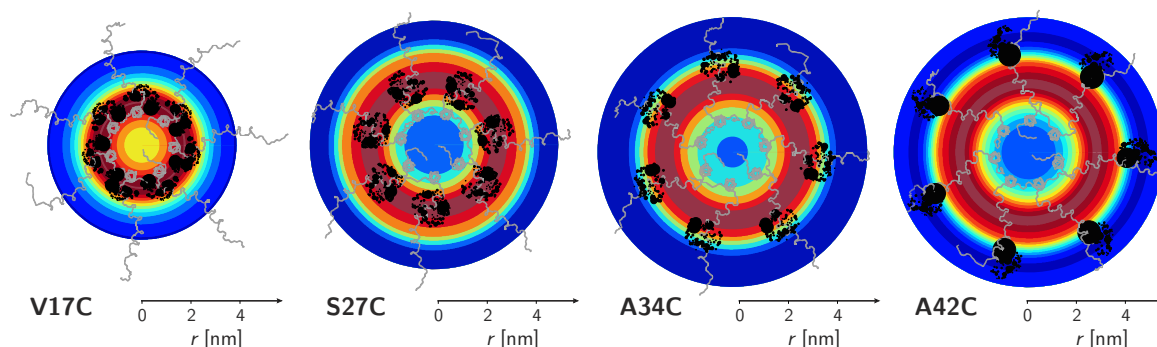


FIGURE 6.15: Contour plots of the root-mean-square deviations for DEER traces simulated for a range of displacements of the rotamer distributions from the centre of the TatA complex. The structure of the seven-membered TatA complex is shown in grey with NO midpoint positions highlighted in black with sizes depending on the rotamer populations predicted by MMM [214]. The root-mean square deviations between experimental and calculated DEER traces as a function of radius are shown in the background. The red regions correspond to the lowest and the blue regions to the highest RMSD values.

distributions from the centre of the complex and the root-mean-square deviations between calculation and experiment were computed. The results of this analysis are shown in the contour plots of figure 6.15. The rotamer distributions for the seven-membered complex are superimposed for comparison. The rotamer distributions for spin labelling positions V17C and S27C fall into the region of displacements with the lowest RMSD values, but the rotamer distributions of the NMR model for the A34C and A42C labelling positions fall at larger displacements with respect to the position giving the lowest RMSD values.

The root-mean-square deviations of the DEER traces are plotted as a function of the average NO positions with respect to the centre of the complex, determined from the rotamer distribution of each TatA subunit, in figure 6.16 A. The average NO positions with the lowest RMSD values were used to construct a model of the TatA complex in better agreement with the experimental DEER results. The TatA subunits were divided into three helices modelled as rigid cylinders, the transmembrane helix and the proximal and distal segments of the amphipathic helix. The rotamer distributions were defined with respect to this simple model of the protein. The angles between the helices were optimised in a least mean square fitting routine to give the best agreement with the average NO positions determined from the RMSD analysis. The resulting model structure is compared to the NMR structure in

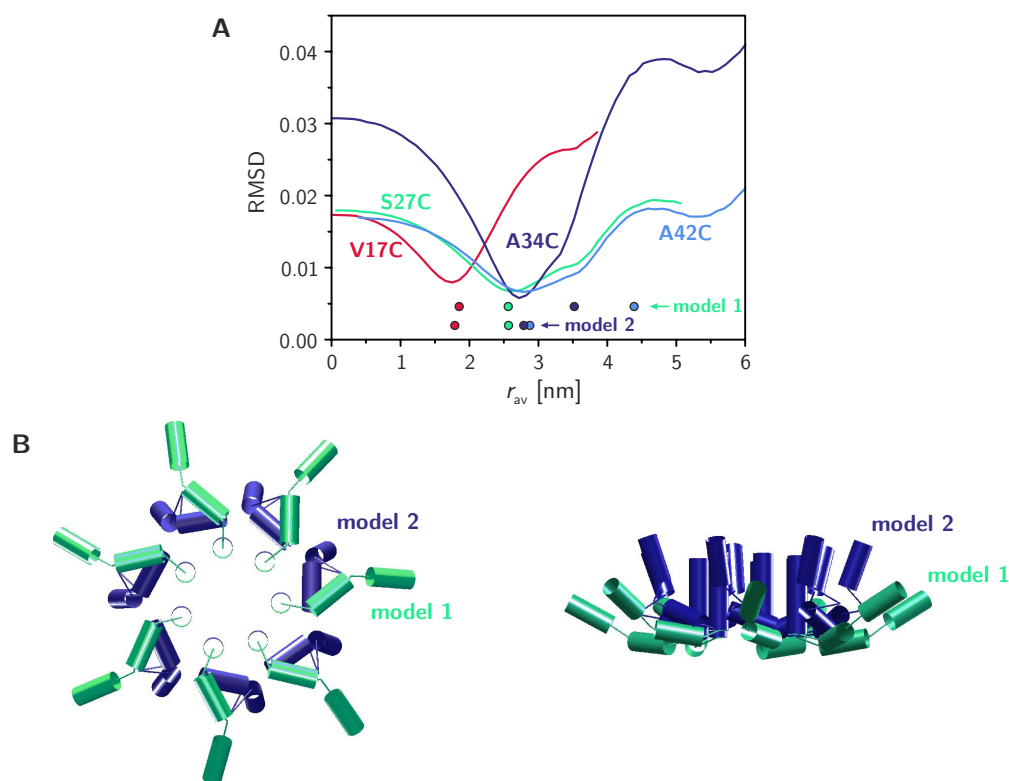


FIGURE 6.16: (A) RMSDs between experimental DEER traces and traces calculated for different displacements of the average NO position from the centre of the complex for the four different spin labelling positions. The average NO positions predicted for the model structures shown in B are indicated on the bottom. (B) Comparison of the NMR model structure (model 1, green) and the structure obtained by fitting to the average NO positions with the lowest RMSD values (model 2, blue) with overlaid transmembrane helices viewed from the top (left) and from the side (right).

figure 6.16 B and the DEER traces calculated for the two models are compared to the experimental data in figure 6.17.

The DEER results predict a shorter inter-spin distance between neighbouring nitroxide spin labels at positions A34C and A42C with respect to the inter-spin distances in the NMR model structure. The average inter-spin distances for labelling positions S27C, A34C and A42C determined from the DEER data are quite similar. If these observations are a true reflection of the inter-spin distances in the TatA complex rather than size restriction effects in micelles, this would suggest a structure with the distal segment of the amphipathic helix approximately parallel to the transmembrane helix, as only in this case the same inter-spin distances would be expected for spin labels on the proximal and on the distal segment of the amphipathic helix. These inter-spin distances are larger than the inter-spin distance for the V17C labelling position, hence the amphipathic helices would be required to form a ring with a larger radius compared to the transmembrane helix. The least mean square fitting predicts a double channel structure with an inner channel formed by the transmembrane helices and an outer channel mainly formed by the distal segment of the amphipathic helix. The angle between the proximal and distal segments of the amphipathic helix predicted in this model is about 30° , which is within the range of angles determined in the molecular dynamics simulations described in reference [233] (ca. 20 - 100°) and therefore feasible. A series of double channel structures have previously already been proposed for the TatA complex [238, 239, 241] and would also be consistent with the

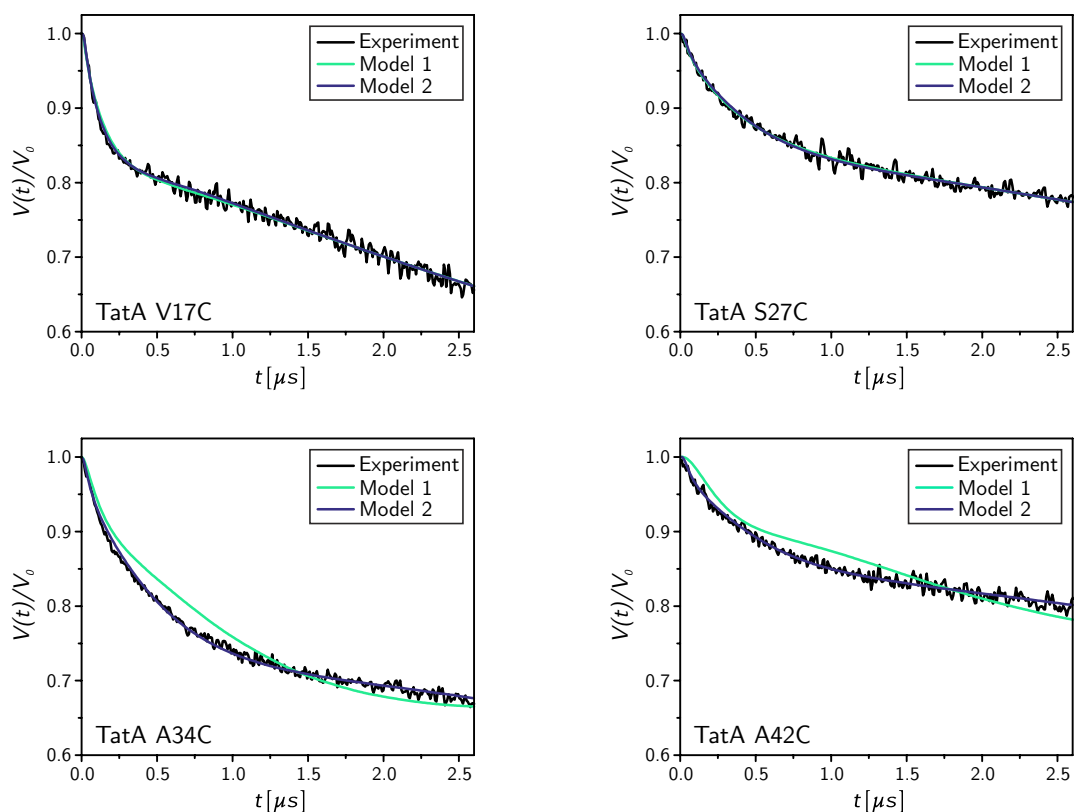


FIGURE 6.17: Comparison of the experimental 4:1 WT:SL DEER traces for the TatA V17C, S27C, A34C and A42C variants with traces simulated for seven-membered complexes with the structures shown in figure 6.16 B.

electron microscopy results [232], although typically the amphipathic helix was proposed to form the inner lining of the TatA channel, which would give smaller inter-spin distances for variants S27C, A34C and A42C compared to V17C. Complete insertion of the amphipathic helix into the membrane does however seem unlikely due to the presence of some polar and charged residues in the distal part of the amphipathic helix. Still, the structure of the complex in micelles might deviate from the structure expected in lipid bilayers, as the size of the micelle could potentially influence the complex structure.

The uncertainties related to the experimental DEER results do not allow any definite conclusions on the exact structure of the TatA complex in DPC micelles beyond the fact that the amphipathic helices are located on the outer part of the channel complex, while the transmembrane helices form the inner lining. The model proposed based on the EPR results could be verified by intramolecular distance measurements on TatA subunits labelled on both the transmembrane helix and the distal part of the amphipathic helix. The biological relevance of the structure of the complex determined in DPC micelles would have to be verified by comparison of these measurements with measurements performed in other detergents and potentially in liposomes. The use of the rigid spin label RX attached at two positions on adjacent helical turns [242] could also provide an improvement in the quality of the DEER results in terms of observed dipolar modulations.

6.4 CONCLUSIONS

The structure of the channel complex of the Tat translocation system was studied by cw-EPR and a combination of three- and four-pulse DEER as part of a larger investigation also including NMR, electron microscopy and molecular dynamics. Some of the results of this study have been published in conjunction with the corresponding NMR data in reference [233].

The observation of dipolar broadening in the cw-EPR spectrum of the TatA variant labelled on the transmembrane helix, but not in the spectra of the TatA variants labelled at different positions along the amphipathic helix and the observation of shorter inter-spin distances for the transmembrane labelling position confirmed a model structure with the amphipathic helix pointing outwards with respect to the transmembrane helix.

A quantification of the number of TatA subunits in each channel complex under the experimental conditions of this investigation was attempted based on considerations on the modulation depth and a series of simulations based on model structures with different numbers of TatA protein units per complex. The results of this analysis suggest the presence of about seven- to nine-membered oligomeric TatA complexes in DPC micelles.

While good agreement between the model structure proposed by NMR was obtained for the labelling positions on or close to the transmembrane helix, larger deviations were observed for the labelling positions on the distal part of the amphipathic helix. Molecular dynamics simulations described in reference [233] have shown that different conformations of the amphipathic helix (straight or kinked) are possible and might also influence the relative positions of the spin labels and their flexibility.

A simple modelling approach was used to determine a structure of the TatA complex that would be in agreement with the experimental results for all spin labelling positions. The proposed structure corresponds to a double-helical channel with an internal channel lined by the transmembrane helices and with the distal part of the amphipathic helices approximately parallel to the complex normal. This proposed structure would be in agreement both with the experimental continuous wave EPR and DEER results, as well as with the determined spin label mobilities based on room temperature cw-EPR measurements, which indicate higher mobilities for the spin label position on the proximal part of the amphipathic helix with respect to the labelling positions on the distal part.

The quality of the experimental data that could be achieved and the uncertainties due to the nature of the investigated system do not allow any further definite conclusions on the channel complex structure. Further investigations based on doubly-labelled TatA units and performed at higher magnetic fields could potentially provide further insight. The sensitivity of the function of the TatA protein with respect to mutations of residues on the amphipathic helix of the TatA subunits indicate its importance in the TatA translocation mechanism. Information on the static structure as well as on the conformational space explored by the TatA protein under different conditions could be important in determining the mechanism of translocation of folded proteins, which could so far not be determined at molecular resolution.

7 ORIENTATION-SELECTIVE DEER ON A FERREDOXIN-CYTOCHROME P450 COMPLEX

The work presented in this chapter is part of an ongoing collaboration between Prof. Christiane R. Timmel's and Prof. Luet L. Wong's research groups, aimed at determining the structure of protein complexes involved in the P450 electron transport chain. Previous members of the group have investigated the structure of ferredoxin reductase-ferredoxin and ferredoxin-cytochrome P450 complexes of *Rhodopseudomonas palustris* by orientation-selective DEER [230, 243–245]. The investigation has been extended here to one of the ferredoxin-cytochrome P450 complexes of *Novosphingobium aromaticivorans*, in order to compare the features of related protein complexes in different organisms.

The structural characterisation was based on the results of orientation-selective DEER measurements between a nitroxide spin label on the cytochrome P450 and the iron-sulfur cluster of the ferredoxin. The data analysis was performed using the OriDEERSim program previously developed in the group and described in section 5.3.5. The experimental results were compared to simulations performed for model structures obtained by protein-protein docking and the structures were further refined using a pattern search algorithm, in analogy to the procedure used by Dr. Jeffrey Harmer and Dr. Alice M. Bowen for the resolution of the ferredoxin-cytochrome P450 complex structure of *Rhodopseudomonas palustris* [244, 245].

7.1 CYTOCHROMES AND THE ELECTRON TRANSPORT CHAIN

Cytochrome or P450 enzymes are heme-based mono-oxygenases present in most organisms and involved in a wide range of biological processes including carbon source assimilation, biosynthesis of steroids, degradation of exogenous compounds and metabolism of medicines [246–249]. The main function of cytochromes is the activation of molecular oxygen for regio- and stereospecific oxidation of organic compounds. The enzymatic activity of cytochromes requires two electrons, which originate from oxidation of NADH (reduced nicotinamide adenine dinucleotide) and are transferred to the cytochrome by electron transport proteins. Several different classes of P450 electron transport chains exist; the principal ones are class I and class II transport chains. Class I transport chains are mainly present in prokaryotes and are based on an oxygenase-coupled NAD(P)H-dependent ferredoxin reductase with an FAD (flavin adenine dinucleotide) cofactor and an iron-sulfur cluster based ferredoxin

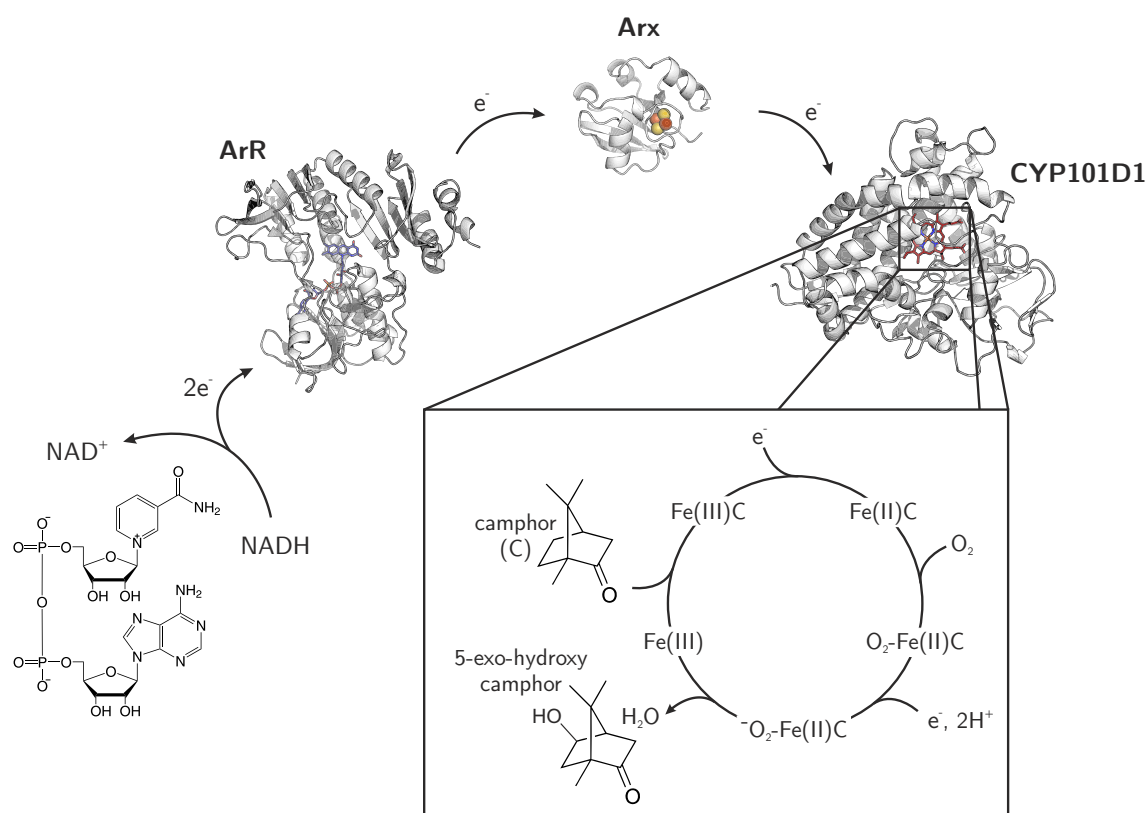


FIGURE 7.1: Schematic representation of the electron transport chain for the hydroxylation of organic substrates including a ferredoxin reductase (ArR, 3LXD.pdb), a ferredoxin (Arx, 3LXF.pdb) and a P450 cytochrome (CYP101D1, 3LXH.pdb). The catalytic cycle involving the Fe ion in the heme group of the cytochrome is also shown.

(see figure 7.1). Class II transport chains are characteristic of eukaryotes and are based on a single cytochrome P450 reductase containing both an FAD and an FMN (flavin mononucleotide) cofactor [247].

The ferredoxin-cytochrome P450 system investigated here is part of a class I electron transport chain in the gram-negative bacterium *Novosphingobium aromaticivorans*. The class I P450 electron transport systems are typically characterised by redox partner specificity, which suggests the presence of specific interactions between the proteins involved in the electron transfer process.

The crystal structures of a series of isolated cytochromes and ferredoxins are available, including the proteins investigated here [249], but the complexes are difficult to crystallise and hence only limited information on the structure of the active protein complexes is available. The structures of three different ferredoxin-cytochrome P450 pairs were determined by crystallisation of the proteins connected by a covalent linker [250–252], the structure of one of these complexes was determined in a study combining X-ray and paramagnetic NMR as well [253] and the structure of a fifth complex was resolved using spin labelling and DEER in this group [244, 245]. The advantage of structural characterisations using DEER over X-ray analysis of cross-linked complexes is that perturbation of the complex structure by the linker is avoided.

The cytochrome P450 of *Pseudomonas putida* (CYP101A1) and its complex with the corresponding ferredoxin (putidaredoxin, Pdx) is one of the most investigated and it is known that Pdx acts as an effector of cytochrome enzymatic activity [252, 254–260]. Recently a cross-linked crystal structure of the complex has been solved, which not only identified a series of residues directly involved in protein recognition and binding, but also showed that the binding of Pdx leads to a conformational change that allows establishment of a water-mediated H-bonded network promoting the electron transfer from the ferredoxin to the heme [252]. A combined X-ray and NMR study published at the same time led to an almost identical complex structure, and also showed that there were only negligible differences between the solution and crystal structure [253]. Further studies have shown that in solution the conformation of CYP101A1 depends on the oxidation state of the ferredoxin [260].

The ferredoxin-cytochrome P450 pair (CYP199A2-HaPux) previously investigated in the group is part of the electron transport chain in *Rhodopseudomonas palustris*. The structure of this protein complex was determined using X-band orientation-selective DEER measurements, protein-protein docking and molecular dynamics simulations [244, 245].

The structural features of the electron transport chain proteins of *Novosphingobium aromaticivorans* are similar to those of *Pseudomonas putida* and *Rhodopseudomonas palustris*, however significant differences in the proposed protein-protein interactions exist [249, 261].

The cytochrome CYP101D1 protein of *Novosphingobium aromaticivorans* shares 44% sequence identity with CYP101A1 and 24% sequence identity with CYP199A2. The root-mean-square deviations for the C $_{\alpha}$ atoms amount to about 0.5 and 0.3 nm, respectively. While for most cytochromes X-ray structures revealed the presence of an open or closed conformation, respectively, in the absence and presence of substrate, no difference was observed between the substrate-free and camphor-bound CYP101D1 structures [249]. In both cases the CYP101D1 protein structure resembles the closed conformation of related proteins. CYP101A1 and CYP101D1 further differ significantly in terms of electrostatic potentials, with a more positively charged interaction face for CYP101D1, due to the presence of a series of basic arginine residues (see figure 7.2). The electrostatic surface potential for CYP199A2 is similarly positively charged.

The main ferredoxin protein of *Novosphingobium aromaticivorans*, Arx (aromaticivoransredoxin), is related to Pdx of *Pseudomonas putida* and Pux of *Rhodopseudomonas putida* by 39% and 36% sequence identity, respectively. The secondary structure motifs are highly conserved in all three ferredoxins, and the iron-sulfur cluster binding loop is almost identical. In contrast to many other ferredoxins, Arx is characterised by a predominately negative electrostatic potential surface on the proposed binding interface [249] (see figure 7.2). The protein region in the immediate proximity of the iron-sulfur cluster is dominated by neutral hydrophobic residues. Compared to Pdx and Pux, Arx also has a significantly different C-terminal region, which is known to be involved in protein recognition and electron transfer [249].

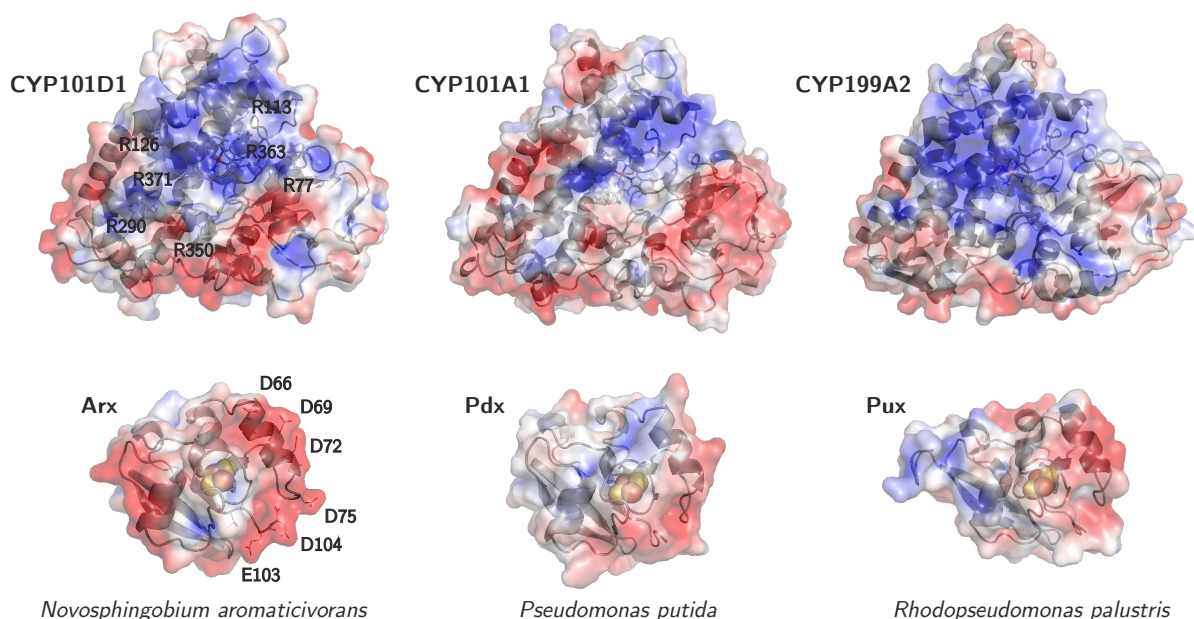


FIGURE 7.2: Electrostatic surface potential maps for the interaction faces of the ferredoxin-cytochrome P450 pairs of *Novosphingobium aromaticivorans* investigated in this work (3LXH.pdb and 3LXF.pdb [249]), of *Pseudomonas putida* (cross-linked complex structure 4JX1.pdb [252]) and of *Rhodopseudomonas palustris* (4DNJ.pdb and 3HUI.pdb [262]). Negatively and positively charged regions are coloured in red and blue, respectively. The charged residues on the interaction face are highlighted for the CYP101D1-Arx pair (R = arginine, D = aspartate, E = glutamate). The electrostatic surface potentials were generated using the Adaptive Poisson-Boltzmann Solver (APBS) plugin in PyMol [263].

The significant and approximately complementary surface charge potentials in the CYP101D1-Arx pair of *Novosphingobium aromaticivorans*, as well as an ion-strength-dependent activity of the complex, suggest the importance of electrostatic interactions in binding for this ferredoxin-cytochrome P450 system [249, 261]. The differences in the C-terminal region of the ferredoxin further suggest that the mechanism of protein recognition and electron transfer, as well as the structure of the complex, might differ from that of the CYP101A1-Pdx and CYP199A2-Pux systems.

The understanding of the structural features promoting complex formation and electron transfer is one of the first steps towards design of protein structures with improved activity for potential application in the synthesis of pharmaceuticals and fine chemicals under mild conditions and with high specificity. Since Arx was shown to efficiently transfer electrons not only to CYP101D1, but also to at least four other cytochrome proteins [261], structural information on the CYP101D1-Arx complex would further the understanding of the factors determining redox-partner specificity.

Both the cytochrome P450 and the ferredoxin contain paramagnetic species, the heme iron in the cytochrome P450 and the iron-sulfur cluster in the ferredoxin, so in theory no external modification of the system would be required for distance measurements using EPR. However, it has been shown that due to the fast relaxation of the two spin centres and the orientation selection characterising these spin systems, distance measurements are not feasible between the heme and the iron-sulfur

cluster directly [243]. Therefore the P450 protein is modified by site-directed mutagenesis and spin labelled at several positions distributed over the whole protein. The distances between those spin labels and the iron-sulfur cluster in the ferredoxin are measured.

The iron-sulfur cluster in ferredoxins is a $[\text{Fe}_2\text{S}_2]$ centre ligated by four cysteine residues and is prepared in an $S = \frac{1}{2}$ state by reduction with NADH. The paramagnetic $S = \frac{1}{2}$ state arises from anti-ferromagnetic coupling between a high-spin Fe^{3+} ($S = \frac{5}{2}$) and a high-spin Fe^{2+} ($S = 2$) ion [264]. These types of iron-sulfur clusters can be characterised by rhombic or axial g -tensors, depending on the surrounding protein structure [265]. The g -tensor orientation in the ferredoxin Pux of *Rhodopseudomonas putida* has been determined previously in the group based on ^1H ENDOR and HYSCORE measurements [230]. Due to the broad signal of the iron-sulfur cluster, orientation-selective DEER measurements [176] are possible and provide additional orientational constraints for the structure determination.

7.2 EXPERIMENTAL METHODS

7.2.1 SAMPLE PREPARATION

The cloning, mutation, expression and purification of the CYP101D1, the ArR and the Arx proteins of *Novosphingobium aromaticivorans* DSM12444 were carried out by Dr. Eachan Johnson and Mr. Yang Cao in Prof. Luet L. Wong's research group.

The sites for mutation and spin labelling were chosen on secondary structure elements with relatively limited mobility and based on considerations of solvent accessibility and distances between the nitroxide label and the iron-sulfur cluster in the proposed complex structure that fall within the range of high sensitivity by DEER [266]. The spin labelling sites were evenly distributed around the proposed binding site to facilitate characterisation of the protein complex structure. The selected labelling sites are highlighted in figure 7.3. The activity of each of the mutants was assessed and found to be similar to the activity of the wild type protein in most cases, except for lower activity of the T71C mutant and very low activity of the T135C mutant.

The CYP101D1 protein with a single cysteine mutation was exchanged into Tris-HCl buffer (pH = 7.4) using centrifugal ultrafiltration through filter membranes. A quantification of the protein concentration was performed by UV-Vis (based on the peak at 450 nm in the subtraction spectrum of CYP101D1 reduced with sodium hydrosulfite and the same solution after capping of the heme with CO, $\epsilon = 91000 \text{ M}^{-1} \text{ cm}^{-1}$). The spin labelling was performed with a 50-100-fold excess of MTSL dissolved in DMSO in a 10 μM solution of CYP101D1 containing 1 mM of camphor and 1% DMSO. The solution was kept at 4°C in the dark for 5-12 h. The solutions were then concentrated and washed two to three times with Tris-HCl buffer in D_2O with 1 mM of camphor (pD = 7.8) to remove excess spin label. The solution was then concentrated to a volume of 100-200 μl . The Arx and ArR protein solutions were similarly washed with the Tris-HCl buffer in D_2O , concentrated and the concentration quantified by

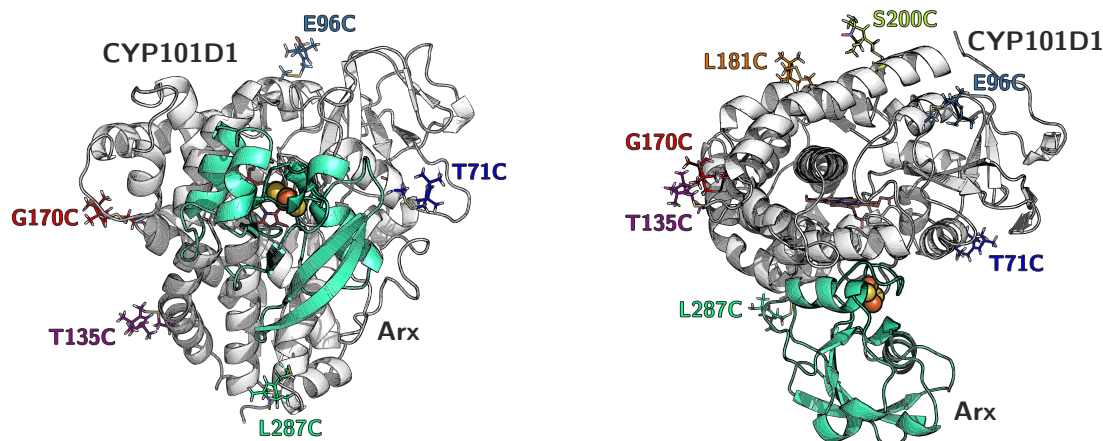


FIGURE 7.3: Top and side view of the structure of the CYP101D1-Arx complex proposed based on the structure determined for the CYP199A2-Pux complex [244] with highlighted spin labelling positions. The nitroxide rotamer with the highest population from calculations with MMM [214] is shown for each position.

UV-Vis (based on the absorbance at 415 and 455 nm, respectively, $\epsilon = 10000 \text{ M}^{-1} \text{ cm}^{-1}$ for both). A NADH solution was prepared in D_2O and the concentration was also quantified by UV-Vis based on the absorbance at 340 nm ($\epsilon = 6290 \text{ M}^{-1} \text{ cm}^{-1}$). Then CO was bubbled through the solution and it was transferred to the glove box. Equimolar amounts of concentrated CYP101D1 and Arx were mixed to a final volume of 200-300 μl and 0.01 equivalents of ArR were added. The solution was well mixed and left to incubate for about ten minutes before bubbling CO through the solution and transferring it to the glove box. In the glove box, 20-30% of glycerol- d_8 were mixed into the protein solution. Then 0.8-1.6 equivalents of NADH were added to reduce the heme and the iron-sulfur cluster and a colour change of the solution was observed after mixing. The amount of NADH added differs as it was adjusted based on the relative signal intensities of the nitroxide and iron-sulfur cluster signals in the EPR spectrum in samples prepared consecutively. The solution was then transferred to 3 mm OD X-band and 1.6 mm OD Q-band tubes and frozen in liquid nitrogen outside the glove box.

Doubly nitroxide-labelled CYP101D1 samples were prepared for the detection of potential conformational changes upon ferredoxin binding (see section 7.3.1). Twice the amount of MTSL was used for spin labelling of these samples. One sample was prepared following the same procedure as above, but adding only 0.5 equivalents of NADH to reduce the heme, but not the iron-sulfur cluster. A second sample was prepared by adding catalytic amounts (0.01 equivalents) of Arx and ArR to the CYP101D1 solution and by again using only 0.5 equivalents of NADH.

The final samples contained an EPR-silent CO-capped heme Fe(II) and a reduced $S = \frac{1}{2}$ iron-sulfur cluster. The silencing of the heme iron helped to avoid the increased relaxation rates and interference signals in the distance measurements [243].

7.2.2 PULSE EPR

X-band DEER measurements were performed at 25 K on a Bruker E680 spectrometer with a Bruker 3 mm split-ring resonator (ER 4118X_MS3) equipped with a liquid helium cryostat and a variable temperature unit from Oxford Instruments. Four-pulse DEER experiments were performed with the sequence $\frac{\pi}{2} - \tau_1 - \pi - \tau_1 - \tau_2 - \pi - \tau_2 - echo$ at the observer frequency ν_A , and a single π pulse at the pump frequency ν_B is moved within the section $\tau_1 - \tau_2$ of the observer sequence in 8 ns steps. A two-step phase cycle was employed for the first observer pulse in both cases. Deuterium nuclear modulations were averaged by increasing τ_1 from 400 to 792 ns in 56 ns steps. The pulse lengths were 40 ns for the detection and 20 ns for the pump pulses. The shot repetition time was set to 10 μ s. Orientation-selective measurements were performed by pumping at different positions in the EPR spectrum of the iron-sulfur cluster and by detecting at the maximum of the nitroxide signal. The frequency offsets $\Delta\nu$ used in the measurements were +330 MHz, +250 MHz, +165 MHz, +85 MHz and -65 MHz. Data were accumulated for 12-48 h at each pump position depending on spin concentration.

Q-band pulsed DEER measurements were performed on a Bruker E580 spectrometer with a Bruker EN 5107D2 resonator. The nitroxide-nitroxide four-pulse DEER measurements were performed at 50 K by pumping on the maximum of the nitroxide signal and detecting at a 150 MHz lower frequency. The pulse lengths were 24 ns for the detection and 12 ns for the pump pulse. The orientation-selective nitroxide-iron-sulfur cluster DEER measurements were performed at 25 K by pumping on the maximum of the nitroxide signal and detecting at two positions of the iron-sulfur cluster EPR spectrum. The detection frequencies were offset by +250 MHz and -180 MHz. Pulse lengths of 16 ns and 32 ns were used for the pump and detection pulses, respectively. For both types of measurements deuterium nuclear modulations were averaged out by increasing τ_1 from 180 to 292 ns in 16 ns steps. The signals from unwanted echoes were removed by an eight-step phase cycle. The shot repetition time was set to 5 μ s for the nitroxide-nitroxide and to 8 μ s for the nitroxide-iron-sulfur cluster measurements.

7.2.3 PROTEIN-PROTEIN DOCKING

The structure of the ferredoxin-cytochrome P450 complex was determined by comparing the experimental DEER results with simulations based on a series of model structures using a procedure analogous to that described in Dr. Alice Bowen's thesis [244] and discussed in more detail in section 7.3.3. The model structures for the protein complex were generated using the docking software PatchDock and FireDock [267–269].

The PatchDock software performs geometry-based rigid-body docking of two proteins. The surface of each of the proteins is described in terms of convex, concave or flat patches and the docking program generates a series of structures based on complementarity of these patches on the two proteins [267]. In the present case, the surface regions of the P450 and ferredoxin proteins involved in binding are known based on previous studies [249] and hence the docking was guided by defining a series of residues on the binding interface (CYP101D1: R78, R114, R127, R291, R351, R364 and R372;

Arx: L39, D67, D70, D73, D76, E104 and D105, see figure 7.2). The FireDock software allows further refinement of the docked protein complex structures by side-chain optimisation followed by further rigid-body minimisation [268, 269]. A total of about 15000 model structures was generated using these two programs and then evaluated in terms of agreement with the experimental DEER data as described in detail in section 7.3.3.

7.3 RESULTS AND DISCUSSION

7.3.1 CONFORMATION OF THE P450 PROTEIN

Most of the cytochrome P450 proteins can exist in two different conformations: an open or substrate-free and a closed or substrate-bound conformation. The conformational change from the closed to the open state involves a shift of the helices F and G on the distal site of the heme and increased disorder for the B' helix, revealing the substrate access channel [252] (see figure 7.4). Additional movements of smaller magnitude also occur on the I helix, close to the O₂ binding site, and the C helix on the ferredoxin binding site [252].

Crystal structures of both open and closed conformations of CYP101A1 and CYP199A2 are available, however crystallisation of CYP101D1, investigated here, led to structures with closed conformations in the absence and presence of substrate, suggesting that no such conformational change occurs for this cytochrome P450 protein [249, 270].

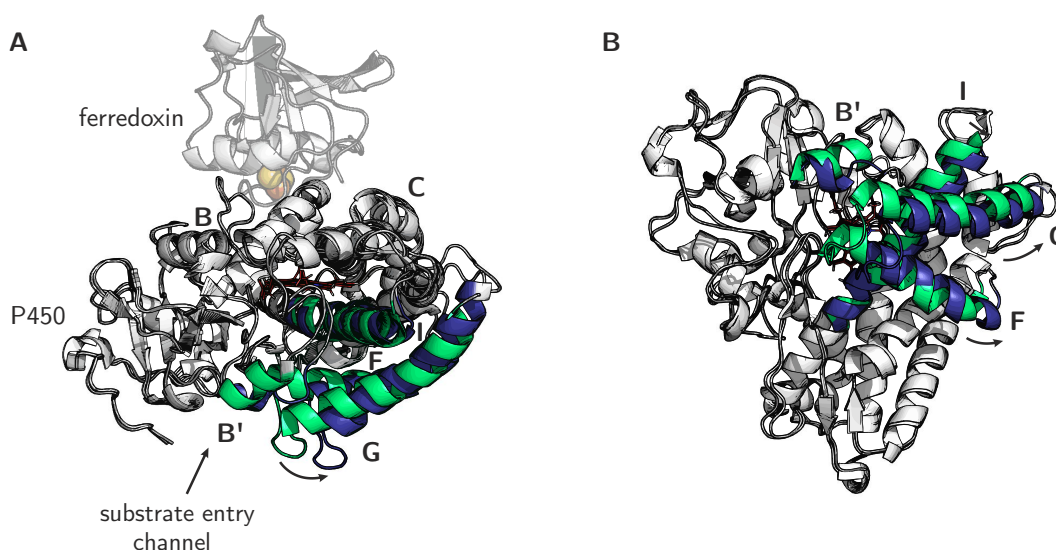


FIGURE 7.4: Superposition of the closed (green) and open (blue) conformations of the cytochrome of *Pseudomonas putida* (CYP101A1) viewed from the side with the bound ferredoxin (A) and from the substrate entry channel (B) (3L63.pdb (closed) and 4JX1.pdb (open, ferredoxin bound)). The helices involved in the conformational change, B', F, G and I, are highlighted and the directions of the helix movements from a closed to an open conformation are indicated by arrows.

A recent study by Tripathi et al. on a cross-linked CYP101A1-Pdx complex revealed that, in contrast to previous theories, the cytochrome is present in its open conformation in complex with the ferredoxin [252]. Based on this result it was suggested that the effector role of Pdx consists in shifting CYP101A1 towards the open conformation, where a water-mediated H-bonded network promotes the proton-coupled electron transfer and oxygen activation [252]. A further study using nitroxide-nitroxide DEER suggested that in solution the conformation of CYP101A1 depends on the oxidation state of both the heme iron and the iron-sulfur cluster of the ferredoxin [260].

Based on these results from the literature, it would be possible that, even though X-ray structure resolution indicates the presence of only the closed conformation of CYP101D1 of *Novosphingobium aromaticivorans*, an open conformation may be visited in the presence of Arx. This prompted additional intra-protein distance measurements on CYP101D1, as previously used to monitor the conformational change of CYP101A1 in solution upon substrate binding by X-band DEER [271].

The nitroxide spin labels for the intramolecular measurement were introduced at a position on the G helix (S200C), which is involved in the conformational change observed for other cytochrome P450s, and at a position on the opposite side of the protein (T71C) not involved in the conformational change (see figure 7.5 A). Q-band four-pulse DEER measurements were performed on a sample prepared with only catalytic amounts of Arx (CYP T71C/S200C) to determine the conformation of free CYP101D1 and to confirm the validity of the X-ray structure. The same measurements were repeated on a sample prepared with the same procedure used for the DEER measurements on the protein complex (CYP T71C/S200C + Arx), in order to detect potential conformational changes occurring upon binding of the ferredoxin Arx. The results of the measurements on the two samples are shown in figure 7.5 C and the pump and detection positions used are indicated by arrows on the spectrum in figure 7.5 B. Test measurements for different frequency offsets ($\Delta\nu = 80\text{--}200\text{ MHz}$) revealed only minor orientation selection effects (data not shown). The DEER traces recorded for the two samples are characterised by the same modulation frequency, indicating that CYP101D1 assumes the same conformation under both conditions.

The agreement of the experimental results with the crystal structure was verified by simulations. The rotamer distributions at both spin labelling positions were calculated using the MMM program [214] and the corresponding NO midpoint positions are shown in figure 7.5 A. The calculation of rotamer distributions for a temperature of 298 K yielded a marginally better agreement with the experimental results compared to the distributions obtained for 175 K and thus MMM rotamer distributions computed for 298 K were used in the rest of the analysis. DEER traces for each pair of rotamers with a probability exceeding 0.005 were computed using the OriDEERSim program and the weighted sum was calculated. The simulations were compared to the experimental data after adjusting the modulation depth and adding a background decay function for each of the two traces individually (see figure 7.5 C). The corresponding distance distribution is shown in figure 7.5 D. The calculations were repeated based on a structure of CYP101D1 in the open conformation obtained by homology modelling (data not shown). A shift of the maximum of the distance distribution from 4.25 to 4.65 nm would be expected in case of a change from the closed to the open conformation. The agreement

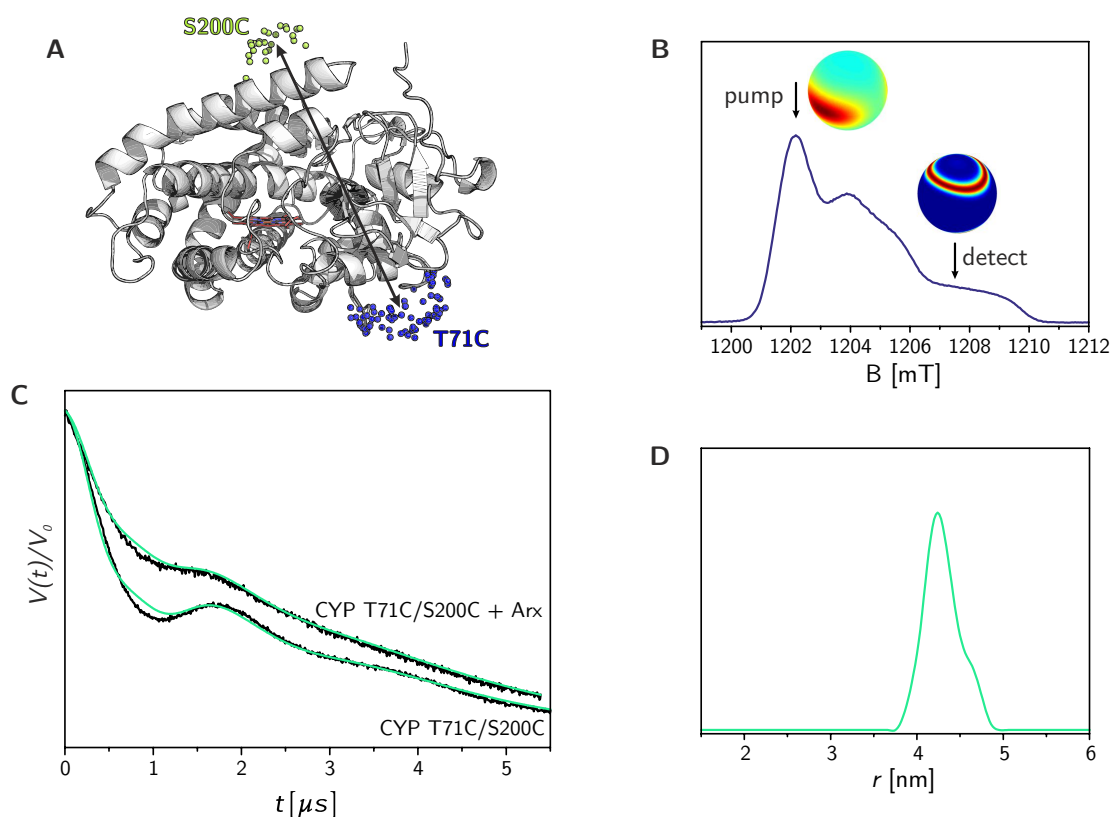


FIGURE 7.5: (A) Structure of CYP101D1 (3LXH.pdb) with NO midpoint positions predicted for the labelling sites T71C and S200C by MMM [214]. (B) Experimental Q-band nitroxide spectrum recorded at 50 K for the CYP T71C/S200C sample at a microwave frequency of about 33.7 GHz. The pump and detection positions and the corresponding orientations excited by a 12 and 24 ns pulse, respectively, are also shown. (C) Experimental Q-band four-pulse DEER traces recorded at 50 K for CYP T71C/S200C and CYP T71C/S200C-Arx. The experimental details are described in section 7.2.2. Simulations performed using the OriDEERSim program [227] based on MMM rotamer distributions are compared to the experimental results. The simulated form factors were multiplied by an exponential decay to compare them to the experimental data. The background and modulation depth were fitted individually to each of the two DEER traces. (D) Distance distribution resulting from the MMM rotamer distributions (computed for the X-ray structure of CYP101D1 (3LXH.pdb) at 298 K) at the labelling positions T71C and S200C and used for the calculation of the DEER simulations shown in C.

between simulations for the closed conformation and experiment is quite good for both samples, confirming that the solution structure is in agreement with the crystal structure and thus justifying rigid-body docking for the determination of the structure of the ferredoxin-cytochrome P450 complex.

The same two labelling positions used for the intramolecular nitroxide-nitroxide distance measurements were also used individually for the intermolecular nitroxide-iron-sulfur cluster measurements described in the next section. DEER measurements for the T71C mutant unfortunately did not yield any modulations, while, based on the EPR spectrum, nitroxide spin label and reduced iron-sulfur cluster were both present in the sample at approximately equal concentrations. These results indicate that either the distance is too short (<1.7 nm) to be observed by DEER or that the presence of the spin label prevents binding of Arx to CYP101D1. This casts doubts on the results of this conformational analysis and a potential change to an open conformation upon ferredoxin binding cannot be excluded with absolute certainty. Additional mutations should be introduced at positions located at a

greater distance from the binding interface and the measurements should be repeated to be able to conclusively prove the absence of a conformational change in CYP101D1.

7.3.2 DEER MEASUREMENTS ON THE CYP101D1-ARX COMPLEX

The determination of the structure of the CYP101D1-Arx complex was based on orientation-selective four-pulse DEER measurements performed at X-band and partly at Q-band frequencies. Orientation selection is provided by the broad iron-sulfur cluster signal characterised by an axial g -tensor with the principal values $g_{\perp} = 1.933$ and $g_{\parallel} = 2.023$. The X-band DEER measurements were performed by pumping at different positions of the iron-sulfur cluster spectrum and by detecting at the maximum of the nitroxide signal, corresponding to a g -value of about 2.005. The pump positions are distributed over the whole iron-sulfur cluster spectrum and excite different orientations ranging from the $g_{\perp}$ plane to the $g_{\parallel}$ axis as shown in figure 7.6. The detection on the maximum of the nitroxide signal leads to an almost isotropic excitation of this spin and typically no orientational effects are observed at X-band for flexible spin labels leading to a distribution of rotamers in frozen solution [176]. The Q-band DEER measurements were performed by pumping on the maximum of the nitroxide signal, in order to achieve an almost isotropic excitation of this spin centre, and by detecting on the iron-sulfur cluster. At Q-band the spectrum of the iron-sulfur cluster extends over a wider field range and due to the limited bandwidth of the resonator only two detection positions on either side of the nitroxide signal could be used. The orientations selected at these positions are shown in figure 7.6.

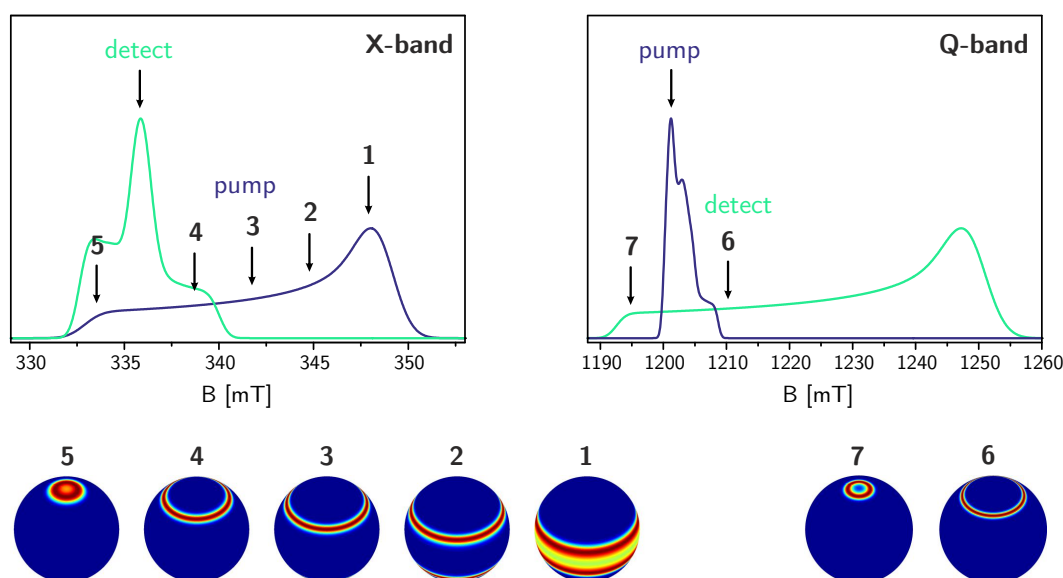


FIGURE 7.6: Detection and pump pulse positions with respect to simulated nitroxide and iron-sulfur cluster EPR spectra at X- and Q-band. Orientations of the iron-sulfur cluster excited at each position ($t_{\pi} = 20$ ns at X-band and $t_{\pi} = 32$ ns at Q-band) are indicated on a unit sphere (red - intensely excited, blue - not excited). At X-band the detection frequencies were offset by 330, 250, 165, 80 and -65 MHz from the pump frequency, at Q-band the frequency offsets correspond to 250 and -180 MHz.

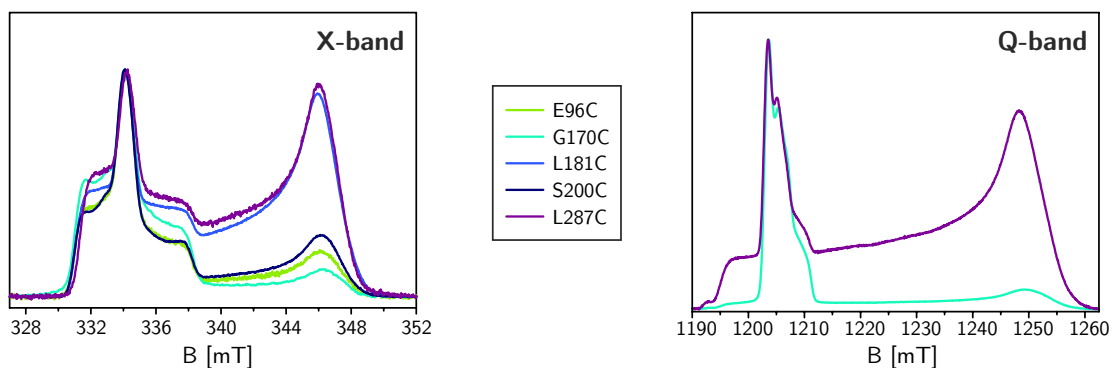


FIGURE 7.7: Experimental echo-detected X- and Q-band EPR spectra of the mutants considered for the structure determination recorded at 25 K ($\nu_{mw} = 9.45$ and 33.8 GHz). The spectra are normalised to the maximum of the nitroxide signal.

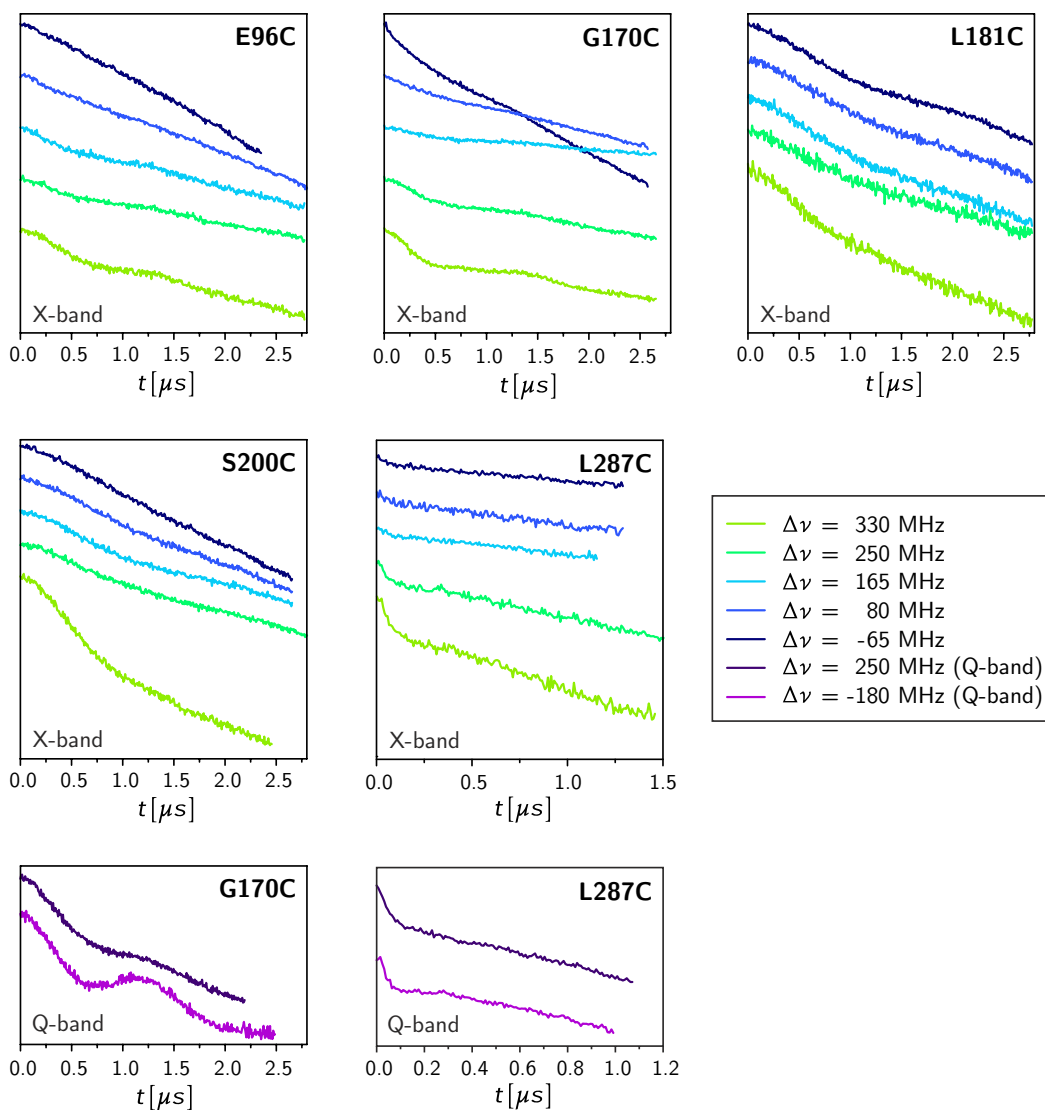


FIGURE 7.8: Experimental orientation-selective X- and Q-band four-pulse DEER data recorded at 25 K for the CYP101D1-Arx complex with nitroxide spin labels introduced at positions E96C, G170C, L181C, S200C and L287C on CYP101D1. Experimental details can be found in section 7.2.2. The traces are offset for better visualisation.

The echo-detected EPR spectra recorded at X- and Q-band for the samples used for the DEER measurements are shown in figure 7.7 and the corresponding experimental DEER traces are shown in figure 7.8. The different relative intensities of the nitroxide and iron-sulfur cluster signals can be attributed to the difficulty in reliably reducing the iron-sulfur cluster while avoiding reduction of the nitroxide spin label. The differences in the spectra are reflected in the sample-to-sample variability of the DEER modulation depths and signal-to-noise ratios. The spectra of the T71C and T135C mutants are not shown as they did not give any modulations in the DEER measurements, although the signals of both spin centres were present in the echo-detected EPR spectra.

The different DEER traces recorded for each mutant are characterised by significantly different modulation depths, depending both on the intensity of the iron-sulfur cluster signal at the pump frequency as well as on the experimental measurement conditions. Close inspection of the experimental traces reveals orientation selection effects of varying magnitude in the initial decay of the DEER signal for all mutants. More pronounced effects, such as a clearly visible shift in modulation frequency for G170C, are observable for the Q-band DEER data.

7.3.3 DETERMINATION OF THE CYP101D1-ARX COMPLEX STRUCTURE

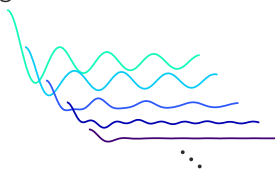
The orientational selectivity of the DEER measurements performed on the ferredoxin-cytochrome P450 complex provides orientational information in addition to distance information and allows determination of the structure of the complex with a reduced number of spin labelling positions. In order to exploit this additional information, orientation selection needs to be taken into account in the simulation of the experimental data. The procedure for the extraction of structural information on the CYP101D1-Arx complex from the orientation-selective DEER data is based on a similar study on the CYP199A2-Pux complex [244] and is summarised in figure 7.9.

The orientation-selective DEER traces were calculated using the OriDEERSim program previously developed in the group [227, 244] (see section 5.3.5 for a description of the program). Repeating the calculation of DEER traces for every rotamer of each mutant in each of the model structures would be very time-consuming. For this reason, a library of DEER traces for distances from 1.5 to 6.3 nm with a 0.1 nm spacing and for orientations of the nitroxide NO midpoint distributed on a spherical grid with 30 knots in the g -tensor frame of the iron-sulfur cluster was generated. For the calculation of the DEER traces for each model structure, the position and orientation of the NO midpoint in the $[\text{Fe}_2\text{S}_2]$ g -tensor frame for each nitroxide- $[\text{Fe}_2\text{S}_2]$ pair was then determined and the simulated traces for the closest point on the positional and orientational grid were loaded from the library. The simulations were based on the spin system parameters determined for the nitroxide and iron-sulfur cluster and the experimental frequency offsets between detection and pump frequencies. In all of the simulations, both for X- and Q-band, the nitroxide was assumed to be isotropically excited.

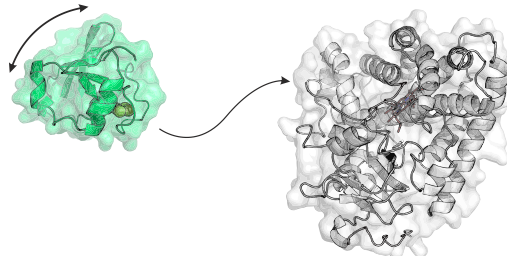
The simulation of orientation-selective DEER between a nitroxide spin label and the iron-sulfur cluster of Arx requires the knowledge of the orientation of the $[\text{Fe}_2\text{S}_2]$ g -tensor with respect to its structure and the distribution of spin density over the iron-sulfur cluster and adjacent binding cysteines. The

- 1 Calculation of a library of orientation-selective DEER traces for the experimental frequency offsets and magnetic fields, for distances ranging between 1.5 and 6.3 nm and angles, θ and ϕ , evenly distributed on a sphere.

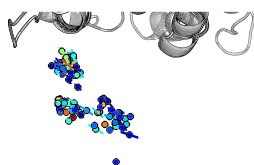
$r, \theta, \phi, \Delta\nu$



- 2 Generation of a set of model structures by protein-protein docking, guided by information on the interaction interface region, using the PatchDock and FireDock programs.

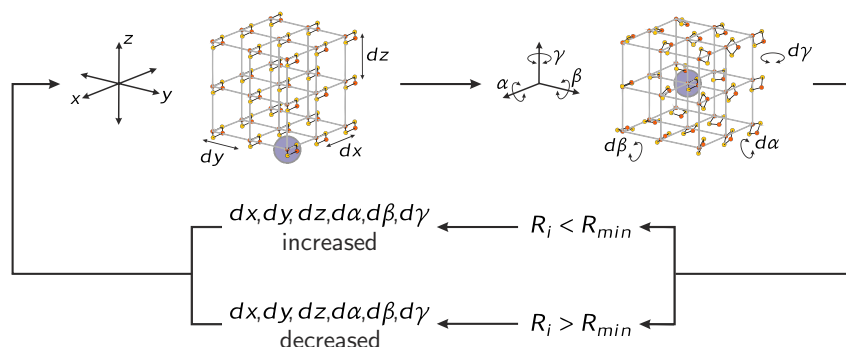


- 3 Calculation of the DEER traces for the model structures using the library of orientation-selective DEER traces calculated with OriDEERSim. Rating of each of the model structures based on the weighted root-mean-square deviation between the simulations and the experimental data for each mutant and frequency offset.



$$R_m = \omega_m \sum_{n=1}^{N(\Delta\nu)} \sqrt{\frac{\sum_{i=1}^N (V_{m,n}^{exp} - V_{m,n}^{sim})^2}{N}}$$

- 4 Optimisation of the top-ranking model structures by translation in three directions and rotation around three axes of Arx with respect to CYP101D1 using a pattern search algorithm.



- 5 Analysis of the optimised structures in terms of known requirements for biological function and determination of the structural properties of the CYP101D1-Arx complex.

FIGURE 7.9: Outline of the procedure used for the determination of the CYP101D1-Arx structure based on the experimental orientation-selective DEER data.

iron-sulfur cluster of the ferredoxin Pux of *Rhodopseudomonas palustris* was characterised in detail using ENDOR and HYSCORE by previous members of the group [230]. The ferredoxins Pux and Arx have highly conserved secondary structure motifs (figure 7.10 A), leading to a root mean square deviation of only 0.033 nm over the $[\text{Fe}_2\text{S}_2]$ cluster and the adjacent residues shown in figure 7.10 B. This pronounced similarity justifies the assumption of the validity of the $\mathbf{g}$ -tensor orientation and spin

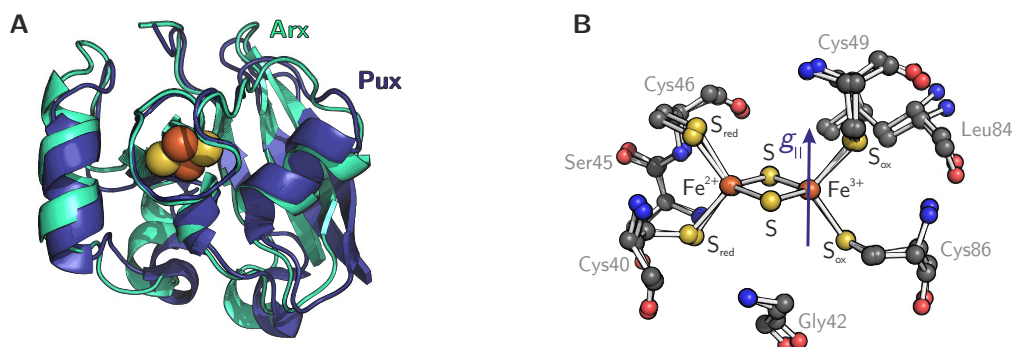


FIGURE 7.10: (A) X-ray structures of the ferredoxins Arx (green) and Pux (blue) of *Novosphingobium aromaticivorans* and *Rhodopseudomonas palustris*, respectively (3LXF.pdb and 3HUI.pdb, aligned on the [Fe₂S₂] cluster). (B) Comparison of the active sites of the two ferredoxins and orientation of $g_{||}$ as determined for Pux by ENDOR and HYSCORE in reference [230].

density distribution determined for Pux also for the ferredoxin Arx used in this study. The orientation of the g -tensor in the molecular frame of Arx is depicted in figure 7.10 B. The $g_{||}$ axis was determined to lie at an angle of approximately 26° with respect to the axis perpendicular to the plane of the [Fe₂S₂] cluster. The spin density was considered to be distributed over the iron-sulfur cluster as well as the sulfur atoms of the four ligating cysteines according to $\rho_{\text{Fe}^{3+}} = +1.555$, $\rho_{\text{Fe}^{2+}} = -0.841$, $\rho_{\text{S}} = +0.083$, $\rho_{\text{S}_{\text{ox}}} = +0.089$ and $\rho_{\text{S}_{\text{red}}} = -0.030$ [230].

Protein-protein docking was used as described in section 7.2.3 to generate a large amount of potential structures of the complex. Simulations of the DEER traces were performed for each of the model structures by adding rotamer distributions computed using MMM for each of the spin labelling positions (see figure 7.11) to the coordinates of CYP101D1. The structures were then transformed into the g -tensor frame of the iron-sulfur cluster and the library of DEER traces was used to calculate

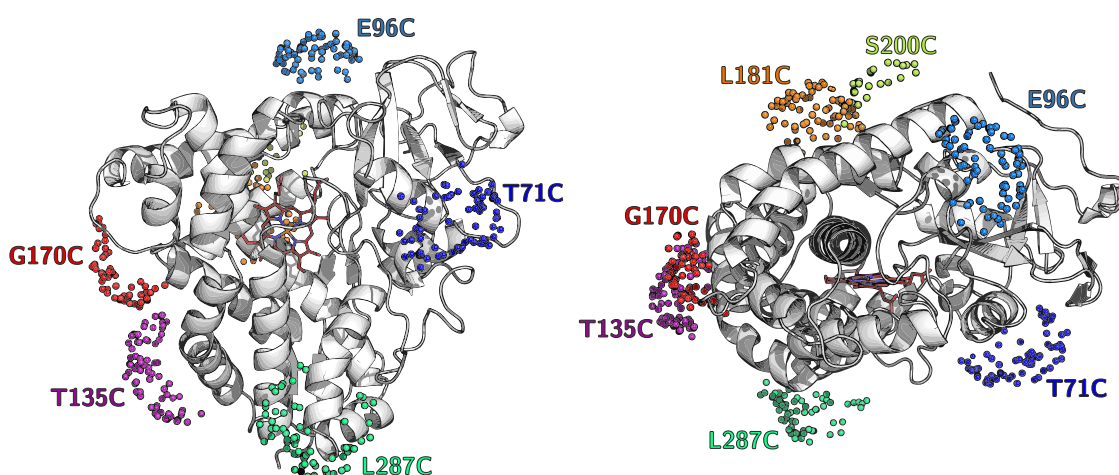


FIGURE 7.11: Top and side view of the structure of CYP101D1 with positions of the NO midpoints for the rotamers with a weighting > 0.005 , as calculated using the program MMM [214].

the overall DEER trace averaged over the rotamer distribution. A background function determined by exponential fitting of the experimental DEER data was added to the simulated form factor and the modulation depth was fitted to obtain the best agreement with the experimental data. The root-mean-square deviation between simulation and experiment was calculated for the initial part of each of the DEER traces up to a cutoff value of 2 μ s for the E96C, G170C and S200C mutants, of 1.5 μ s for the L181C mutant and of 0.8 μ s for the L287C mutant. The overall RMSD value was calculated as:

$$R_{tot} = \frac{\sum_{m=1}^M R_m}{\sum_{m=1}^M w_m} \quad R_m = w_m \sum_{n=1}^{N(\Delta\nu)} \sqrt{\frac{\sum_{i=1}^N (V_{m,n}^{exp} - V_{m,n}^{sim})^2}{N}} \quad (7.1)$$

where the indices m , n and i run over the M different mutants, the $N(\Delta\nu)$ different frequency offsets and the N number of points of the DEER trace $V_{m,n}$, and w_m is the weight for the experimental data of the mutant m . A five times larger weight was used for the G170C and L287C mutants, due to the better quality of the experimental data and since the corresponding labelling positions are located on parts of the protein close to the interface and thus not involved in significant conformational changes in any of the known cytochrome P450s. In the mutants E96C, L181C and S200C the nitroxide spin labels are located on the B', F and G helices, respectively, which are involved in the conformational change leading to opening of the substrate access channel in other P450 proteins. Even though no indication of a change in conformation for CYP101D1 was apparent either in X-ray studies [249, 270] or in the DEER measurements described in section 7.3.1, problems with the labelling positions used for the DEER measurement prevented a definite conclusion. The decreased degree of confidence in the DEER measurements corresponding to the E96C, L181C and S200C mutants is reflected by the lower weighting.

The model structures from protein-protein docking were ranked based on the calculated RMSD values. The curve representing the RMSD values in order of increasing magnitude is characterised by a relatively steep initial rise, followed by a shallow central region and a second steep rise for the largest RMSD values. This means that a small set of docked structures is in relatively good agreement with the experimental data, while most of the considered structures are in only moderate agreement and another small set of structures does not agree with the experimental data at all. The cutoff for the structures considered in the further analysis was chosen in the region of the curve where the slope had dropped to 20% of its initial value. The structures with RMSD values below this cutoff value were analysed in terms of the structure of the complex and the orientation of $g_{||}$ of the iron-sulfur cluster (see figure 7.12).

The solutions with the lowest RMSD values cluster into three different groups with similar positions of the iron-sulfur cluster with respect to CYP101D1 and with similar orientations of $g_{||}$, as shown in figure 7.12 A. The largest set of solutions (41%, group 1 in figure 7.12 A) is characterised by an average distance of 1.86 nm between the centre of the iron-sulfur cluster and the heme iron, while the other two sets have the iron-sulfur cluster at a significantly larger distance from the heme iron (2.61 nm on average). The second largest set (30%, group 2 in figure 7.12 A) contains solutions with

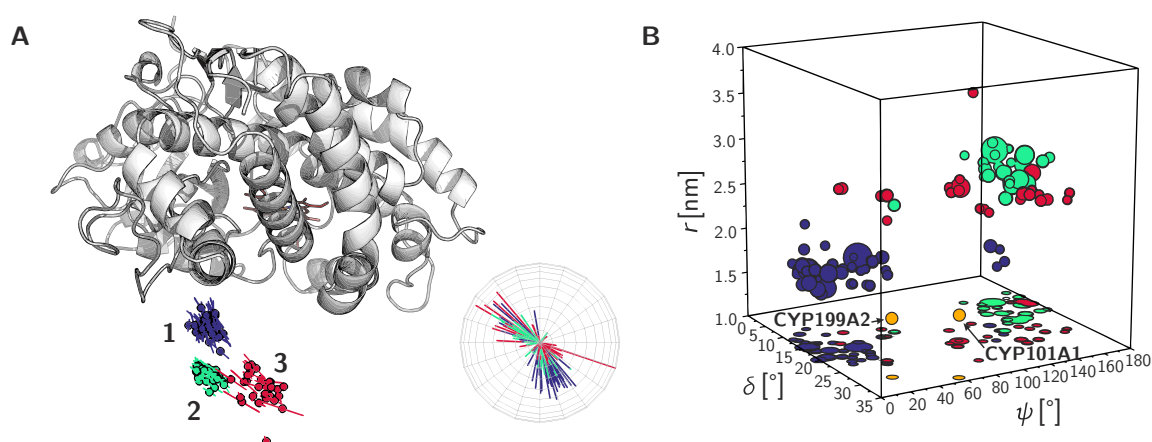


FIGURE 7.12: (A) Positions of the Fe^{3+} atoms of the $[\text{Fe}_2\text{S}_2]$ cluster and orientations of g_{II} with respect to CYP101D1 for the solutions with the lowest RMSD values. The g_{II} orientations are also shown as vectors in a half-sphere (in the same coordinate system of the CYP101D1 protein shown). (B) 3D plot showing the values of the structural parameters r , δ and ψ , defined in figure 7.13, for the model structures in best agreement with the experimental DEER results. The sizes of the dots reflect the RMSD values (large = low RMSD value, small = large RMSD value). The values determined for the known structures of CYP101A1-Pdx (X-ray/NMR) and CYP199A2-Pux (DEER) are shown for comparison in yellow.

very similar g_{II} orientations, while the remaining set is characterised by a larger variability. Almost all of the g_{II} orientations are grouped into two regions related by a mirror plane. The clustering of the g -tensor orientations into a limited region of the sphere indicates that orientation selection effects significantly aid the structure determination for this protein-protein complex.

The electron transfer between ferredoxin and cytochrome P450 occurs through proton-coupled electron transfer and the distance between the two centres required for this to occur lies in the range of about 1.5 to 2.0 nm [272]. Model structures with larger donor-acceptor distances most probably do not reflect the structure of the active complex, which would exclude the solutions in groups 2 and 3 of figure 7.12. A close inspection of the simulated DEER traces for these solutions reveals that the modulations of the traces recorded for the E96C and S200C mutants are not correctly reproduced. This disagreement does not significantly affect the calculated RMSD values due to the small modulation depths of the experimental DEER traces.

In order to further analyse the solutions in terms of complex structure and interaction interface, the following parameters were defined (figure 7.13):

- the distance r between the heme iron and the centre of the $[\text{Fe}_2\text{S}_2]$ cluster;
- the angle δ between the distance vector r and the heme plane normal;
- the angle ψ between the midpoints of helix $\alpha 2$ of the ferredoxin (residues D67 to S74, green in figure 7.13) and helix C of CYP101D1 (residues T111 to K119, blue in figure 7.13) (the midpoints are projected onto a plane perpendicular to the distance vector for the determination of the angle).

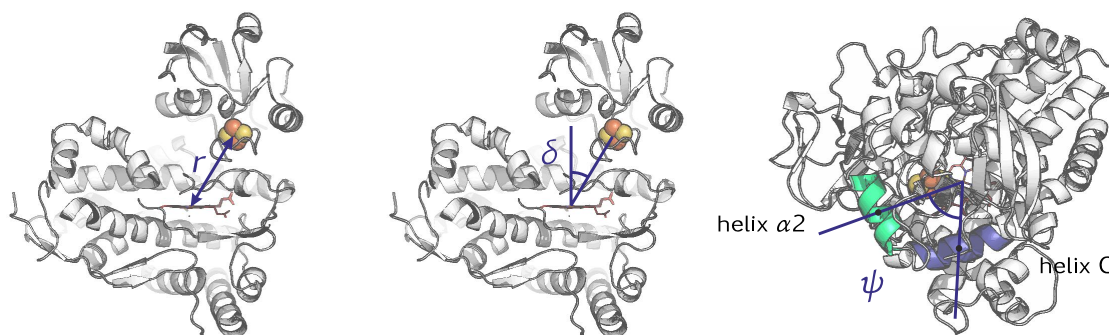


FIGURE 7.13: Definitions of the structural parameters r , δ and ψ used for the description of the structure of a ferredoxin-cytochrome P450 complex.

The first two parameters describe the position of the iron-sulfur cluster of the ferredoxin with respect to the heme group of the cytochrome P450, which determines the feasibility of electron transfer between the two centres. The third parameter is used to describe the interaction interface between the two proteins. The helix $\alpha 2$ of Arx is characterised by significant negative charges (see section 7.1 and figure 7.2), and the binding is believed to be driven by interaction with a positively charged region on CYP101D1.

The parameters determined as an average for each one of the three groups of solutions from the initial protein-protein docking are summarised in table 7.1 and compared to the corresponding values for known structures of ferredoxin-cytochrome P450 complexes. The r , δ and ψ values determined for each individual model structure are also plotted in figure 7.12 B. The solutions are clustered into two regions of the parameter space, distinguished by the parameters r and ψ , while δ falls in the range from 10° to 25° for almost all of the solutions. The set of solutions with the smaller r values is characterised by an angle ψ of approximately 20° between the $\alpha 2$ helix of Arx and helix C of CYP101D1, indicating a potential interaction between the residues of these two helices. For the structures in the second set of solutions, with larger distances r , the angle ψ between the midpoints of these two helices is much larger, excluding an interaction between them. In order to identify the presence of interactions with other parts of the CYP101D1 binding interface the individual model structures were inspected, revealing a position of the iron-sulfur cluster considerably removed from the interaction interface and providing an additional reason for the exclusion of these structures from the subsequent analysis.

The structures with the lowest RMSD values resulting from the initial docking were further refined using a pattern search algorithm based on a procedure proposed by Dr. Jeffrey Harmer. First a 3x3 grid of positional and angular deviations from the original position of the ferredoxin with respect to CYP101D1 was defined. The RMSD values between experimental DEER traces and simulations based on the structure corresponding to each grid point were calculated and the point corresponding to the lowest value was identified and defined as the next start position (see figure 7.9, step 4). In each iteration the position was optimised first, followed by optimisation of the orientation. If a structure with

TABLE 7.1: Average values of the structural parameters r , δ and ψ for the different sets of initial solutions for the CYP101D1-Arx complex, for the model structures resulting from the analysis and for the X-ray/NMR/DEER structures of other ferredoxin-cytochrome P450 complexes.

Structure	Method	r [nm]	δ	ψ
Model structures from protein-protein docking				
CYP101D1-Arx: group 1	DEER	1.87 ± 0.11	$18^\circ \pm 4^\circ$	$28^\circ \pm 34^\circ$
CYP101D1-Arx: group 2	DEER	2.63 ± 0.09	$14^\circ \pm 3^\circ$	$153^\circ \pm 21^\circ$
CYP101D1-Arx: group 3	DEER	2.60 ± 0.26	$20^\circ \pm 5^\circ$	$112^\circ \pm 48^\circ$
Model structures after optimisation				
CYP101D1-Arx: model 1	DEER	1.85	25°	29°
CYP101D1-Arx: model 2	DEER	1.78	19°	6°
CYP101D1-Arx: model 3	DEER	1.67	17°	15°
Structures of complexes known from the literature				
CYP101A1-Pdx [252]	X-ray	1.66	34°	58°
CYP101A1-Pdx [253]	NMR	1.70	31°	59°
CYP101A1-Pdx [253]	X-ray	1.70	34°	60°
CYP199A2-HaPux [245]	DEER	1.63	30°	23°

a lower RMSD value could be identified on the grid, the step size for the next iteration was increased, while the step size was decreased if no solution with a lower RMSD value was found. Convergence was signalled when the step size for both translational and rotational optimisations decreased below a predefined cutoff value, corresponding to 0.001 nm and 0.1° , respectively.

The optimised structures were analysed analogously to the initial structures from protein-protein docking and the results are summarised in figure 7.14. Even though optimisation of the solutions of groups 2 and 3 was attempted, the initial structures were too far removed from the minimum and the experimental data did not provide sufficiently strong constraints to achieve convergence to a single global minimum.

The RMSD values calculated for the optimised structures and visual inspection of the agreement between the corresponding simulated DEER traces and the experimental data were used to identify the best model structures, which are highlighted in figure 7.14. The final solutions are characterised by an average distance of 1.82 nm between heme iron and the iron-sulfur cluster. Two groups of solutions with slightly different values of the parameters δ and ψ were found. The average values for r , δ and ψ for the two groups correspond to 1.78 nm, 18° and 6° and to 1.86 nm, 24° and 30° . Since the simulated DEER traces agree with the experimental data equally well, a model structure representative of each of the two sets of solutions was considered in the final analysis (model 1 and model 2). In all of the model structures, the distance between the heme iron and the iron-sulfur cluster is slightly larger compared to the distance determined for the CYP101A1-Pdx and CYP199A2-HaPux complexes by X-ray and NMR and DEER, respectively. For this reason, the solution with the shortest distance r was also considered in the analysis (model 3). The three proposed model structures of the CYP101D1-Arx complex are compared in figure 7.15 and the corresponding structural parameters can be found in table 7.1. The DEER simulations corresponding to model 1 are shown in figure 7.16 and the simulations for models 2 and 3 are shown in figures D.1 and D.2 in appendix D.

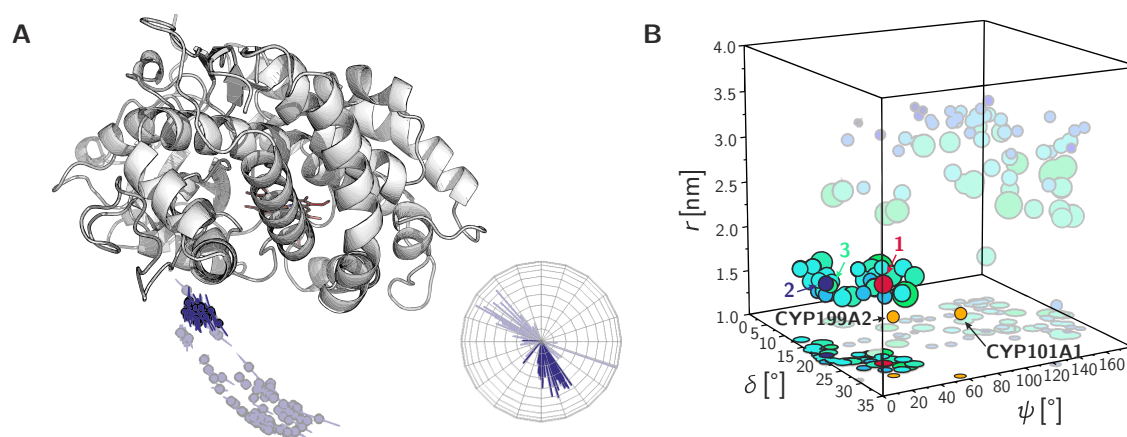


FIGURE 7.14: (A) Positions of the Fe^{3+} atoms of the $[\text{Fe}_2\text{S}_2]$ cluster and orientations of $g_{||}$ with respect to CYP101D1 for the model structures optimised using a pattern search algorithm. The $g_{||}$ orientations are also shown as vectors in a half-sphere (in the same coordinate system of the CYP101D1 protein shown). (B) 3D plot showing the values of the structural parameters r , δ and ψ , defined in figure 7.13, for the optimised model structures. The colours and sizes of the dots reflect the RMSD values, green and large corresponds to the lowest RMSD values, dark blue and small to the largest. The values for the structures of CYP101A1-Pdx (X-ray/NMR) and CYP199A2-Pux (DEER) are shown for comparison in yellow. The solutions shown in the background, arising mainly from the optimisation of models in groups 2 and 3, were not considered in the final analysis. The points referring to models 1, 2 and 3 considered in the further analysis are indicated by arrows and highlighted in red, blue and green, respectively.

The three model structures are characterised by similar positions of the iron-sulfur cluster with respect to CYP101D1, as well as similar positions of the interfacial helix $\alpha 2$ of the ferredoxin. Larger differences can be observed in the distal region of the protein. The experimental distance constraints clearly define the most likely position of the iron-sulfur cluster with respect to CYP101D1, while the orientational constraints based on the available experimental data are not strong enough to clearly

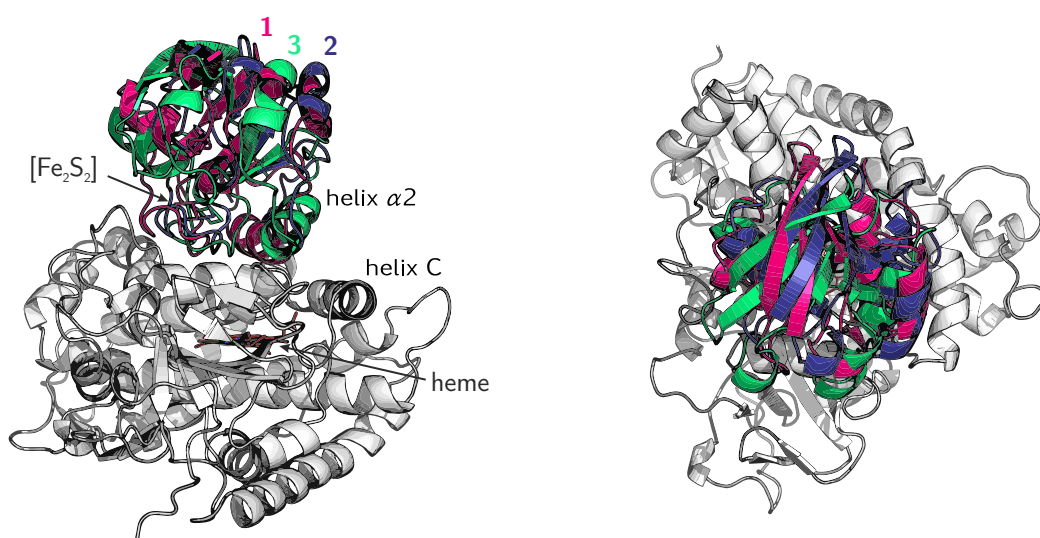


FIGURE 7.15: Comparison of the structures of models 1, 2 and 3 of the CYP101D1-Arx complex obtained by protein-protein docking followed by optimisation with a pattern search algorithm.

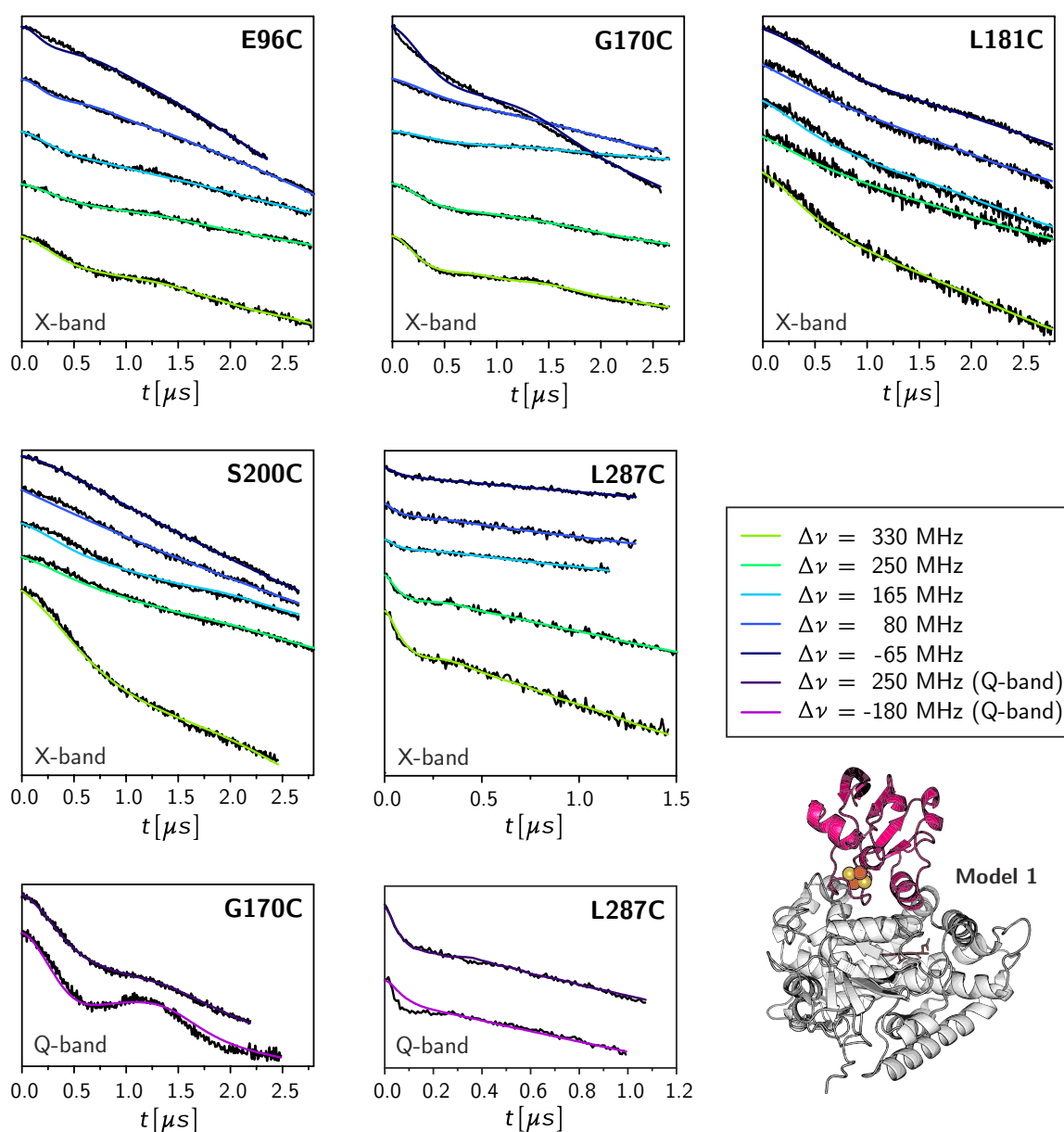


FIGURE 7.16: Experimental and simulated orientation-selective X-band and Q-band four-pulse DEER data for model 1 of the CYP101D1-Arx complex with nitroxide spin labels introduced at positions E96C, G170C, L181C, S200C and L287C on CYP101D1. Experimental details can be found in section 7.2.2 and details on the simulations performed are described in the text. A background function and modulation depth correction was added to the simulated form factors for comparison with the experimental data.

define the exact orientation of the ferredoxin. Measurements on a larger number of mutants and potentially the insertion of a nitroxide spin label on the distal region of the ferredoxin could provide the additional constraints required to better define the structure of the complex.

The three model structures for the CYP101D1-Arx complex are characterised by slightly larger distances between the heme iron and the iron-sulfur cluster compared to the known structures of the CYP199A2-HaPux (DEER) and CYP101A1-Pdx (X-ray/NMR) complexes. In addition to this, while the relative position of the iron-sulfur cluster with respect to the heme normal, described by the angle δ , is

similar for both known structures of ferredoxin-cytochrome P450 complexes, all three of the proposed model structures for this complex are characterised by a slightly different value of δ . In terms of interaction surface, the proposed models share quite significant similarities with the CYP199A2-HaPux structure, while larger differences are observed with respect to the CYP101A1-Pdx structure. The similarities in the electrostatic surface potentials of CYP101D1 and CYP199A2, but not CYP101A1 (see figure 7.2), could be the reason for this observation. Electrostatic interactions are believed to play an important role in the complex formation of both CYP101D1-Arx and CYP199A2-Pux [249, 261]. The high redox-partner specificity in the CYP101A1-Pdx complex has often been believed to be an indication of significant functional differences [270] and might explain the differences in the determined complex structures.

DEER simulations for models based on the CYP199A2-HaPux (DEER) and CYP101A1-Pdx (X-ray) structures were also performed and are shown in figures D.3 and D.4 of appendix D. RMSD values of 0.0080 and 0.0083 are obtained for the CYP199A2-HaPux and CYP101A1-Pdx model structures compared to 0.0053-0.0055 for models 1-3. The simulations are in worse agreement with the experimental data in terms of both distances and orientation selection effects, indicating differences in the binding of Arx to CYP101D1 compared to the other two complexes.

The analysis would be significantly simplified, and the confidence in the final model structure notably increased, by the availability of additional mutants with nitroxide spin labels attached closer to the interaction interface and more evenly distributed with respect to the mutants that could be used for the DEER measurements. The spin label introduced at residue T71 could have provided such an additional constraint, but unfortunately did not show any appreciable DEER modulations. Inspection of the model structures reveals that this spin labelling position is close to the binding interface, potentially interfering with complex formation. Calculations of the corresponding DEER traces for the proposed model structures of the CYP101D1-Arx complex have shown that most of the inter-spin distances are expected to be smaller than 1.7 nm, thus potentially preventing detection due to limited excitation bandwidth [169]. The presence of large dipolar frequencies can be detected as dipolar broadening of the cw-EPR spectrum and thus cw-EPR measurements were performed for the CYP101D1-Arx complex with the T71C mutant and, as a reference, also for the E96C mutant. A slight broadening was observed for the T71C mutant, in particular for the $m_I = -1$ transition (see figure D.5 in appendix D), but the overlap of the nitroxide EPR signal with that of the iron-sulfur cluster and the different relative intensities of the two signals in different samples unfortunately do not allow any definite conclusion to be drawn.

7.3.4 ELECTRON TRANSFER IN THE CYP101D1-ARX COMPLEX

The ferredoxin-cytochrome P450 complexes are formed for the reduction of the heme through electron transfer from the iron-sulfur cluster of the ferredoxin. The proposed model structures of the complex were thus analysed in terms of electron transfer efficiency.

Intra- and inter-protein electron transfer has been the object of extensive investigations aimed at determining the factors influencing its efficiency [273–275]. The rate of non-adiabatic electron transfer can be expressed in terms of Fermi's golden rule as [273]:

$$k_{ET} = \frac{2\pi}{\hbar} |V_{DA}|^2 \frac{\exp\left[-(\Delta G + \lambda)^2 (4\lambda k_B T)^{-1}\right]}{\sqrt{4\pi\lambda k_B T}} \quad (7.2)$$

where V_{DA} is the electronic coupling matrix of donor and acceptor of the electron transfer and the term on the right is the Franck-Condon factor, depending on the driving force ΔG and the reorganisation energy λ . The electronic coupling matrix V_{DA} determines the decay of k_{ET} with the distance between electron donor and acceptor. An approximate model allowing evaluation of V_{DA} for a given protein system was developed by Beratan, Onuchic et al. [274] and implemented in the program PATHWAYS. The electron transfer in proteins is assumed to be mediated by three different types of interactions: covalent bonds, hydrogen bonds and through-space interactions. In the program, the proposed electron transfer pathway is divided into different segments assigned to one of the three types of interactions and the electronic coupling element V_{DA} is assumed to be proportional to the product of decay factors for each segment [274]:

$$V_{DA} \propto \prod_i \epsilon_C(i) \prod_j \epsilon_H(j) \prod_k \epsilon_S(k) \quad (7.3)$$

where ϵ_C , ϵ_H and ϵ_S correspond to the covalent bond, hydrogen bond and through-space decay factors. The decay factors are usually parametrised as follows [274]:

$$\begin{aligned} \epsilon_C &= 0.6 \\ \epsilon_H &= \epsilon_C \exp[-1.7(r - 2.8)] \\ \epsilon_S &= \epsilon_C \exp[-1.7(r - 1.4)] \end{aligned} \quad (7.4)$$

where r is the distance between the atoms in Å and hydrogen atoms are not included in the analysis. The program PATHWAYS, included in the HARLEM molecular modelling package [276], calculates the optimal electron transfer pathway given a donor and an acceptor group in a particular protein structure by minimising the product in equation 7.3. The final model structures for the CYP101D1-Arx complex were analysed with this program and the predicted optimal electron transfer pathways are highlighted in figure 7.17. The whole iron-sulfur cluster was defined as donor and the whole heme group as acceptor. The electron transfer pathways in CYP199A2-HaPux and CYP101A1-Pdx are shown for comparison. Predicted electronic couplings and maximum electron transfer rates calculated using the PATHWAYS program are summarised in table 7.2. A limitation of the program is that it only considers the static input structure, while side chain optimisations might lead to the detection of a more efficient electron transfer pathway.

The structures determined for the CYP101A1-Pdx (by X-ray/NMR) and CYP199A2-HaPux (by DEER) complexes are characterised by much higher electron transfer efficiencies as well as similarities in the residues mediating the electron transfer. The different position of the iron-sulfur cluster with respect

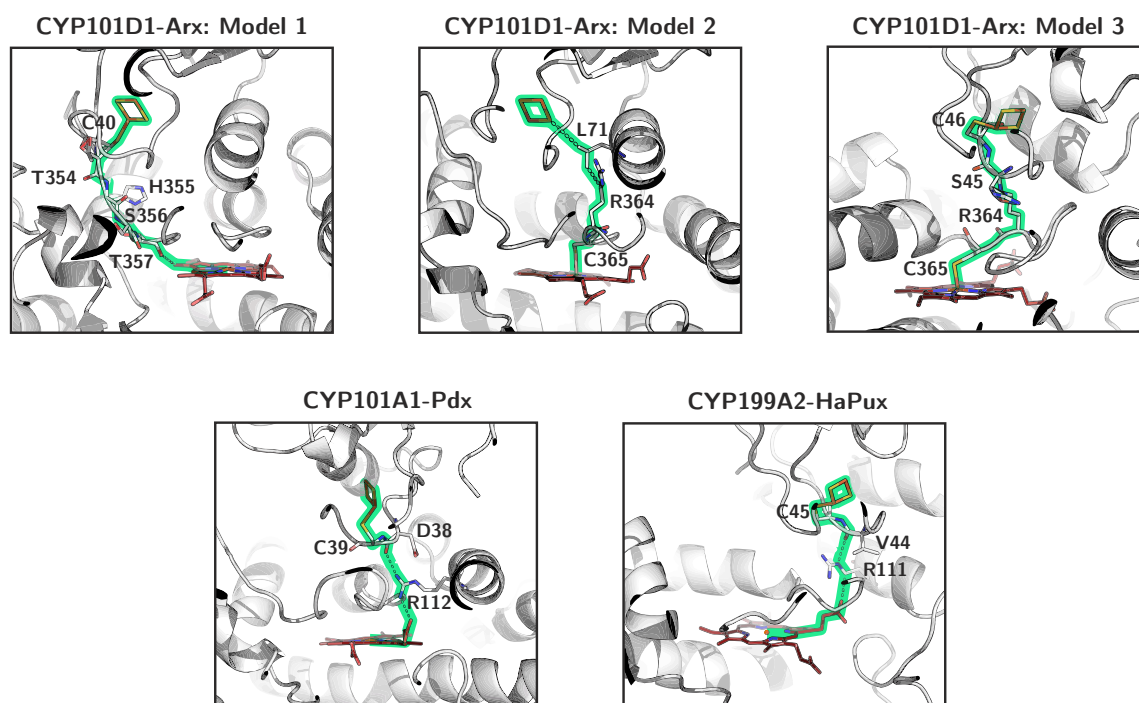


FIGURE 7.17: Structure of the ferredoxin-cytochrome P450 interaction interface with the best electron transfer pathway, calculated with the program HARLEM [276] for the different models of the CYP101D1-Arx complex and for the CYP101A1-Pdx and CYP199A2-Pux complexes determined by X-ray/NMR and DEER, respectively, highlighted in green. The amino acid residues mediating the electron transfer are also indicated.

to the heme group in the model structures of the CYP101D1-Arx complex resulting from the analysis presented here requires a different pathway involving an increased number of residues. The decrease in electronic coupling is related to the larger edge-to-edge distances in these model structures. The predicted electron transfer rates are nevertheless more than an order of magnitude larger than the product formation rates [277], not excluding the proposed complex structures.

Analysis of the protein-protein interaction interface revealed several interactions involving the iron-sulfur cluster binding loop and some interactions involving helix C of CYP101D1. Significantly more interactions between interface residues are present in models 1 and 2 compared to model 3. A more accurate analysis of the interactions and the electron transfer pathways would require molecular

TABLE 7.2: Edge-to-edge distance, electronic coupling V_{DA} and maximum electron transfer rate k_{max} calculated for different complex structures using the PATHWAYS program as implemented in HARLEM [276].

Structure	edge-to-edge distance [nm]	V_{DA}	k_{max} [s^{-1}]
CYP101D1-Arx: model 1	1.53	$8.1 \cdot 10^{-6}$	$6.5 \cdot 10^3$
CYP101D1-Arx: model 2	1.54	$7.7 \cdot 10^{-6}$	$5.9 \cdot 10^3$
CYP101D1-Arx: model 3	1.48	$6.0 \cdot 10^{-5}$	$3.6 \cdot 10^5$
CYP101A1-Pdx [252]	1.21	$1.9 \cdot 10^{-4}$	$3.5 \cdot 10^6$
CYP199A2-HaPux [245]	1.07	$2.3 \cdot 10^{-4}$	$5.2 \cdot 10^6$

dynamics simulations to remove side-chain clashes in the docked structures and to predict structural rearrangements or conformational changes accompanying complex formation.

The decreased electron transfer efficiency in the model structures in best agreement with the experimental DEER data compared to known structures of related complexes could potentially be explained by assuming the determined structure corresponds to an encounter complex, rather than to the active docked complex [278]. Encounter complexes are formed in the first stage of protein-protein binding and limit the conformational space that needs to be explored in order to form the final electron-transfer active complex [278]. This initial stage in protein complex formation is particularly important for the transient complexes formed by proteins of the electron transport chain [253, 278]. An encounter complex between CYP101A1 and Pdx was detected by paramagnetic NMR measurements [253], and the position of the iron-sulfur cluster with respect to the heme group in this complex is similar to that observed in models 1-3 of the CYP101D1-Arx complex. If the structures of the three models correspond to such an encounter complex, minor rearrangements could lead to a structure with a more efficient electron transfer pathway. The presence in frozen solution of a distribution of positions and orientations of the ferredoxin with respect to CYP101D1, including an initial encounter complex as well as the final electron-transfer active complex, also cannot be excluded. Measurements on additional labelling positions and further modelling taking distributions of complex structures into account might help determine the exact nature of the CYP101D1-Arx complex characterised in the DEER measurements.

7.4 CONCLUSIONS

Determination of the structure of the CYP101D1-Arx complex of *Novosphingobium aromaticivorans* was attempted through orientation-selective DEER measurements between the iron-sulfur cluster on the ferredoxin and nitroxide spin labels introduced on the cytochrome P450. Distance and orientation information can be extracted from the experimental data through simulations taking orientation selection into account. A model for the structure of the protein-protein complex was derived through protein-protein docking followed by structure optimisation guided by simulation of the experimental DEER traces. The quality of the experimental data and the number and position of mutants was unfortunately not sufficient for the analysis to converge to a single clear solution. Nevertheless, the use of biochemical information allowed identification of three potential models for the protein-protein complex.

The model structures were analysed in terms of protein-protein interactions and electron transfer pathways and seem to be reasonable, although significant differences to known structures of related complexes exist. These differences could be a further indication of the low state of refinement of the complex structure and cannot be interpreted in terms of different binding or electron transfer mechanism with any certainty at this stage. A more accurate structural characterisation would require several additional constraints provided by nitroxide spin labels introduced in particular in the region of helix B (residues R69-R77, see figure 7.4) of CYP101D1. The structure resolution of a related

protein complex, CYP199A2-HaPux, through an analogous procedure required introduction of an additional nitroxide spin label on the ferredoxin to remove ambiguity on the exact orientation of the ferredoxin with respect to the cytochrome P450. A similar measurement would also be useful in the case of the CYP101D1-Arx complex. The additional measurements performed at Q-band for some of the mutants have shown that the inclusion of Q-band data in the analysis can provide further strong constraints through the more pronounced orientation selection and elimination of the overlap of the nitroxide and iron-sulfur cluster spectrum.

A TRIplet STATE DELOCALISATION IN FREE BASE PORPHYRINS

As explained in section 4.3.1 of chapter 4, EPR measurements on the free base porphyrins were performed to verify a hypothesis for the explanation of the spin polarisations in the spectra of the corresponding zinc porphyrins. This required confirmation that the triplet state delocalisation in the free base porphyrins parallels that observed in the zinc porphyrins, specifically the reorientation of the ZFS tensor from *l*-P1 to *l*-P2. Hence magnetophotoselection and ENDOR measurements were performed and the results are shown below.

A.1 TIME-RESOLVED EPR

The time-resolved EPR spectra recorded for the free base porphyrin oligomers with one to four porphyrin units are shown in figure A.1 and the zero-field splitting parameters and relative sublevel populations determined by simulation are reported in table A.1.

The zero-field splitting parameters D and E for the free base oligomers with different lengths change in a similar way as observed for the zinc porphyrin oligomers. The D value increases from free base *l*-P1 to *l*-P2, but by a much smaller amount compared to the zinc porphyrins (+24% for the zinc and +3% for the free base porphyrins). However, in both cases, the relative increase in D predicted by DFT, of +26% and +2% for the zinc and free base porphyrins, respectively, is close to the change observed experimentally. The D values for the longer oligomers show a small increase, similarly to the behaviour observed for the zinc porphyrins. The E/D ratios for the free base monomer and

TABLE A.1: Zero-field splitting parameters and relative zero-field sublevel populations for free base *l*-P1 and linear oligomers *l*-P2 to *l*-P6 determined through simulation of the time-resolved EPR spectra recorded after excitation at 532 nm and shown in figure A.1.

	$ D $ [MHz]	$ E $ [MHz]	E/D	$P_X : P_Y : P_Z$
<i>l</i> -P1	1024 ± 3	144 ± 2	0.14	0.53 : 0.47 : 0.00
<i>l</i> -P2	1053 ± 3	311 ± 4	0.29	0.27 : 0.00 : 0.73
<i>l</i> -P3	1087 ± 3	321 ± 2	0.30	0.14 : 0.00 : 0.86
<i>l</i> -P4	1116 ± 6	308 ± 5	0.28	0.07 : 0.00 : 0.93

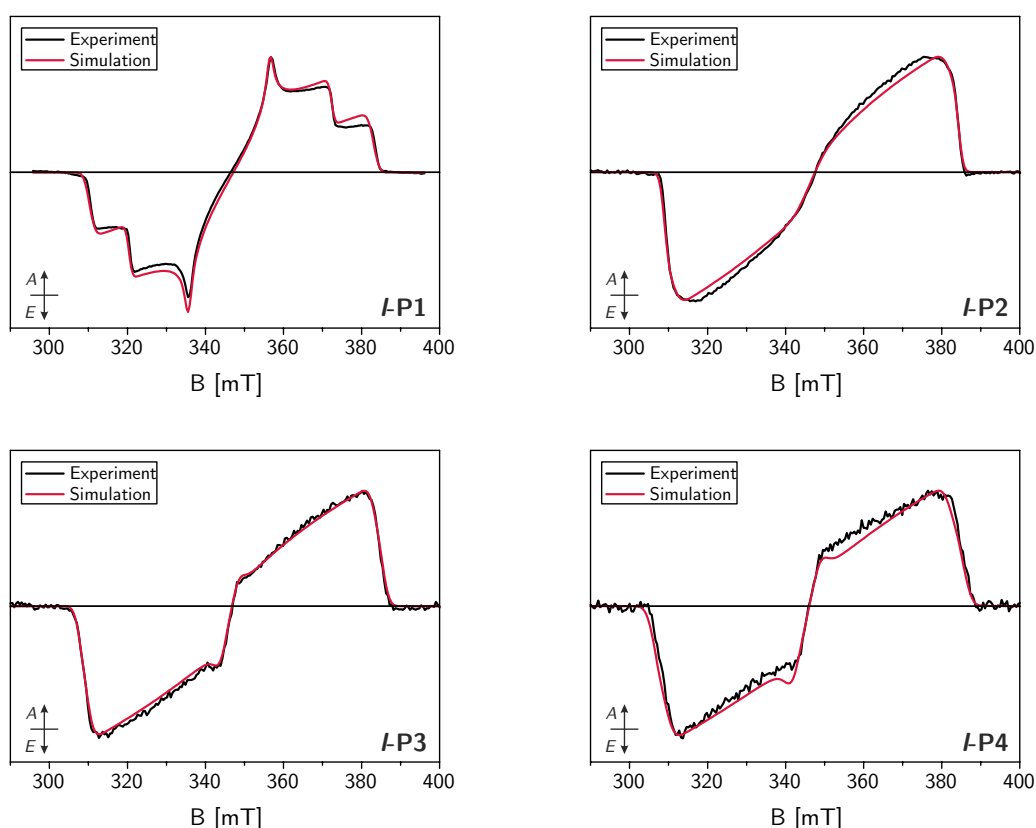


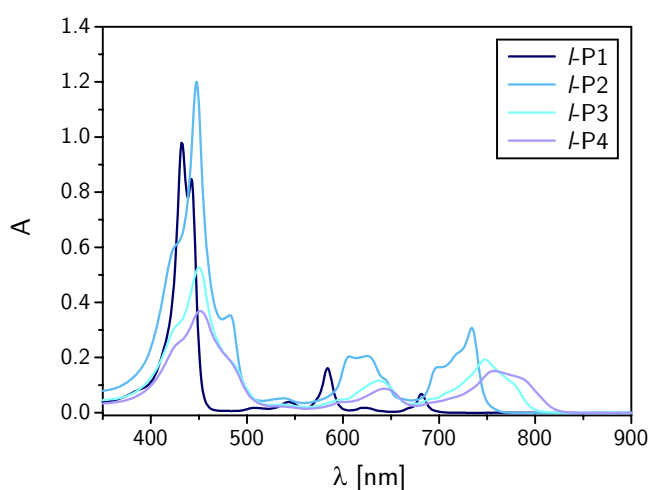
FIGURE A.1: Experimental time-resolved EPR spectra (black) of the free base linear oligomers with one to four porphyrin units recorded at 20 K and averaged over the first 2 μ s after the laser flash ($\lambda = 532$ nm) and the corresponding simulations performed with the parameters given in table A.1.

oligomers are very similar to those of the zinc porphyrins with a significant increase between *l*-P1 and *l*-P2 and smaller changes for the longer oligomers.

The changes in relative sublevel populations are discussed in detail in section 4.3.1. The almost equal population of the two in-plane sublevels, X and Y, for the free base porphyrin monomer changes to an increasing population of the Z sublevel for the longer oligomers. This again suggests that Z becomes the sublevel along the long axis of the molecule for the porphyrin dimer and longer oligomers due to a reorientation of the ZFS tensor as the spin density distribution changes from an oblate to a prolate symmetry.

A.2 MAGNETOPHOTOSELECTION

Magnetophotoselection experiments were again performed to confirm the reorientation of the ZFS tensor proposed based on the changes in the time-resolved EPR spectra. The UV-Vis spectra of the free base porphyrins with one to four units are shown in figure A.2. The appearance of the free base UV-Vis spectra is similar to that of the zinc porphyrin UV-Vis spectra; the Q bands are however more clearly split into two contributions in the free base porphyrins.

FIGURE A.2: Room temperature UV-Vis spectra of free base *l*-P1, *l*-P2, *l*-P3 and *l*-P4 in MeTHF.

The results of magnetophotoselection experiments on the porphyrin monomer with excitation in the Q band region are shown in figure A.3. The UV-Vis spectrum of the free base porphyrin in this wavelength region is usually explained by two transitions, Q_X and Q_Y , and their vibronic progression [152]. The polarisation index for the Z transitions is negative in the whole Q band region, identifying the Z axis as the out-of-plane axis and confirming assignment of a positive D value for the free base monomer. The polarisation indices for the X and Y transitions are both positive over most of this wavelength region. This could either be due to extensive overlap of the Q_X and Q_Y absorption bands

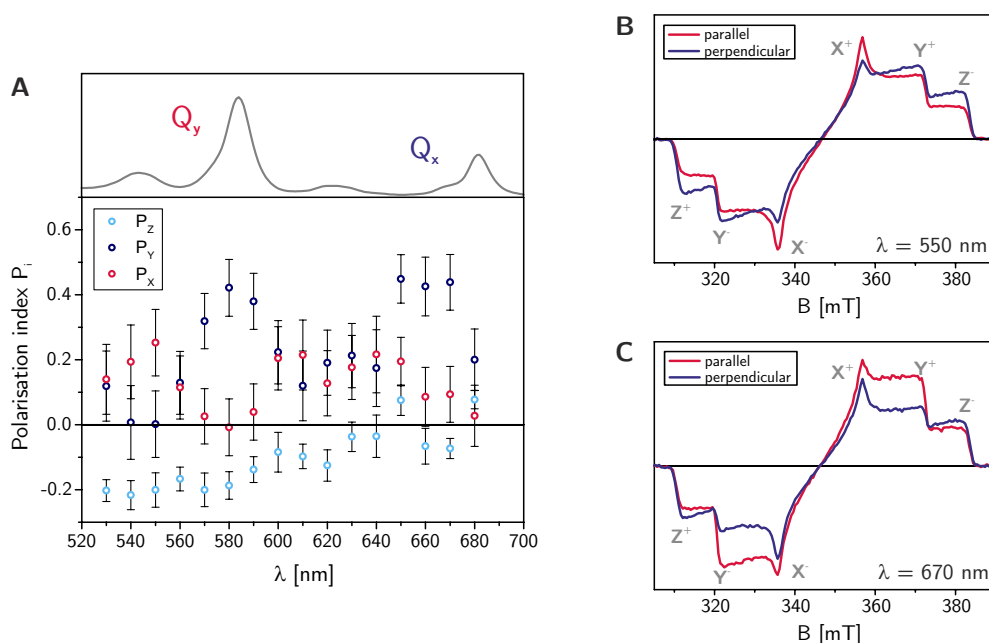


FIGURE A.3: (A) Polarisation indices for the three canonical orientations calculated for free base *l*-P1 as a function of excitation wavelength in the Q band region of the porphyrin absorption spectrum. The corresponding region of the room temperature UV-Vis spectrum is shown above the graph. The polarisation indices were calculated as described in section 4.2.4. (B, C) Time-resolved EPR spectra of *l*-P1 recorded with light polarised parallel and perpendicular to the magnetic field at the indicated excitation wavelengths.

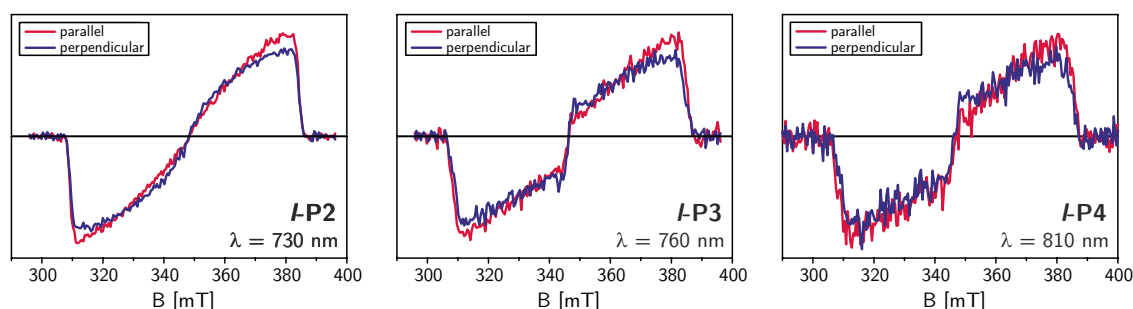


FIGURE A.4: Time-resolved EPR spectra of free base *l*-P2, *l*-P3 and *l*-P4 recorded with light polarised parallel and perpendicular to the magnetic field at the indicated excitation wavelengths corresponding approximately to the maximum of the Q band absorption.

or could indicate that the optical transition moments and in-plane ZFS axes are rotated by 35-55° with respect to each other (see section 4.3.2). The presence of the core NH protons in free base *l*-P1 lowers the symmetry of the molecule and hence the ZFS tensor and the optical transition moments are not expected to be along the same axes as in the zinc porphyrin monomer. DFT and TDDFT calculations predict a rotation of -13.3° for the in-plane ZFS axes with respect to those of the zinc porphyrin and of +23° for the optical transition moments. These results would be in agreement with the positive polarisation indices P_X and P_Y . The changes in polarisation indices would seem to suggest a different assignment of the different peaks to the two Q transitions with respect to the literature, however this method is not sufficiently precise to confirm this. Fluorescence anisotropy measurements would be required to test this hypothesis.

The analysis of the magnetophotoselection experiments on the longer free base oligomers is complicated by the particular type of spin polarisation, characterised by a significant contribution of the Z transition over most of the field range. However, the EPR intensity of the outermost transitions in the EPR spectra is always higher for light polarised parallel to the magnetic field with respect to light polarised perpendicular to it (see figure A.4), indicating a positive polarisation index P_Z and hence identifying Z as an in-plane axis for the free base porphyrin dimer and longer oligomers. The results of the magnetophotoselection experiments thus confirm a sign change of D and a reorientation of the ZFS tensor between the free base monomer and dimer, in analogy to the results obtained for the corresponding zinc porphyrins.

A.3 CHARACTERISATION OF THE PROTON HYPERFINE INTERACTION

The delocalisation in the free base porphyrin oligomers was investigated by ^1H ENDOR. The measurements and analysis mirror those described for the zinc porphyrins in section 4.3.3.

The results of ENDOR measurements on free base *l*-P1 at the six canonical field positions are shown in figure A.5 C. The ENDOR spectra recorded for the Z canonical orientation show a series of clearly resolved hyperfine peaks. A comparison with the results of DFT calculations (A.5 B and E) allows assignment of the -5.4 MHz hyperfine coupling to one set of H_1 protons and of the narrow peak at

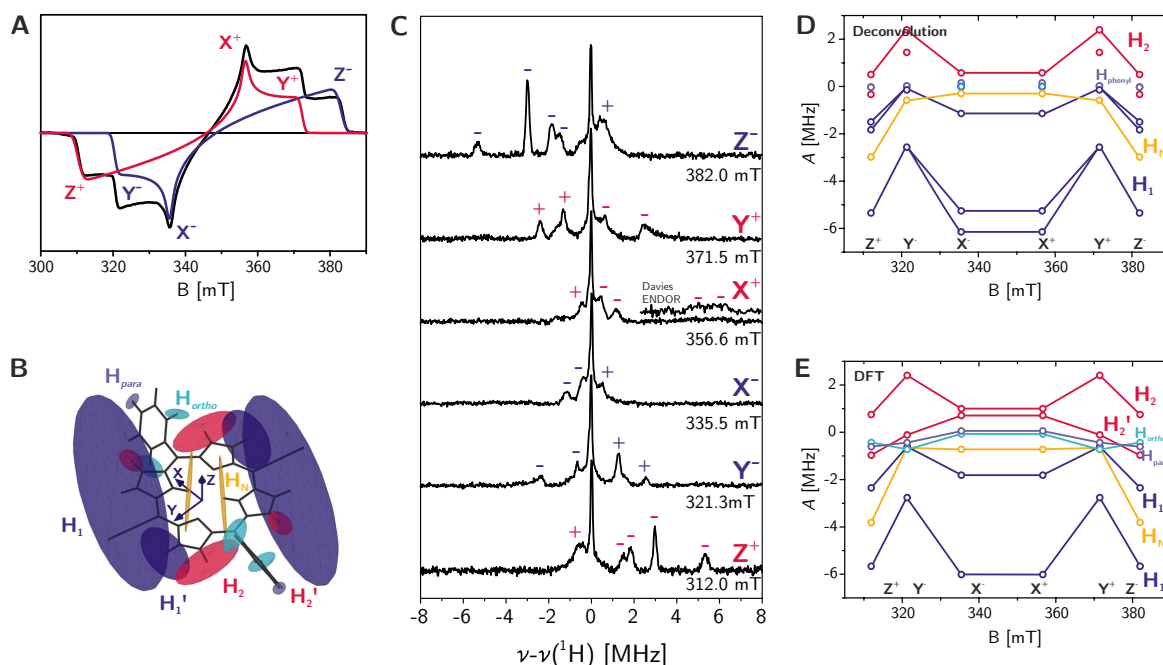


FIGURE A.5: (A) Experimental time-resolved free base *l*-P1 EPR spectrum and simulations for the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions. (B) Proton hyperfine tensor ellipsoids calculated for the free base porphyrin monomer using B3LYP/EPRII and orientation of the ZFS tensor in the molecular frame. (C) Experimental Mims ENDOR spectra of the free base porphyrin monomer recorded at the canonical field positions. The signs of the hyperfine coupling constants of the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions are shown above the corresponding ENDOR peaks. For the X^- orientation the inset shows part of a Davies ENDOR spectrum recorded to better characterise the larger hyperfine coupling. (D) Field-frequency plots for the peak positions obtained by deconvolution of the experimental ENDOR spectra as described in section 4.2.4. The hyperfine couplings were tentatively assigned to different types of protons based on comparison with DFT calculations. (E) Plot of the hyperfine couplings along the ZFS tensor axes as predicted by B3LYP/EPRII calculations on free base *l*-P1.

-3.0 MHz to the hyperfine coupling of the NH protons. The ENDOR spectra corresponding to the Y orientation only show peaks corresponding to relatively small hyperfine couplings, as also observed for the zinc porphyrins. At first only small hyperfine couplings and only a small number of peaks could be identified for the X orientation, but based on the DFT results a large negative hyperfine coupling would also be expected. Since the sensitivity of Mims ENDOR for large hyperfine couplings is reduced, Davies ENDOR was performed to verify the presence of peaks with larger hyperfine couplings. At the X^+ field position a broad peak ranging from about 5 to 7 MHz was observed and assigned to the H_1 hyperfine coupling in the X direction of the ZFS tensor. The hyperfine couplings along the ZFS tensor axes were determined by deconvolution of the experimental ENDOR spectra and the results are shown in the field-frequency plot of figure A.5 D. The hyperfine couplings along the ZFS tensor axes predicted by DFT are plotted in figure A.5 E and a comparison with the graph in figure A.5 D shows that there is reasonable agreement between the experimental and calculated results.

The results of ENDOR measurements on the free base porphyrin dimer are shown in figure A.6. The time-resolved EPR spectrum is compared to the corresponding echo-detected field sweep in figure

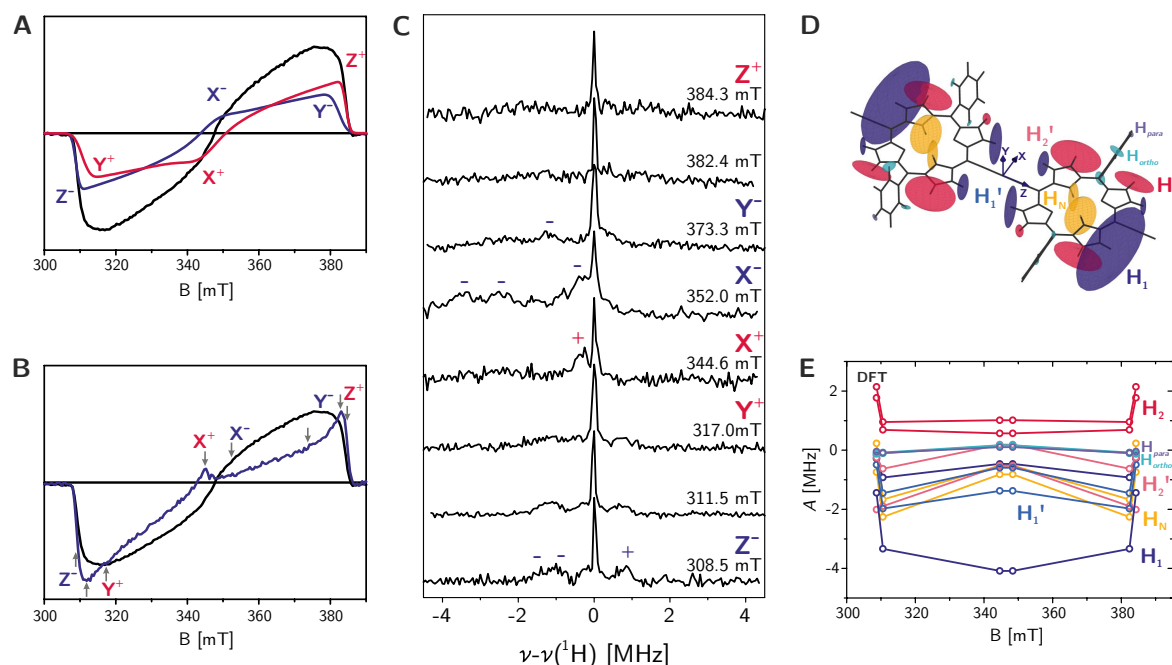


FIGURE A.6: (A) Experimental time-resolved free base *l*-P2 EPR spectrum and simulations for the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions. (B) Comparison of the time-resolved EPR and echo-detected field sweep spectra recorded with a τ value of 120 ns and a presaturation pulse to eliminate the central radical signal. The field positions used for ENDOR measurements are indicated by grey arrows. (C) Experimental Mims ENDOR spectra of the free base porphyrin monomer recorded at the field positions indicated in B. The signs of the hyperfine coupling constants of the $m_S=0 \rightarrow m_S=+1$ (red) and the $m_S=-1 \rightarrow m_S=0$ (blue) transitions are shown above the corresponding ENDOR peaks where possible. (D) Proton hyperfine tensor ellipsoids calculated for the free base porphyrin dimer using B3LYP/EPRII and orientation of the ZFS tensor in the molecular frame. (E) Plot of the hyperfine couplings along the ZFS tensor axes as predicted by B3LYP/EPRII calculations on free base *l*-P2.

A.6 B. The echo intensity is significantly increased at the Z canonical field positions, due to the increased relaxation for orientations not aligned with a ZFS tensor axis [123]. Additional distortions attributed to the modulation of the echo intensity by nitrogen hyperfine couplings are also present. Notably the X^+ signal seems to become absorptive, while it is emissive in the time-resolved EPR spectrum. A potential explanation for the observed effect is a combination of the modulations by the nitrogen hyperfine couplings and an overlap of the X^+ and X^- transitions that lead to a positive echo at the X^+ field position. The ENDOR spectra recorded for free base *l*-P2 only show clearly resolved hyperfine peaks for the Z^- orientation. The same peaks are present in most of the ENDOR spectra recorded at higher field positions, indicating the significant contribution of the Z transition in an extensive field range, as can also be seen from figure A.6 A and B. The hyperfine couplings determined from these peaks are quite small (<2 MHz), indicating a reduction of the hyperfine couplings with respect to the free base monomer. The X transitions also show additional peaks, but the spectra for the two different triplet transitions corresponding to this orientation are very similar, with the main hyperfine peaks at frequencies lower than the Larmor frequency. The ENDOR spectrum recorded for the X^- transition shows more clearly resolved peaks and weak and broad peaks are also present on the same side of the Larmor frequency for higher magnetic field positions. These peaks would thus

indicate negative hyperfine couplings from -2.5 to -3.5 MHz, i.e. about half the hyperfine coupling observed for the same orientation in the porphyrin monomer. The hyperfine couplings predicted by DFT are shown in figure A.6 D and E. Due to the limited number of peaks that could be detected experimentally a direct comparison was not possible, nevertheless the results are sufficiently different from those of the corresponding monomer to confirm delocalisation in the free base dimer.

The combination of the results from the magnetophotoselection and ^1H ENDOR experiments confirms that the changes observed in the zinc porphyrin systems are reflected also in the free base porphyrins. This analysis validates the use of the time-resolved EPR data recorded for the free base porphyrins for the interpretation of the spin polarisation of the corresponding zinc porphyrin spectra.

B TRIPLET STATE ESEEM AND HYSCORE SIMULATIONS

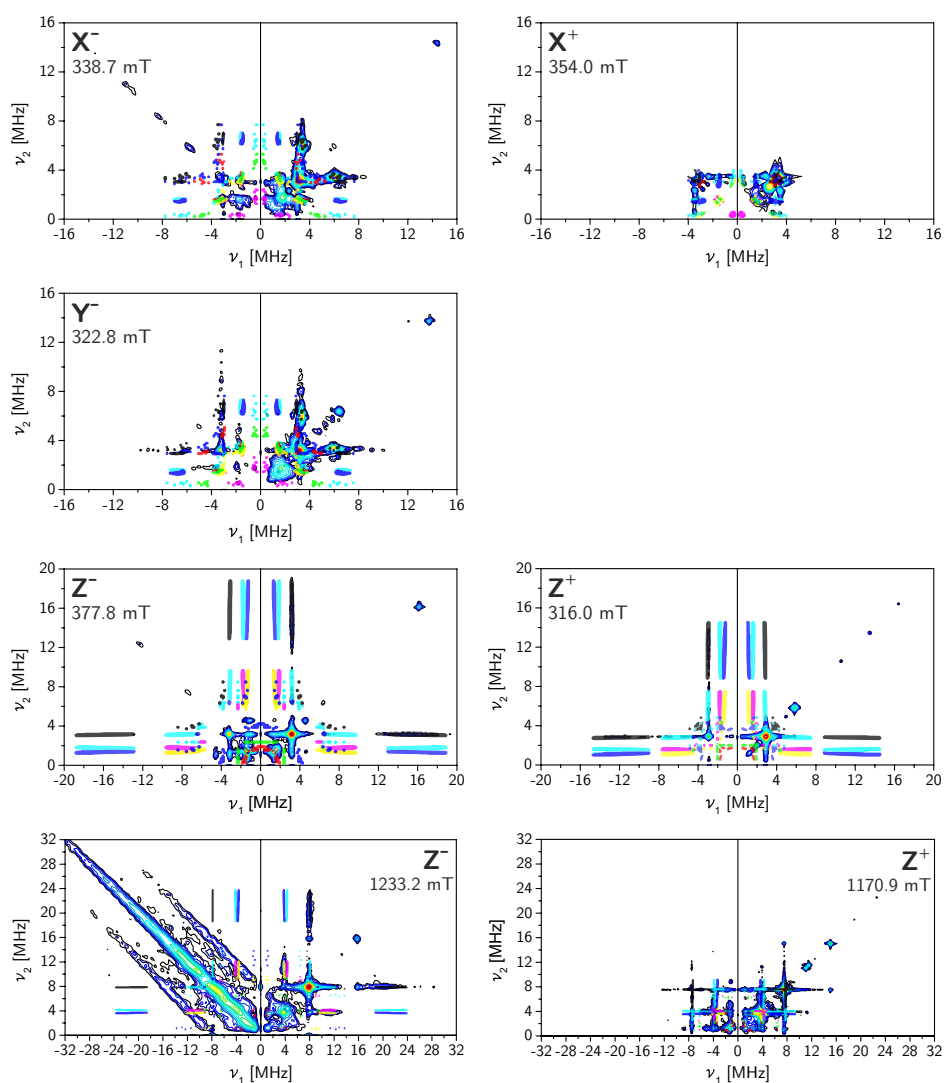


FIGURE B.1: Full set of HYSCORE spectra for *l*-P1 and calculated HYSCORE cross-peak positions for the optimisation assuming both porphyrin and pyridine nitrogens contribute to the strongest cross peaks of the X and Y positions. The simulations include the contribution of pyridine nitrogens based on DFT calculations for different pyridine-zinc distances. The calculated HYSCORE cross peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

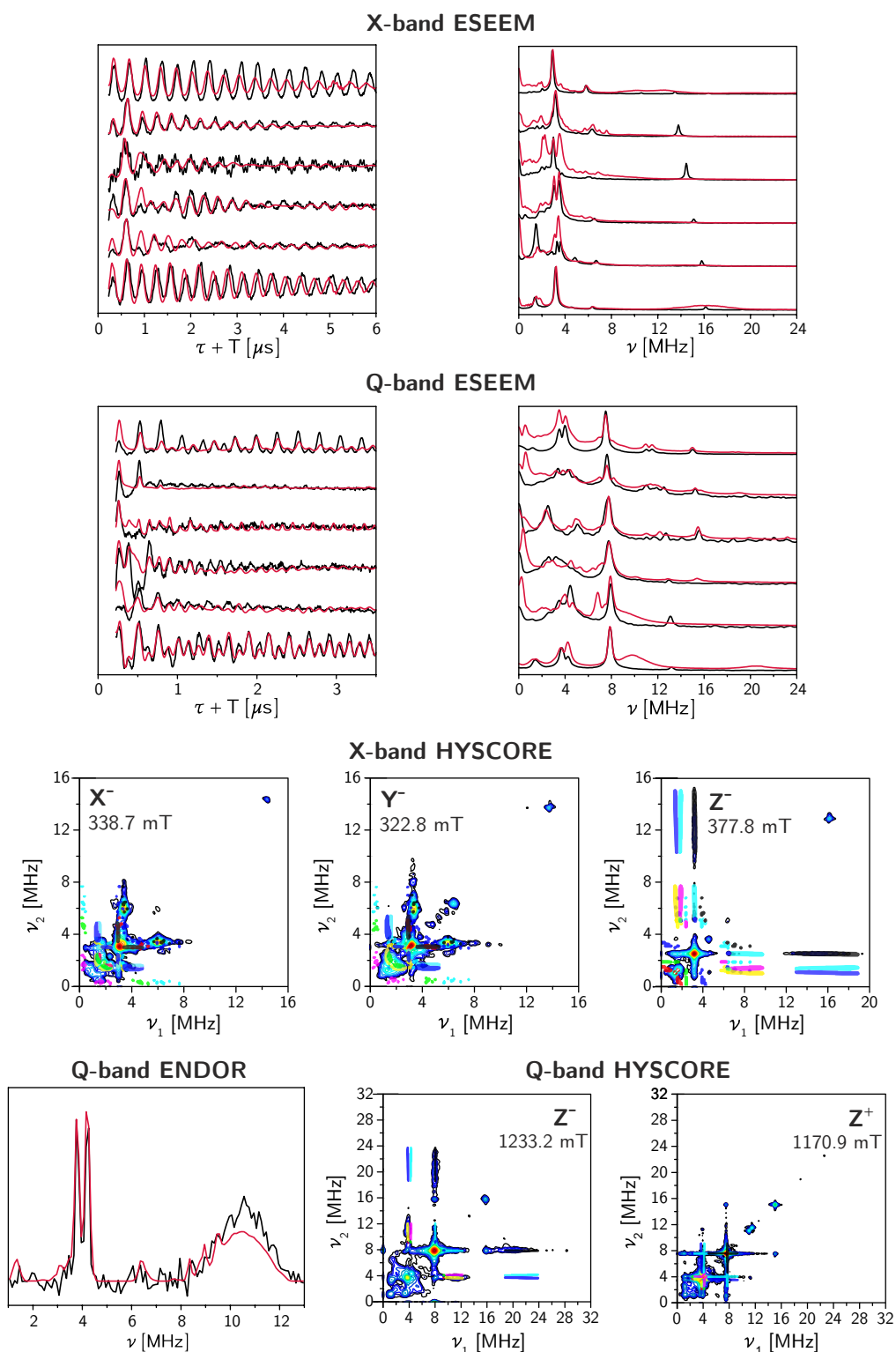


FIGURE B.2: Experimental and simulated *l*-P1 ^{14}N ESEEM and ENDOR data and calculated HYSCORE cross peak positions for the second set of optimised parameters of table 4.3 (O2, optimisation assuming only pyridine nitrogens contribute to the strongest cross peaks in the HYSCORE spectra of the X and Y positions). All simulations except the time-domain ESEEM simulations include the contribution of pyridine nitrogens based on DFT calculations for a distribution of different pyridine-zinc distances. The full set of HYSCORE spectra is shown in appendix B. The calculated HYSCORE cross peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

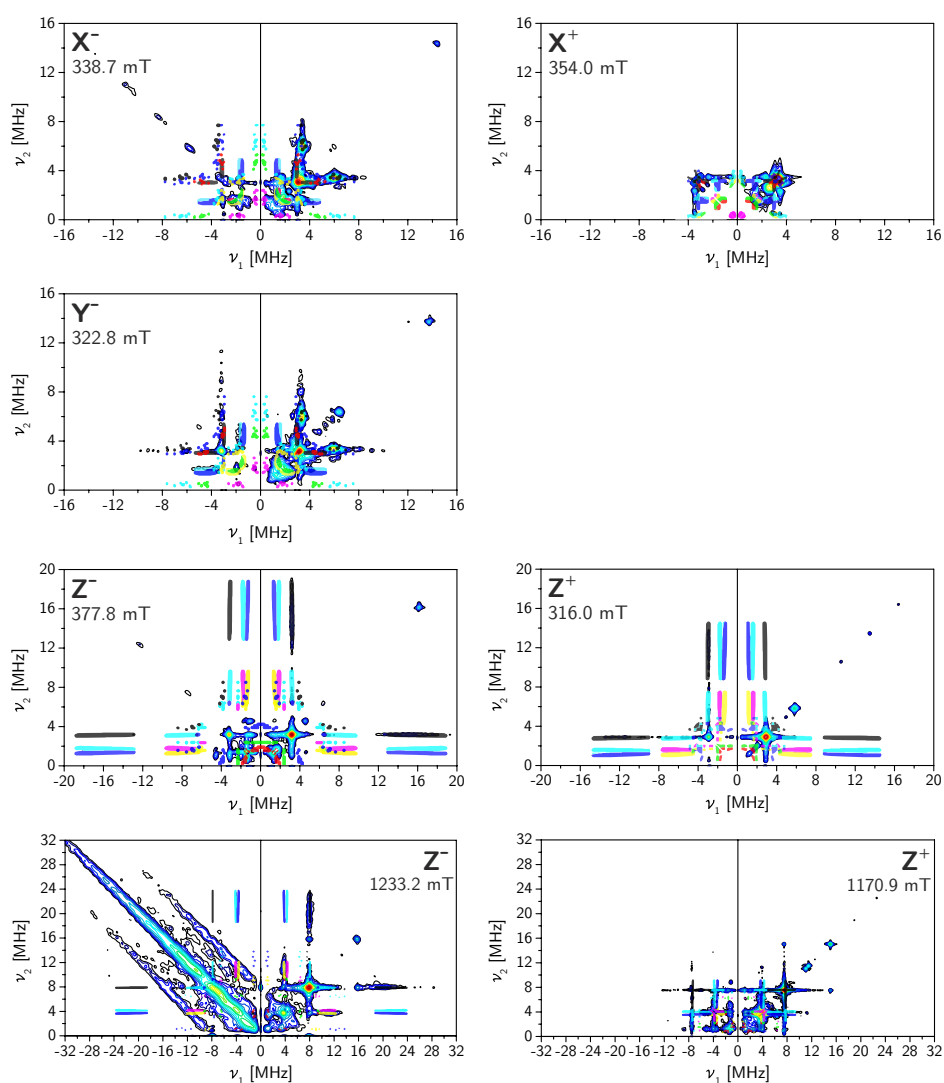


FIGURE B.3: Full set of HYSCORE spectra for *l*-P1 and calculated HYSCORE cross-peak positions for the optimisation assuming only pyridine nitrogens contribute to the strongest cross peaks of the X and Y positions. The simulations include the contribution of pyridine nitrogens based on DFT calculations for different pyridine-zinc distances. The calculated HYSCORE cross peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

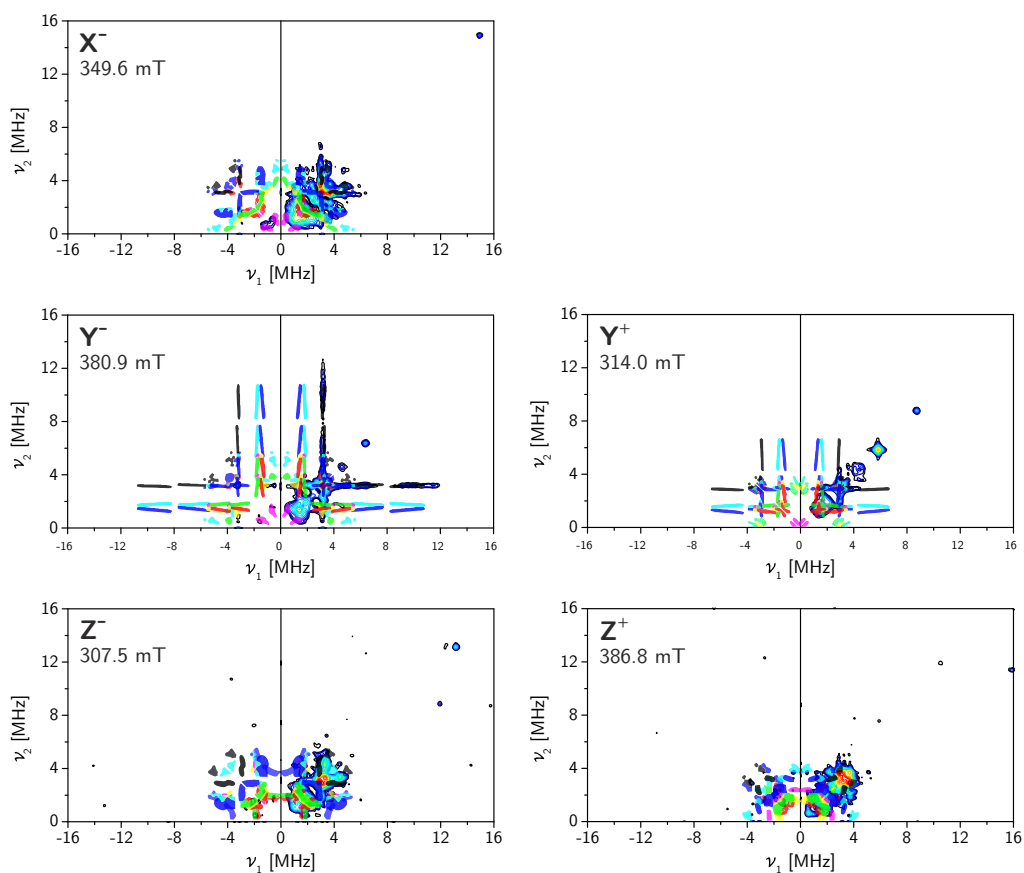


FIGURE B.4: Full set of HYSCORE spectra for *l*-P2 and calculated HYSCORE cross-peak positions for the optimisation assuming both porphyrin and pyridine nitrogens contribute to the strongest cross peaks of the X and Z positions. The simulations include the contribution of pyridine nitrogens based on DFT calculations for different pyridine-zinc distances. The calculated HYSCORE cross peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

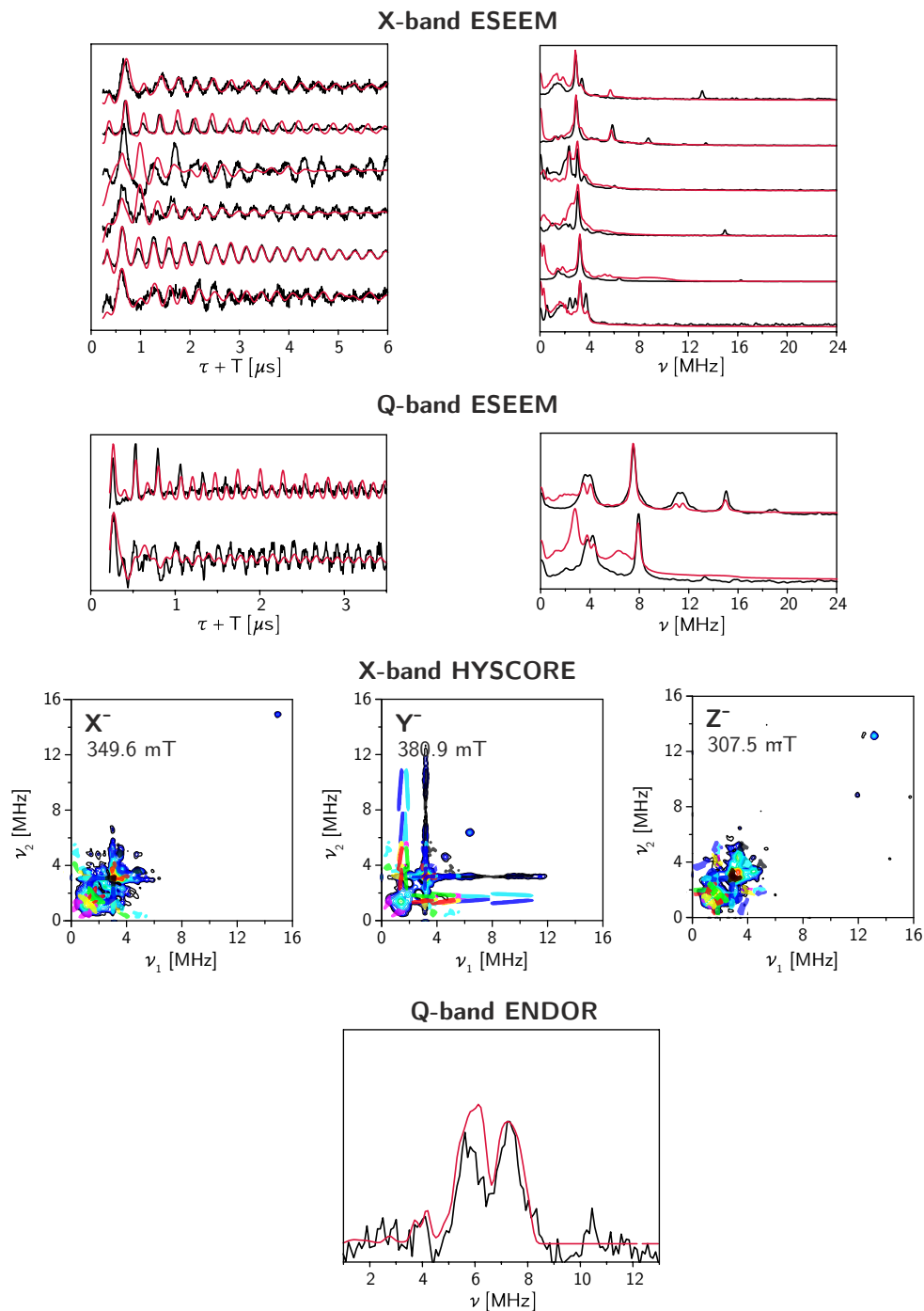


FIGURE B.5: Experimental and simulated I -P2 ^{14}N ESEEM and ENDOR data and calculated HYSCORE cross peak positions for the second set of optimised parameters of table 4.3 (O2, optimisation assuming only pyridine nitrogens contribute to the strongest cross peaks in the HYSCORE spectra of the X and Z positions). All simulations except the time-domain ESEEM simulations include the contribution of pyridine nitrogens based on DFT calculations for a distribution of different pyridine-zinc distances. The full set of HYSCORE spectra is shown in appendix B. The calculated HYSCORE cross peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

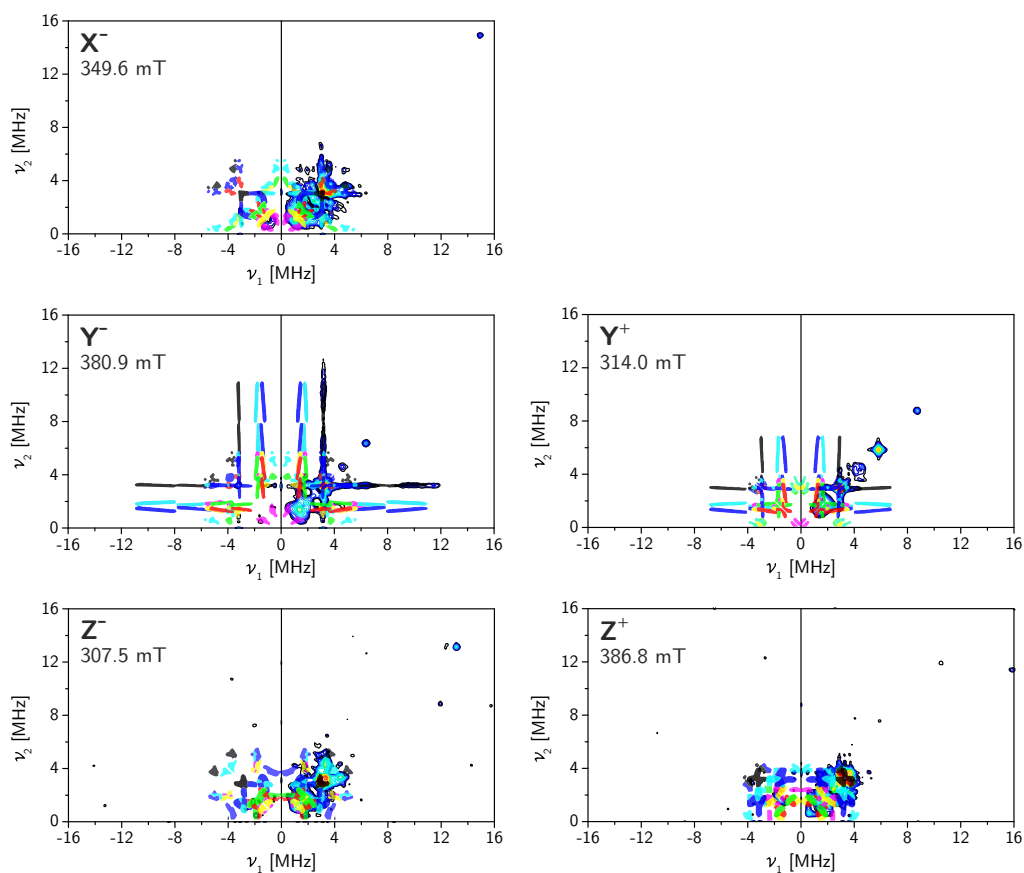


FIGURE B.6: Full set of HYSCORE spectra for *l*-P2 and calculated HYSCORE cross-peak positions for the optimization assuming only pyridine nitrogens contribute to the strongest cross peaks of the X and Z positions. The simulations include the contribution of pyridine nitrogens based on DFT calculations for different pyridine-zinc distances. The calculated HYSCORE cross peaks are colour-coded based on the type of transitions: (ν_{dq}, ν_{dq}) - black, (ν_{dq}, ν_{sq}) - blue/cyan, (ν_{sq}, ν_{sq}) - green/magenta/yellow/red.

C TRIPLET DECAY AND RELAXATION FOR *l*-P1-*l*-P3

The information on the hyperfine couplings allows determination of the extent of triplet state delocalisation, however no information on the mechanism of delocalisation can be obtained from them. A coherent delocalisation and hopping between porphyrin units could potentially be distinguished by characterising the relaxation behaviour. Triplet energy transfer between porphyrin units has been shown to have a strong effect on the decay of the photoexcited triplet state, as it leads to increased spin-lattice relaxation [119].

The triplet state kinetics of the porphyrin monomer were investigated using ESE-detected kinetic curves, obtained by measuring the echo intensity after a two-pulse echo sequence as a function of the delay between the laser flash and the microwave pulses (DAF) (see figure C.1). The time τ between the pulses was kept as short as possible to reduce the effect of relaxation during the inter-pulse delays (100 or 120 ns). The analysis of the triplet state kinetics is much simplified using this pulse method, since the transients recorded using time-resolved EPR are affected by microwave induced relaxation, as the sample is continuously irradiated with microwaves.

The literature on the analysis and interpretation of this type of experiment for triplet states is limited. A general method for the analysis of ESE-detected kinetics is briefly described by Lin [279] and has been used for the analysis of kinetic measurements on single crystals of small organic molecules [31, 280].

The kinetics of triplet excitation and decay are governed by the following set of differential equations [279]:

$$\begin{aligned}\frac{dN_{+1}}{dt} &= -(w_1 + w_3 + k_{+1})N_{+1} + w_1N_0 + w_3N_{-1} + P_{+1}N_S \\ \frac{dN_0}{dt} &= w_1N_{+1} - (w_1 + w_2 + k_0)N_0 + w_2N_{-1} + P_0N_S \\ \frac{dN_{-1}}{dt} &= w_3N_{+1} + w_2N_0 - (w_3 + w_2 + k_{-1})N_{-1} + P_{-1}N_S\end{aligned}\tag{C.1}$$

where N_i are the populations of the triplet state sublevels, N_S is the singlet state population, P_i are the population rates, k_i the decay rates and w_i the spin-lattice relaxation rates (see also figure C.1).

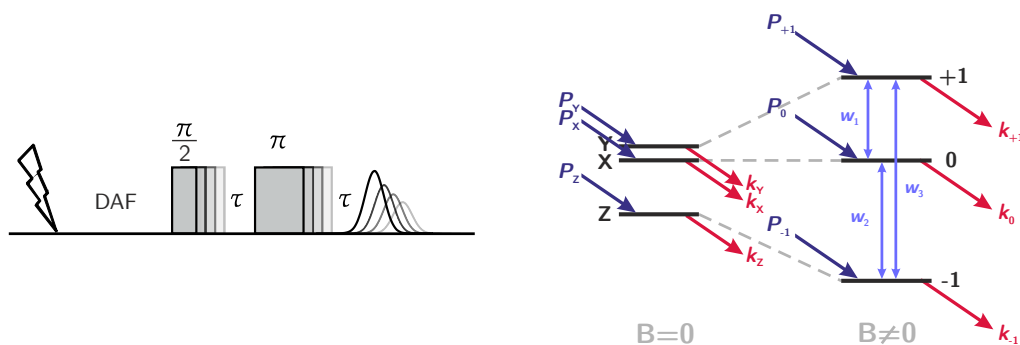


FIGURE C.1: Pulse sequence for ESE-detected kinetic measurements and schematic representation of the triplet state excitation and decay showing the population (P_i) and depopulation (k_i) pathways as well as relaxation between different triplet state sublevels (w_i) in an external magnetic field.

The population and decay rates in the presence of a magnetic field can be expressed as linear combination of the corresponding zero-field values:

$$\begin{aligned} P_0 &= P_Z & P_{\pm 1} &= \frac{1}{2} (P_X + P_Y) \\ k_0 &= k_Z & k_{\pm 1} &= \frac{1}{2} (k_X + k_Y) \end{aligned} \quad (\text{C.2})$$

The analysis is much simplified, if the spin relaxation processes are negligible compared to the triplet state decay rates. In that case, the transients can be fitted with a biexponential decay, depending only on the three decay rate constants, k_X , k_Y and k_Z , since then the population difference changes as a function of time according to

$$\Delta N(t) = [\pm N_{\pm 1}(t=0) \exp(-k_{\pm 1}t)] - [\pm N_0(t=0) \exp(-k_0t)] \quad (\text{C.3})$$

where the signs depend on whether the transitions are absorptive or emissive [281].

In other cases, triplet decay and spin relaxation occur on timescales that are sufficiently different to allow their separate determination by separately fitting the initial and final part of the transients. The initial part represents the decay to Boltzmann equilibrium populations by relaxation and the final part represents the triplet decay after the equilibrium populations have been established [282, 283].

In some papers, the analysis described above is simplified by the high-field and high-temperature approximations [282, 284] allowing the determination of analytical solutions. The high-field approximation assumes that the electron Zeeman interaction is much larger than the zero-field splitting interaction and allows setting $N_{+1}(t=0) = N_{-1}(t=0)$ and $k_{+1} = k_{-1}$. The high-temperature approximation consists in considering the upwards and downwards relaxation rates w as equal, which is equivalent to assuming a Boltzmann factor of one. Under these conditions, the top and bottom level populations are equal at all times, $N_{+1}(t) = N_{-1}(t)$, and the kinetic curves recorded for the low and high field positions of a transition are symmetrical with respect to the time axis. In addition to these assumptions, the population of the triplet levels is considered much faster than the decay and is assumed to occur at the time of the laser flash without influencing the kinetic transients. The differential equations

given in equation C.1 are then simplified to [282]:

$$\begin{aligned}\frac{dN_{+1}}{dt} &= -(w + k_{+1})N_{+1} + w_1N_0 \\ \frac{dN_0}{dt} &= 2wN_{+1} - (2w + k_0)N_0 \\ \frac{dN_{-1}}{dt} &= wN_0 - (w + k_{-1})N_{-1}\end{aligned}\tag{C.4}$$

The analysis of kinetic traces is simplified for single-crystals or for measurements at the Z canonical positions, where the pure decay of one triplet state sublevel is observed [282, 284, 285]. In powder samples, the kinetics are strongly mixed in most parts of the spectrum due to the extensive overlap of different transitions. The most accurate results are in that case obtained by a simulation of the decay of the complete triplet state spectrum and extraction of the transients at the field positions of interest [281].

The ESE-detected kinetic curves recorded for the six canonical transitions of the porphyrin monomer at different temperatures are shown in figure C.2. The traces were recorded in the temperature range from 2.5 K to 90 K, at higher temperatures no measurements could be performed due to the increase in dielectric constant of MeTHF, which prevented tuning. The transients show a pronounced temperature dependence and are in most cases not symmetric with respect to the time axis, with the emissive traces showing a more pronounced decay and becoming absorptive at long times. This behaviour indicates the presence of relaxation towards the Boltzmann equilibrium populations. A comparison of the decay curves for different canonical orientations shows that the differences between the decay along different orientations of the ZFS tensor are quite small. The differences decrease for higher temperatures, indicating that the spin relaxation starts to prevail over the selective triplet decay. The

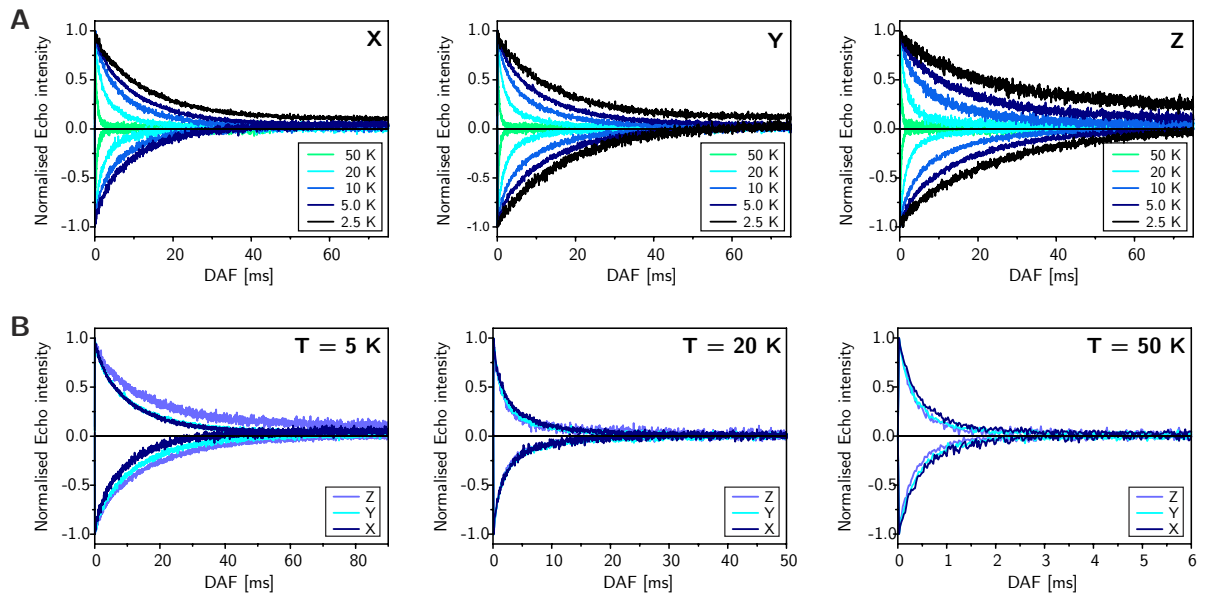


FIGURE C.2: ESE-detected kinetic traces recorded for *l*-P1 at different temperatures at the six canonical field positions. The decay at different temperatures is compared in (A) and the differences in decay for the three triplet sublevels at 5 K, 20 K and 50 K are compared in (B).

order of the decay curves for the X, Y and Z canonical orientations changes between 10 K and 20 K, which might indicate a cross-over between dominating triplet decay and dominating spin lattice relaxation.

The experimental data could not be satisfactorily fitted using a biexponential decay, as expected since the transients are clearly affected by relaxation. In order to determine the triplet decay and spin-lattice relaxation rates at different temperatures, the approach for fitting the experimental data described in reference [282] and used by Bennati to investigate the kinetics of the triplet state of C_{60} at different temperatures [284] was followed. In order to account for the mixing of different transitions, the sublevel populations were calculated by solving the differential equations given in equation C.4 and used to simulate the corresponding triplet state spectrum using EasySpin's *pepper* routine [23]. Transients extracted from the generated 2D surface of signal intensity against field and delay after flash were compared to the experimental data and used in a least-square fitting procedure. The experimental traces were first normalised and then scaled to the relative intensities of the six different transitions determined from the time-resolved EPR spectrum, since the echo-detected spectrum is strongly distorted due to nitrogen modulations. The parameters used in the fitting were the three zero-field decay rates k_X , k_Y and k_Z and three relaxation rates w_X , w_Y , w_Z . The optimised fitting parameters are summarised in table C.1 and the calculated transients are compared to the experimental results in figure C.3.

The fits to the experimental data are reasonable for most of the temperature range, however the fit is considerably worse below 10 K, as the deviations from the high-temperature approximation become more significant. The decay rate and spin lattice relaxation rate constants seem to be of the same order of magnitude for most of the investigated temperature range, preventing an accurate analysis of the two contributions, as their separation is impossible in this case. The results obtained from the fitting are affected by significant errors and hence an unambiguous determination of the triplet decay and relaxation rates is not possible. However, it should still be possible to determine general trends. The fitting results show that the spin-lattice relaxation rate is characterised by a more pronounced temperature dependence, with a significant increase above 20 K, also observed in other systems [284, 285]. In order to verify this experimentally, transient absorption measurements were performed with the help of Dr. Kevin Henbest and the triplet lifetime was determined.

TABLE C.1: Overall triplet decay rate $k_T = \frac{1}{3}(k_X + k_Y + k_Z)$, relaxation rate $3w = w_X + w_Y + w_Z$ ($= \frac{1}{T_1}$) and relative decay and relaxation rates for the three triplet sublevels resulting from least-square fitting of the experimental data of *l*-P1 as described in the text. The errors were evaluated by repeating the fitting for different initial parameter sets and computing the standard deviation, the actual values correspond to the best-fit results.

T [K]	$k_T [10^3 \text{ s}^{-1}]$	$3w [10^3 \text{ s}^{-1}]$	$k_X : k_Y : k_Z$	$w_X : w_Y : w_Z$
2.5	0.015 ± 0.008	0.089 ± 0.028	0.38 : 0.33 : 0.29	0.53 : 0.31 : 0.16
5.0	0.001 ± 0.005	0.137 ± 0.098	0.00 : 0.90 : 0.10	0.50 : 0.35 : 0.15
10	0.004 ± 0.019	0.241 ± 0.200	0.22 : 0.54 : 0.24	0.43 : 0.45 : 0.12
20	0.276 ± 0.128	0.475 ± 0.305	0.34 : 0.35 : 0.32	0.10 : 0.12 : 0.78
50	0.478 ± 1.126	1.819 ± 2.950	0.34 : 0.36 : 0.30	0.24 : 0.33 : 0.43
90	3.485 ± 1.931	9.974 ± 4.396	0.35 : 0.32 : 0.33	0.37 : 0.42 : 0.21

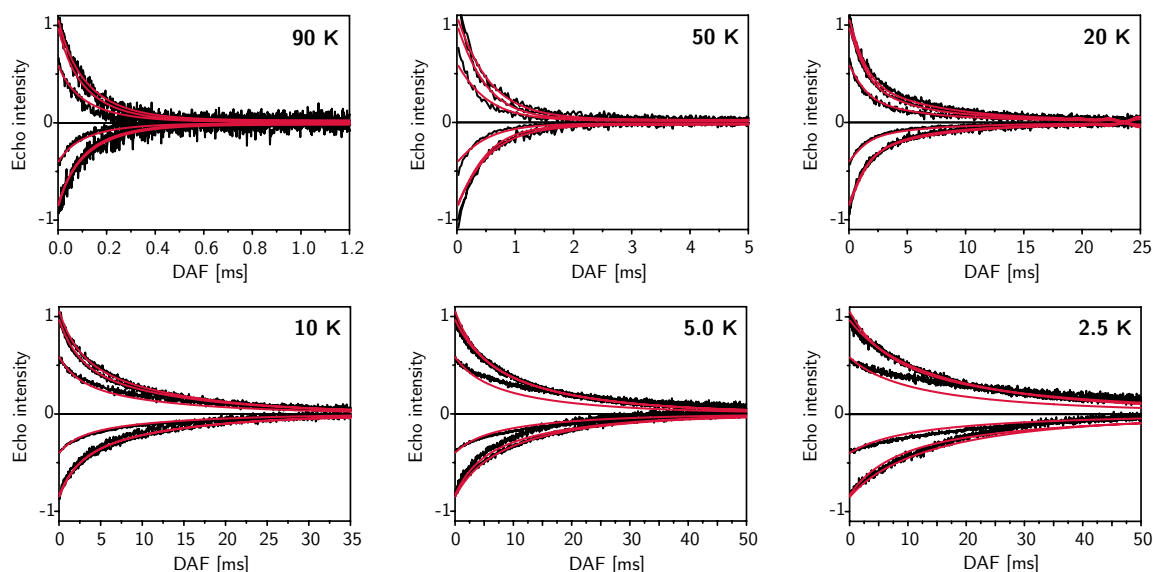


FIGURE C.3: Experimental ESE-detected kinetic traces of *l*-P1 and the corresponding simulated traces following the fitting procedure described in the text. The fitting parameters are given in table C.1.

Transient absorption measurements are performed by laser excitation followed by detection of the decay with a probe light at a series of different wavelengths. The ground state absorption measured without laser excitation is subtracted at each point. The measurements were performed with laser excitation from a Nd:YAG laser at 532 nm and with a Xe arc lamp as probe light. After the sample cell the light passes through a monochromator and is detected by a PMT connected to an oscilloscope. The difference in absorption before and after the laser pulse ΔA is recorded directly.

The transient absorption (TA) measurement was performed at 120 K and the results are shown in figure C.4 A. At lower temperatures no decay of the triplet state was observed on the timescale of the TA experiment (figure C.4 A) and measurements on longer timescales were prevented by significant baseline fluctuations. The transient absorption spectrum for excitation at 532 nm shows ground state bleaching in the region of the Soret and Q bands and a broad triplet absorption with the maximum at higher wavelengths with respect to the ground state absorption. The presence of a triplet absorption band at lower wavelengths with respect to the ground state absorption and one at higher wavelengths is in agreement with literature data on triplet states of zinc tetraphenylporphyrins [286, 287]. In addition to this, a band in the wavelength region from 700 to 900 nm with a peak between 800 and 900 nm is usually also present [286, 287].

The decay of the 120 K TA traces at different wavelengths was found to be monoexponential and allowed a global fit of the TA data over most of the wavelength range with a monoexponential decay and hence determination of the triplet lifetime τ_T . The triplet decay rate constant k_T corresponds to 1.4 ms^{-1} at 120 K. The coefficients of the monoexponential decays at different wavelengths were used to calculate the corresponding TA spectrum for different triplet lifetimes as a comparison with the experimental results (shown in figure C.4 B). A triplet lifetime of about 5 ms would give a similar

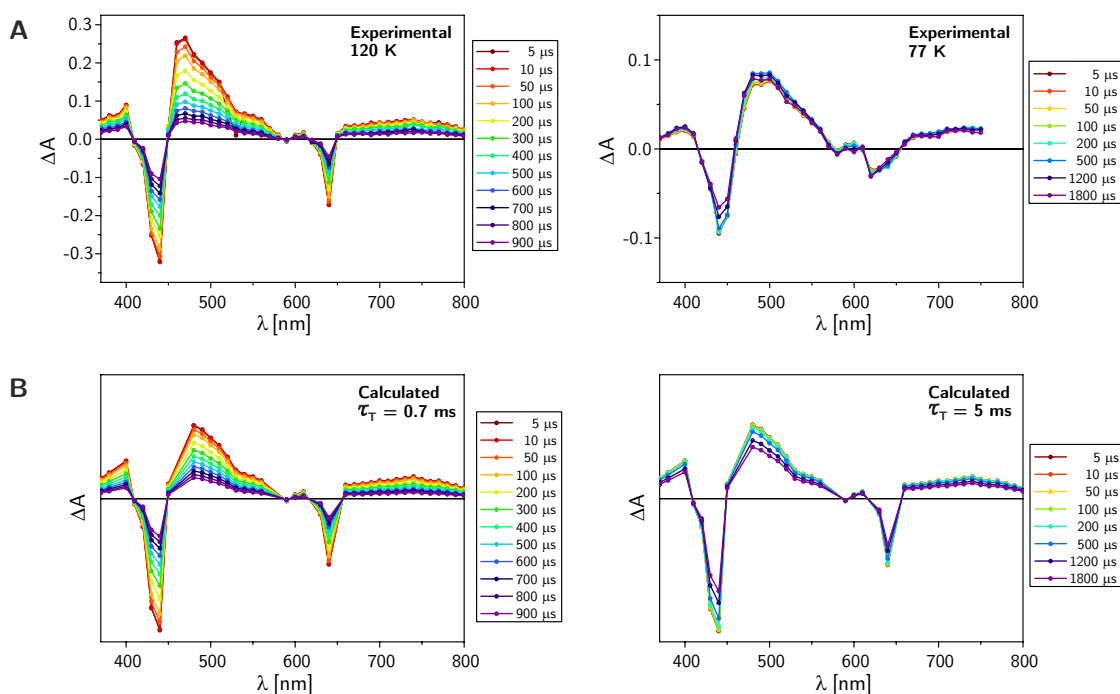


FIGURE C.4: (A) Experimental transient absorption spectra recorded at 120 K and 77 K with laser excitation at 532 nm (the 77 K data shown here was recorded with laser excitation at 450 nm, but the same results were obtained with excitation at 532 nm). (B) Calculated TA spectra for different triplet decay constants τ_T performed using the relative intensities determined from the coefficients of a global monoexponential fit of the 120 K TA spectrum and a monoexponential decay with the given time constant.

TA spectrum to the one observed at 77 K and would agree reasonably well with the results from the EPR kinetic measurements, confirming the prevalence of spin-lattice relaxation at high temperatures.

The relative decay rate constants corresponding to the three zero-field sublevels could provide information on the selectivity of the triplet decay, in the same way as the zero-field populations provided information on the selectivity of the triplet state formation. Due to the presence of both decay and relaxation, the relative values of both decay processes have to be considered. The ratios determined at different temperatures fluctuate significantly, hence using the average is probably most appropriate to attempt an interpretation of the results. The average ratio of relative triplet decay rate constants is $k_X : k_Y : k_Z = 0.27 : 0.47 : 0.26$ and that of the relaxation rates is $w_X : w_Y : w_Z = 0.36 : 0.33 : 0.31$. On average, the spin relaxation rates are approximately the same for the three orientations and hence the ratio of decay rates should reflect the selectivity of the triplet decay. The results show very similar rates for the three sublevels with a slightly larger contribution from the decay of the Y orientation. The population of the triplet state sublevels in *l*-P1 was shown to be almost exclusively selective for the Z sublevel based on the spin polarisation of the time-resolved EPR spectrum, while no strong selectivity seems to be shown by the decay. The observation of different selectivity in the population and depopulation of the triplet state sublevels was also reported for a different zinc porphyrin by van Dorp et al. [137]. In that case population was also observed to occur preferentially to the Z (out-of-plane) sublevel, while the depopulation rates were found to be similar for all three orientations of the ZFS tensor ($k_X : k_Y : k_Z = 0.25 : 0.30 : 0.45$). The results were explained by considering different processes

contributing to triplet population and decay. As explained in section 4.3.1, the selective population of the out-of-plane sublevel in zinc porphyrins arises from a direct spin-orbit coupling term of the type $\langle S_1 | H_{SO} | T_1 \rangle$ with a one-centre contribution on the zinc ion [137]. This term is absent for the ISC from T_1 to S_0 due to the different symmetry of the ground and first excited triplet states (shown for an idealised porphyrin with D_{2h} symmetry by van Dorp et al. in reference [137]). The triplet decay is thus determined only by the vibronic spin-orbit coupling mechanism. As a consequence, the population of the triplet sublevels is highly selective for the out-of-plane sublevel, while the depopulation mechanism shows less anisotropy.

The analysis of triplet state kinetics was extended to the porphyrin dimer and trimer. The ESE-detected kinetic measurements were only performed at 20 K, as most of the other measurements were performed at this temperature and as the results obtained for the porphyrin monomer at this temperature could be fit reasonably well using the high-temperature approximation. A similar fitting procedure to that explained above for the porphyrin monomer was employed to determine triplet decay and relaxation rates as well as relative decay rates from the three zero-field sublevels. The results are shown in figure C.5 and the fitting parameters are summarised in table C.2. Unfortunately the results show that a separation of the triplet decay and spin relaxation processes is not possible and hence an interpretation of the ESE kinetic traces in terms of selective triplet decay, potentially providing further information on the mechanisms driving triplet formation and decay, was not possible.

TABLE C.2: Overall triplet decay rate $k_T = \frac{1}{3}(k_X + k_Y + k_Z)$, relaxation rate $3w = w_X + w_Y + w_Z$ ($= \frac{1}{T_1}$) and relative decay and relaxation rates for the three triplet sublevels resulting from least-square fitting of the experimental data of *l*-P1, *l*-P2 and *l*-P3 as described in the text. The errors were evaluated by repeating the fitting for different initial parameter sets and computing the standard deviation, the actual values correspond to the best-fit results.

	$k_T [10^3 \text{ s}^{-1}]$	$3w [10^3 \text{ s}^{-1}]$	$k_X : k_Y : k_Z$	$w_X : w_Y : w_Z$
<i>l</i> -P1	0.45 ± 0.18	0.83 ± 0.47	0.37 : 0.24 : 0.39	0.41 : 0.58 : 0.01
<i>l</i> -P2	0.19 ± 0.10	1.05 ± 0.32	0.00 : 0.42 : 0.58	0.57 : 0.03 : 0.40
<i>l</i> -P3	0.56 ± 1.40	2.76 ± 8.82	0.31 : 0.34 : 0.35	0.43 : 0.51 : 0.06

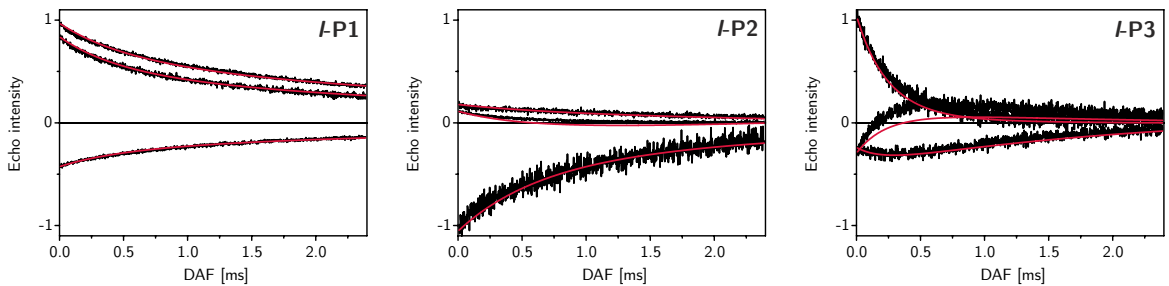


FIGURE C.5: Experimental ESE-detected kinetic traces of *l*-P1, *l*-P2 and *l*-P3 recorded at 20 K and the corresponding simulated traces following the fitting procedure described in the text. The fitting parameters are given in table C.2.

In conclusion, the ESE-detected kinetic measurements on *l*-P1, *l*-P2 and *l*-P3 have shown that triplet decay and spin relaxation rates are of the same order of magnitude in the available temperature range. This and the lack of information on the pure overall triplet decay rate in the temperature range where the EPR experiments were feasible, unfortunately prevented an in-depth quantitative analysis of the triplet state kinetics. In addition to this, both the triplet decay and the relaxation rates are very sensitive to the oxygen concentration in the sample, and while all the samples were degassed and kept under argon or frozen, a small sample-to-sample variability is nevertheless likely. In addition to this, the effect of potential aggregation for the longer oligomers on triplet decay and relaxation is not understood.

Inspection of the experimental data however shows that the timescale of triplet decay is not drastically different for *l*-P2 and *l*-P3 compared to *l*-P1, which supports coherent delocalisation rather than hopping as the triplet state delocalisation mechanism, as in the presence of triplet energy transfer changes in decay rates of up to one order of magnitude were detected for chlorophyll dimers [119].

More precise information on the population and decay rates of the individual triplet state sublevels could potentially be obtained using microwave-induced delayed phosphorescence or fluorescence measurements at extremely low temperatures [137, 288–291]. These experiments are based on the isolation of the three triplet state sublevels at temperatures of 1.1 to 1.3 K, where spin-lattice relaxation is believed to be negligible, and on the observation of changes in the fluorescence or phosphorescence decay after microwave irradiation.

D DEER SIMULATIONS FOR MODEL STRUCTURES OF THE CYP101D1-ARX COMPLEX

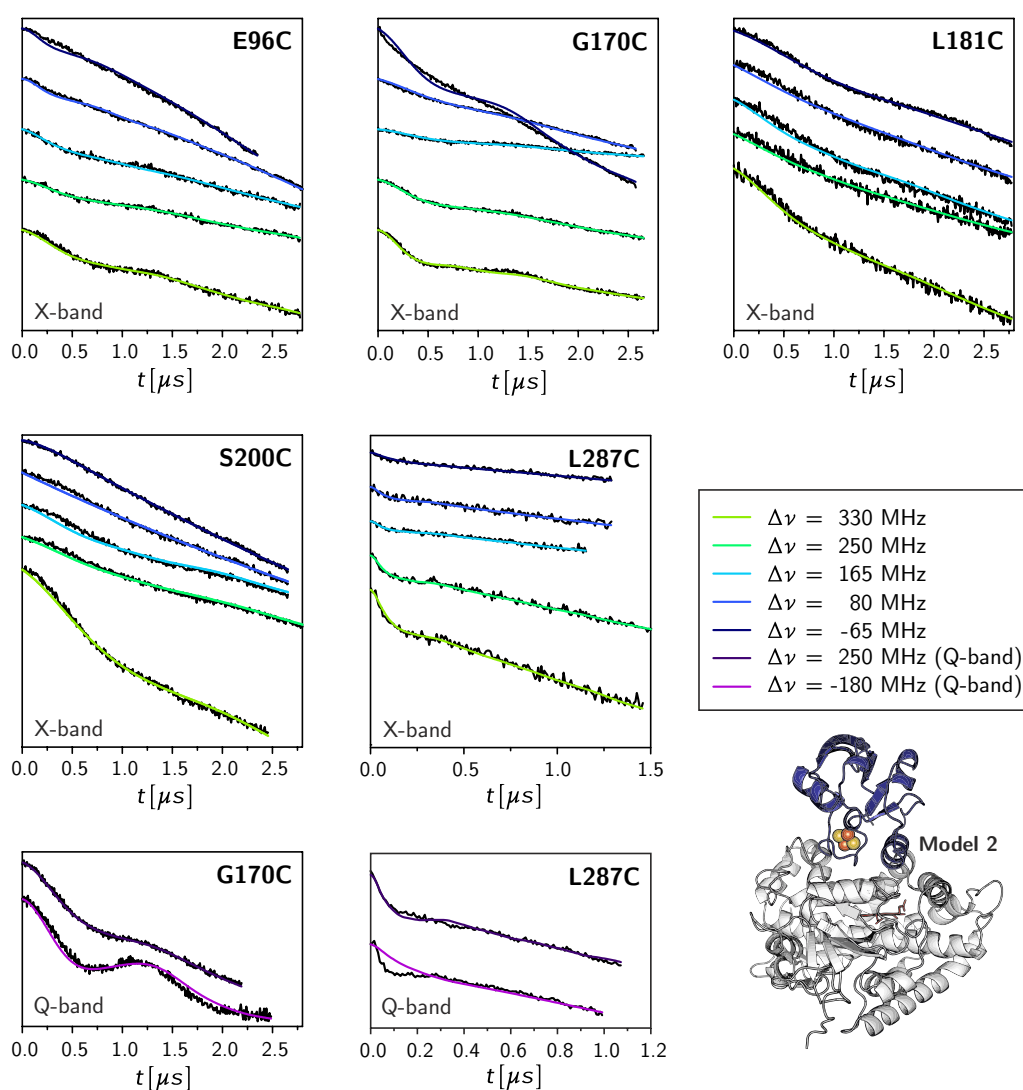


FIGURE D.1: Experimental and simulated orientation-selective X-band and Q-band four-pulse DEER data for model 2 of the CYP101D1-Arx complex with nitroxide spin labels introduced at positions E96C, G170C, L181C, S200C and L287C on CYP101D1. Experimental details can be found in section 7.2.2 and details on the simulations performed are described in the text.

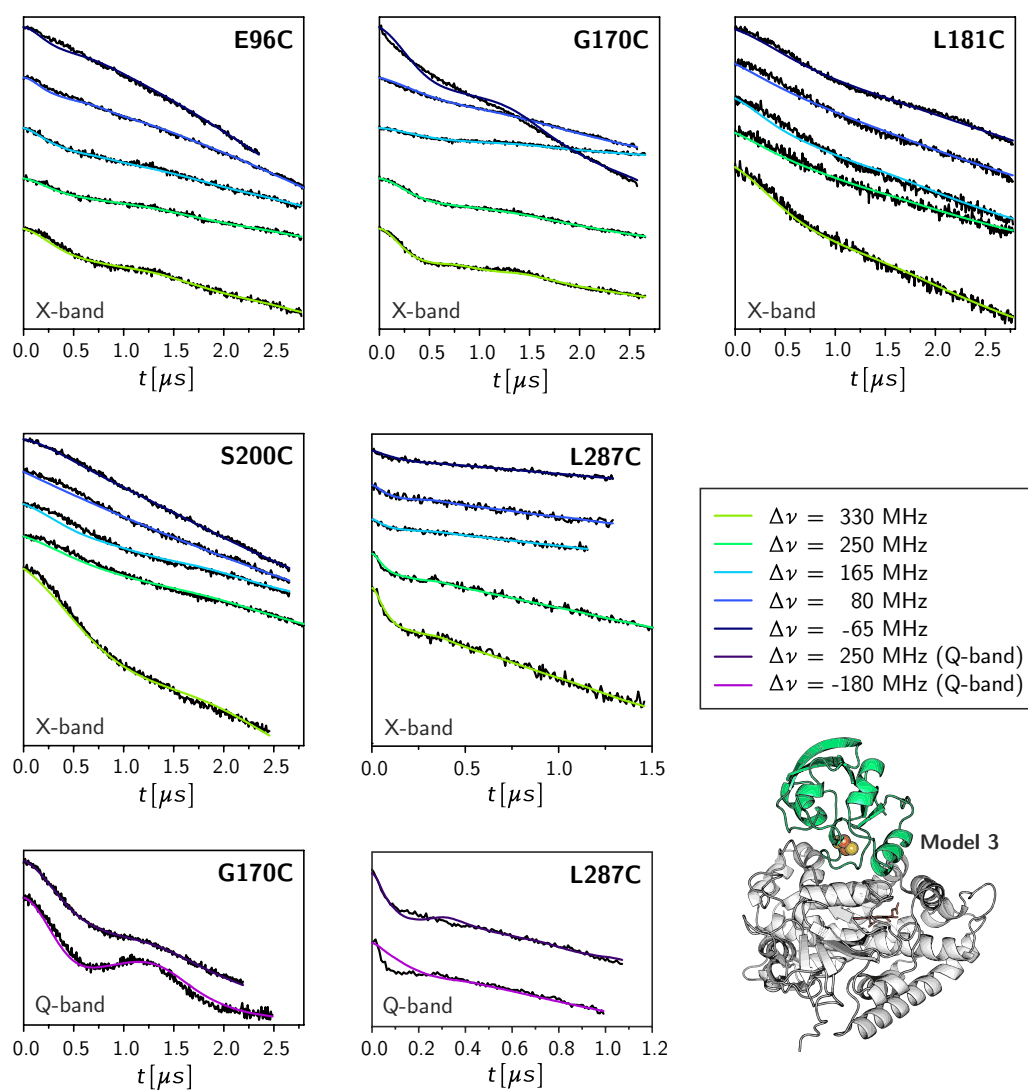


FIGURE D.2: Experimental and simulated orientation-selective X-band and Q-band four-pulse DEER data for model 3 of the CYP101D1-Arx complex with nitroxide spin labels introduced at positions E96C, G170C, L181C, S200C and L287C on CYP101D1. Experimental details can be found in section 7.2.2 and details on the simulations performed are described in the text.

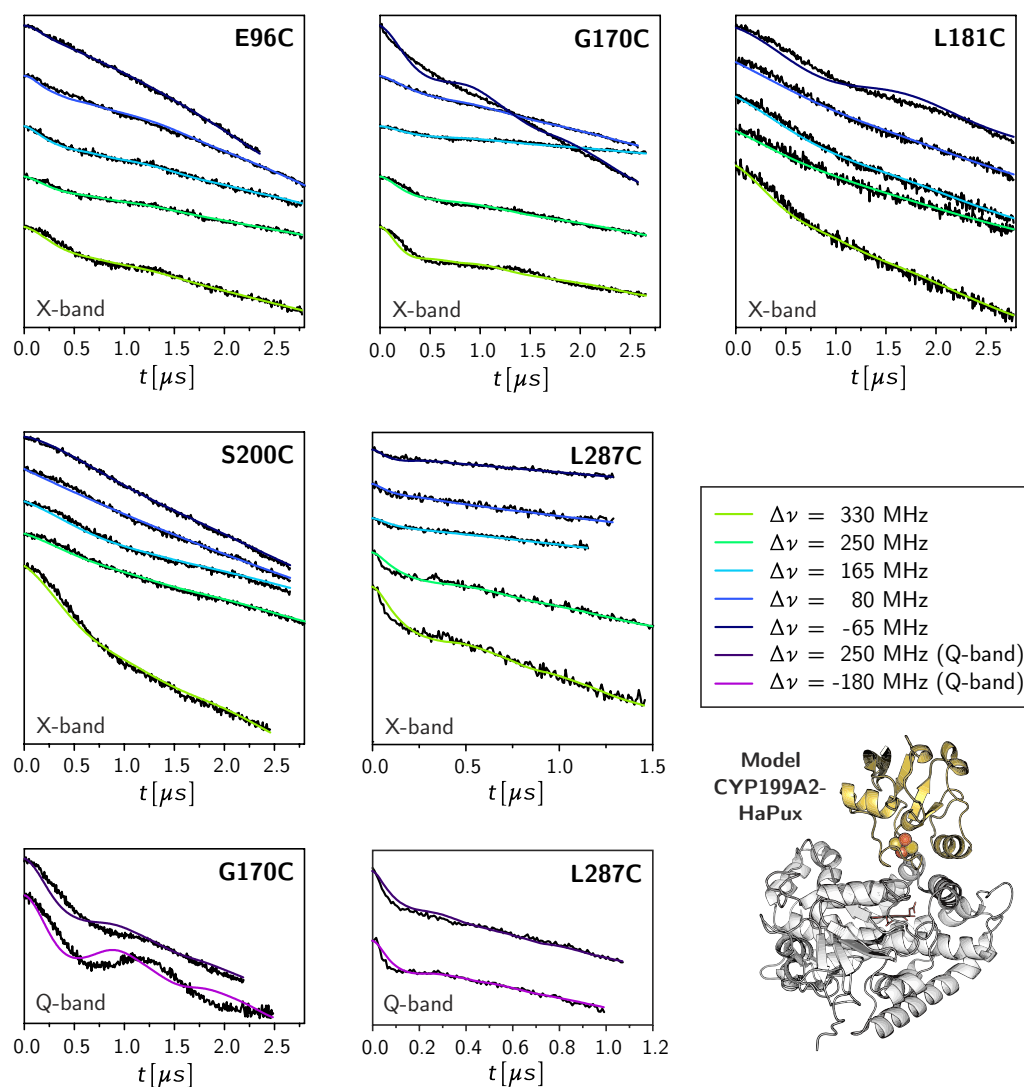


FIGURE D.3: Experimental and simulated orientation-selective X-band and Q-band four-pulse DEER data for a model of the CYP101D1-Arx complex based on the structure of the CYP199A2-HaPux complex [245] with nitroxide spin labels introduced at positions E96C, G170C, L181C, S200C and L287C on CYP101D1. Experimental details can be found in section 7.2.2 and details on the simulations performed are described in the text.

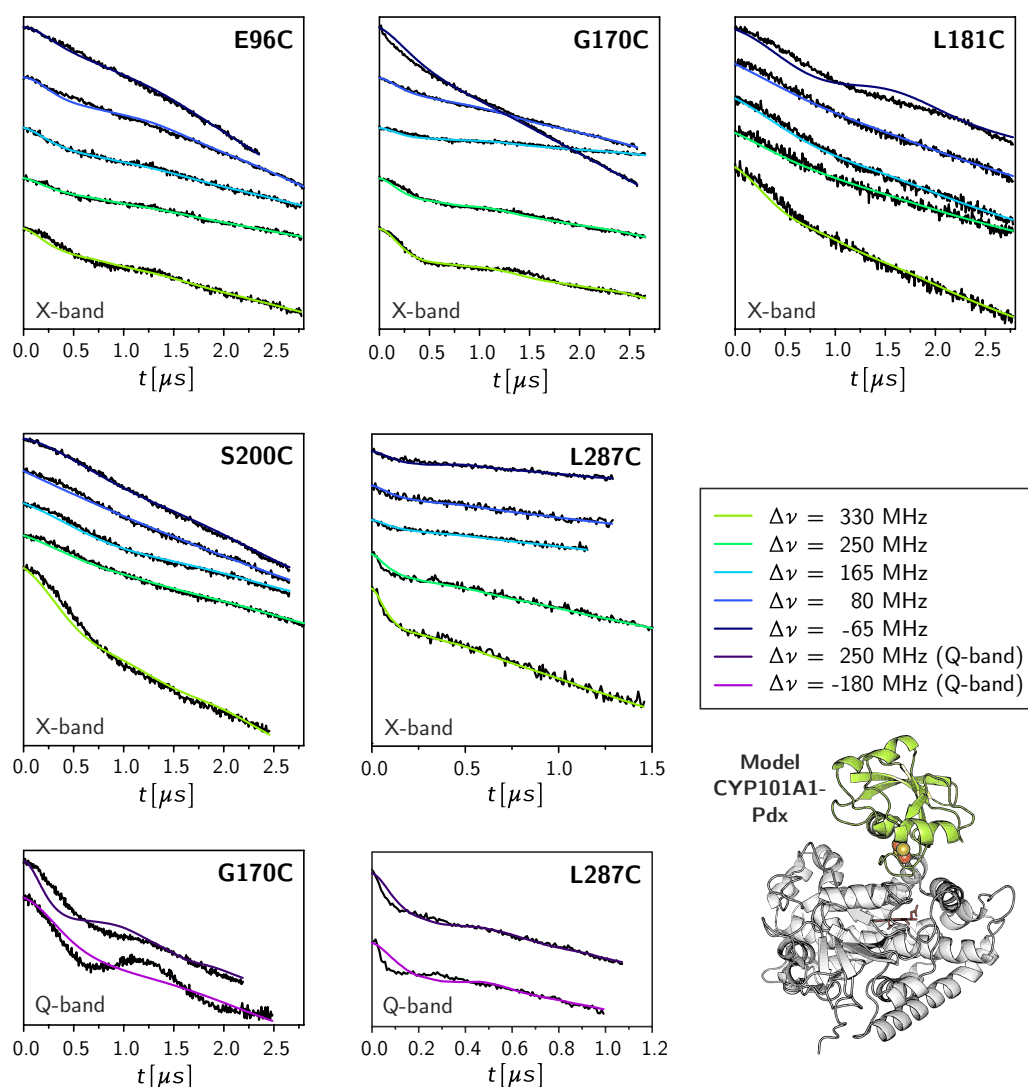


FIGURE D.4: Experimental and simulated orientation-selective X-band and Q-band four-pulse DEER data for a model of the CYP101D1-Arx complex based on the structure of the CYP101A1-Pdx complex [252] with nitroxide spin labels introduced at positions E96C, G170C, L181C, S200C and L287C on CYP101D1. Experimental details can be found in section 7.2.2 and details on the simulations performed are described in the text.

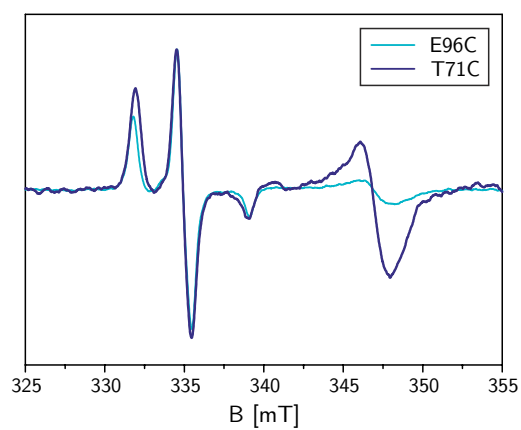


FIGURE D.5: Cw-EPR spectra of the CYP101D1-Arx complex with mutants E96C and T71C recorded at 100 K with a modulation amplitude of 0.1 mT and a microwave power of 0.006325 mW ($\nu_{mw}=9.40$ GHz).

BIBLIOGRAPHY

- [1] N. M. Atherton. *Principles of Electron Spin Resonance*. PTR Prentice Hall, New York, 1993.
- [2] J. A. Weil, J. R. Bolton, and J. E. Wertz. *Electron Paramagnetic Resonance: Elementary Theory and Practical Applications*. John Wiley & Sons, Inc., New York, 1994.
- [3] A. Schweiger and G. Jeschke. *Principles of Pulse Electron Paramagnetic Resonance*. Oxford University Press, 2001.
- [4] N. J. Stone. Table of nuclear magnetic dipole and electric quadrupole moments. *At. Data. Nucl. Data Tables*, 90:75–176, 2005.
- [5] S. Sinnecker and F. Neese. Spin-spin contributions to the zero-field splitting tensor in organic triplets, carbenes and biradicals - A density functional and *ab initio* study. *J. Phys. Chem. A*, 110:12267–12275, 2006.
- [6] A. Bencini and D. Gatteschi. *Electron Paramagnetic Resonance of Exchange-Coupled Systems*. Springer, Heidelberg, 1990.
- [7] G. R. Eaton, S. S. Eaton, D. P. Barr, and R. T. Weber. *Quantitative EPR*. Springer-Verlag, Wien, 2010.
- [8] G. Jeschke. Instrumentation and Experimental Setup. In M. A. Hemminga and L. Berliner, editors, *ESR spectroscopy in Membrane Biophysics*, chapter 2, pages 17–47. Springer, Biological Magnetic Resonance, New York, 2007.
- [9] P. J. Hore, J. Jones, and S. Wimperis. *NMR: The Toolkit*. Oxford University Press, Oxford, 2000.
- [10] S. S. Eaton and G. R. Eaton. Relaxation Times of Organic Radicals and Transition Metal Ions. In L. J. Berliner, S. S. Eaton, and G. R. Eaton, editors, *Distance Measurements in Biological Systems by EPR*, chapter 2, pages 29–154. Kluwer Academic/Plenum Publishers, New York, 2000.
- [11] W. B. Mims. Pulsed ENDOR Experiments. *Proc. R. Soc. A*, 283:452–457, 1965.
- [12] E. R. Davies. A new pulse ENDOR technique. *Phys. Lett. A*, 47:1–2, 1974.
- [13] C. Gemperle and A. Schweiger. Pulsed electron-nuclear double resonance methodology. *Chem. Rev.*, 91:1481–1505, 1991.

- [14] C. Fan, P. E. Doan, C. E. Davoust, and B. M. Hoffman. Quantitative studies of Davies pulsed ENDOR. *J. Magn. Reson.*, 98:62–72, 1992.
- [15] A. V. Astashkin and A. Kawamori. Matrix line in pulsed electron-nuclear double resonance spectra. *J. Magn. Reson.*, 135:406–417, 1998.
- [16] P. E. Doan, C. Fan, C. E. Davoust, and B. M. Hoffman. A simple method for hyperfine-selective heteronuclear pulsed ENDOR via proton suppression. *J. Magn. Reson.*, 200:196–200, 1991.
- [17] P. E. Doan, N. S. Lees, M. Shanmugam, and B. M. Hoffman. Simulating suppression effects in Pulsed ENDOR, and the ‘hole in the middle’ of Mims and Davies ENDOR Spectra. *Appl. Magn. Reson.*, 37:763–779, 2010.
- [18] M. Bennebroek and J. Schmidt. Pulsed ENDOR Spectroscopy at Large Thermal Spin Polarizations and the Absolute Sign of the Hyperfine Interaction. *J. Magn. Reson.*, 128:199–206, 1997.
- [19] B. Epel, P. Manikandan, P. M. H. Kroneck, and D. Goldfarb. High-field ENDOR and the sign of the hyperfine coupling. *Appl. Magn. Reson.*, 21:287–297, 2001.
- [20] B. Epel, A. Pöpl, P. Manikandan, S. Vega, and D. Goldfarb. The effect of spin relaxation on ENDOR spectra recorded at high magnetic fields and low temperatures. *J. Magn. Reson.*, 148:388–397, 2001.
- [21] F. Lenzian, H. van Willigen, S. Sastry, K. Möbius, H. Scheer, and R. Feick. Proton ENDOR study of the photoexcited triplet state P^T in *Rps. sphaeroides* R-26 photosynthetic reaction centres. *Chem. Phys. Lett.*, 118:145–150, 1985.
- [22] C. W. M. Kay, M. Di Valentin, and K. Möbius. A time-resolved Electron Nuclear Double Resonance (ENDOR) study of the photoexcited triplet state of free-base tetraphenylporphyrin. *Sol. Energy Mater. Sol. Cells*, 38:111–118, 1995.
- [23] S. Stoll and A. Schweiger. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.*, 178:42–55, 2006.
- [24] S. Stoll and R. D. Britt. General and efficient simulation of pulse EPR spectra. *Phys. Chem. Chem. Phys.*, 11:6614–6625, 2009.
- [25] W. B. Mims. Envelope modulation in spin-echo experiments. *Phys. Rev. B*, 5:2409–2419, 1972.
- [26] L. G. Rowan, E. L. Hahn, and W. B. Mims. Electron-Spin-Echo Envelope Modulation. *Phys. Rev.*, 137:61–73, 1965.
- [27] S. A. Dikanov and Y. D. Tsvetkov. *Electron Spin Echo Envelope Modulation (ESEEM) Spectroscopy*. CRC Press, Florida, 1992.
- [28] L. Kevan. Modulation of Electron Spin-Echo Decay in Solids. In L. Kevan and R. N. Schwartz, editors, *Time-domain Electron Spin Resonance*, chapter 8, pages 279–341. John Wiley & Sons, Inc., New York, 1979.

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- [29] A. Ponti and A. Schweiger. Echo phenomena in electron paramagnetic resonance spectroscopy. *Appl. Magn. Reson.*, 7:363–403, 1994.
- [30] W. B. Mims. Amplitudes of superhyperfine frequencies displayed in the electron-spin-echo envelope. *Phys. Rev. B*, 6:3543–3545, 1972.
- [31] D. J. Sloop, H. L. Yu, T.-S. Lin, and S. I. Weissman. Electron spin echoes of a photoexcited triplet: Pentacene in p-terphenyl crystals. *J. Chem. Phys.*, 75:3746–3757, 1981.
- [32] A. Coffino and J. Peisach. Nuclear modulation effects in high-spin electron systems with small zero-field splittings. *J. Chem. Phys.*, 97:3072–3090, 1992.
- [33] P. Höfer, A. Grupp, H. Nebenführ, and M. Mehring. Hyperfine sublevel correlation (HYSCORE) spectroscopy: a 2D ESR investigation of the squaric acid radical. *Chem. Phys. Lett.*, 132:279–282, 1986.
- [34] S. Van Doorslaer. Hyperfine Sublevel Correlation Spectroscopy. *EPR Newsl.*, 17:9–12, 2008.
- [35] C. Gemperle, G. Aebli, A. Schweiger, and R. R. Ernst. Phase Cycling in Pulse EPR. *J. Magn. Reson.*, 88:241–256, 1990.
- [36] A. Pöpl and R. Böttcher. Cross peak intensities in two-dimensional four-pulse electron spin echo modulation spectra of deuteriums in single crystals. *Chem. Phys.*, 221:53–66, 1997.
- [37] G. Jeschke, R. Rakhmatullin, and A. Schweiger. Sensitivity enhancement by matched microwave pulses in one- and two-dimensional Electron Spin Echo Envelope Modulation spectroscopy. *J. Magn. Reson.*, 131:261–271, 1998.
- [38] J. Harmer, G. Mitrikas, and A. Schweiger. Advanced Pulse EPR Methods for the Characterization of Metalloproteins. In G. Hanson and L. Berliner, editors, *High Resolution EPR, Biological Magnetic Resonance*, volume 28, pages 13–61. Springer Science, 2009.
- [39] E. J. Reijerse and C. P. Keijzers. Model calculations of frequency-domain ESEEM spectra of disordered systems. *J. Magn. Reson.*, 71:83–96, 1987.
- [40] A. Lai, H. L. Flanagan, and D. J. Singel. Multifrequency electron spin echo envelope modulation in $S = 1/2$, $I = 1/2$ systems: Analysis of the spectral amplitudes, line shapes, and linewidths. *J. Chem. Phys.*, 89:7161–7166, 1988.
- [41] Y. Deligiannakis, M. Louloudi, and N. Hadjiliadis. Electron spin echo envelope modulation (ESEEM) spectroscopy as a tool to investigate the coordination environment of metal centers. *Coord. Chem. Rev.*, 204:1–112, 2000.
- [42] H. L. Flanagan and D. J. Singel. Analysis of ^{14}N ESEEM patterns of randomly oriented solids. *J. Chem. Phys.*, 87:5606–5616, 1987.

- [43] C. Calle, A. Sreekanth, M. V. Fedin, J. Forrer, I. García-Rubio, I. A. Gromov, D. Hinderberger, B. Kasmaj, P. Léger, B. Mancosu, G. Mitrikas, M. G. Santangelo, S. Stoll, A. Schweiger, R. Tschaggel, and J. Harmer. Pulse EPR methods for studying chemical and biological samples containing transition metals. *Helv. Chim. Acta*, 89:2495–2521, 2006.
- [44] S. A. Dikanov and M. K. Bowman. Cross-peak lineshape of two-dimensional ESEEM spectra in disordered $S = 1/2$, $I = 1/2$ spin systems. *J. Magn. Reson. A*, 116:125–128, 1995.
- [45] J. J. Shane, L. P. Liesum, and A. Schweiger. Efficient simulation of ESEEM spectra using gamma. *J. Magn. Reson.*, 134:72–75, 1998.
- [46] Z. L. Madi, S. Van Doorslaer, and A. Schweiger. Numerical simulation of one- and two-dimensional ESEEM experiments. *J. Magn. Reson.*, 154:181–191, 2002.
- [47] S. Stoll, C. Calle, G. Mitrikas, and A. Schweiger. Peak suppression in ESEEM spectra of multinuclear spin systems. *J. Magn. Reson.*, 177:93–101, 2005.
- [48] D. R. Ort and C. F. Yocum, editors. *Oxygenic Photosynthesis: The Light Reactions*. Kluwer Academic Publishers, Dordrecht, 1996.
- [49] S. M. Prince, M. Z. Papiz, A. A. Freer, G. McDermott, A. M. Hawthornthwaite-Lawless, R. J. Cogdell, and N. W. Isaacs. Apoprotein structure in the LH2 complex from *Rhodospseudomonas acidophila* strain 10050: modular assembly and protein pigment interactions. *J. Mol. Biol.*, 268:412–423, 1997.
- [50] G. T. Oostergetel, H. van Amerongen, and E. J. Boekema. The chlorosome: a prototype for efficient light harvesting in photosynthesis. *Photosynth. Res.*, 104:245–255, 2010.
- [51] D. Gust, T. A. Moore, and A. L. Moore. Mimicking photosynthetic solar energy transduction. *Acc. Chem. Res.*, 34:40–48, 2001.
- [52] M.-S. Choi, T. Yamazaki, I. Yamazaki, and T. Aida. Bioinspired molecular design of light-harvesting multiporphyrin arrays. *Angew. Chem. Int. Ed.*, 43:150–158, 2004.
- [53] M. R. Wasielewski. Energy, charge, and spin transport in molecules and self-assembled nanostructures inspired by photosynthesis. *J. Org. Chem.*, 71:5051–5066, 2006.
- [54] Y. Nakamura, N. Aratani, and A. Osuka. Cyclic porphyrin arrays as artificial photosynthetic antenna: synthesis and excitation energy transfer. *Chem. Soc. Rev.*, 36:831–845, 2007.
- [55] N. Aratani, D. Kim, and A. Osuka. Discrete cyclic porphyrin arrays as artificial light-harvesting antenna. *Acc. Chem. Res.*, 42:1922–1934, 2009.
- [56] H. S. Cho, D. H. Jeong, S. Cho, D. Kim, Y. Matsuzaki, K. Tanaka, A. Tsuda, and A. Osuka. Photo-physical properties of porphyrin tapes. *J. Am. Chem. Soc.*, 124:14642–14654, 2002.
- [57] M. O. Senge, M. Fazekas, E. G. A. Notaras, W. J. Blau, M. Zawadzka, O. B. Locos, and E. M. Ni Mhuircheartaigh. Nonlinear optical properties of porphyrins. *Adv. Mater.*, 19:2737–2774, 2007.

- [58] H. L. Anderson. Building molecular wires from the colours of life: conjugated porphyrin oligomers. *Chem. Commun.*, pages 2323–2330, 1999.
- [59] D. K. James and J. M. Tour. Molecular Wires. In L. DeCola, editor, *Molecular Wires: From Design to Properties*, pages 33–59. Springer Verlag, Berlin, 2005.
- [60] F. C. Grozema, C. Houarner-Rassin, P. Prins, L. D. A. Siebbeles, and H. L. Anderson. Supramolecular control of charge transport in molecular wires. *J. Am. Chem. Soc.*, 129:13370–13371, 2007.
- [61] A. M. Brun, S. J. Atherton, A. Harriman, V. Heitz, and J.-P. Sauvage. Photophysics of entwined porphyrin conjugates: competitive exciton annihilation, energy-transfer, electron-transfer, and superexchange processes. *J. Am. Chem. Soc.*, 114:4632–4639, 1992.
- [62] H. L. Anderson. Conjugated porphyrin ladders. *Inorg. Chem.*, 33:972–981, 1994.
- [63] D. Beljonne, G. E. O’Keefe, P. J. Hamer, R. H. Friend, H. L. Anderson, and J. L. Brédas. Investigation of the linear and nonlinear optical response of edge-linked conjugated zinc porphyrin oligomers by optical spectroscopy and configuration interaction techniques. *J. Chem. Phys.*, 106:9439–9460, 1997.
- [64] D. Holten, D. F. Bocian, and J. S. Lindsey. Probing electronic communication in covalently linked multiporphyrin arrays. A guide to the rational design of molecular photonic devices. *Acc. Chem. Res.*, 35:57–69, 2002.
- [65] D. Kim and A. Osuka. Photophysical properties of directly linked linear porphyrin arrays. *J. Phys. Chem. A*, 107:8791–8816, 2003.
- [66] K. Susumu, P. R. Frail, P. J. Angiolillo, and M. J. Therien. Conjugated chromophore arrays with unusually large hole polaron delocalization lengths. *J. Am. Chem. Soc.*, 128:8380–8381, 2006.
- [67] M. U. Winters, J. Kärnbratt, M. Eng, C. J. Wilson, H. L. Anderson, and B. Albinsson. Photophysics of a butadiyne-linked porphyrin dimer: Influence of conformational flexibility in the ground and first singlet excited state. *J. Phys. Chem. C*, 111:7192–7199, 2007.
- [68] T. M. Wilson, T. Hori, M.-C. Yoon, N. Aratani, A. Osuka, D. Kim, and M. R. Wasielewski. Rapid intramolecular hole hopping in *meso-meso* and *meta*-phenylene linked linear and cyclic multiporphyrin arrays. *J. Am. Chem. Soc.*, 132:1383–1388, 2010.
- [69] J. K. Sprafke, D. V. Kondratuk, M. Wykes, A. L. Thompson, M. Hoffmann, R. Drevinskas, W.-H. Chen, C. K. Yong, J. Kärnbratt, J. E. Bullock, M. Malfois, M. R. Wasielewski, B. Albinsson, L. M. Herz, D. Zigmantas, D. Beljonne, and H. L. Anderson. Belt-shaped π -systems: relating geometry to electronic structure in a six-porphyrin nanoring. *J. Am. Chem. Soc.*, 133:17262–17273, 2011.
- [70] J. Yang and D. Kim. Excitation energy migration processes in various multi-porphyrin assemblies. *Philos. Trans. R. Soc. London, Ser. A*, 370:3802–3818, 2012.

- [71] M. C. O'Sullivan, J. K. Sprafke, D. V. Kondratuk, C. Rinfray, T. D. W. Claridge, A. Saywell, M. O. Blunt, J. N. O'Shea, P. H. Beton, M. Malfois, and H. L. Anderson. Vernier templating and synthesis of a 12-porphyrin nano-ring. *Nature*, 469:72–75, 2011.
- [72] P. Neuhaus, A. Clossen, J. Q. Gong, L. M. Herz, and H. L. Anderson. A molecular nanotube with 3D π -conjugation. *Angew. Chem. Int. Ed.*, manuscript accepted, 2015.
- [73] J. R. Norris, R. A. Uphaus, H. L. Crespi, and J. J. Katz. Electron Spin Resonance of chlorophyll and the origin of signal I in photosynthesis. *Proc. Natl. Acad. Sci.*, 68:625–628, 1971.
- [74] W. Lubitz, R. A. Isaacson, E. C. Abresch, and G. Feher. ^{15}N electron nuclear double resonance of the primary donor cation radical P^{+865} in reaction centers of *Rhodospseudomonas sphaeroides*: additional evidence for the dimer model. *Proc. Natl. Acad. Sci.*, 81:7792–7796, 1984.
- [75] W. Lubitz and F. Lendzian. Structural studies of the primary donor cation radical P870^{+} in reaction centers of *Rhodospirillum rubrum* by electron-nuclear double resonance in solution. *Proc. Natl. Acad. Sci.*, 81:1401–1405, 1984.
- [76] F. Lendzian, W. Lubitz, H. Scheer, A. J. Hoff, M. Plato, E. Tränkle, and K. Möbius. ESR, ENDOR and TRIPLE resonance studies of the primary donor radical cation P960^{+} in the photosynthetic bacterium *Rhodospseudomonas viridis*. *Chem. Phys. Lett.*, 148:377–385, 1988.
- [77] D. Kolbasov, N. Srivatsan, N. Ponomarenko, M. Jäger, and J. R. Norris. Modeling charge transfer in oxidized bacterial antenna complexes. *J. Phys. Chem. B*, 107:2386–2393, 2003.
- [78] N. Srivatsan, S. Weber, D. Kolbasov, and J. R. Norris. Exploring charge migration in light-harvesting complexes using Electron Paramagnetic Resonance line narrowing. *J. Phys. Chem. B*, 107:2127–2138, 2003.
- [79] M. Huber, H. Kurreck, B. von Maltzan, M. Plato, and K. Möbius. ENDOR studies of π -electron delocalization in covalently linked porphyrin dimers. Model systems for the primary donor in photosynthesis? *J. Chem. Soc., Faraday Trans.*, 86:1087–1094, 1990.
- [80] J. Seth, V. Palaniappan, T. E. Johnson, S. Prathapan, J. S. Lindsey, and D. F. Bocian. Investigation of electronic communication in multi-porphyrin light-harvesting arrays. *J. Am. Chem. Soc.*, 116: 10578–10592, 1994.
- [81] J. Seth, V. Palaniappan, R. W. Wagner, T. E. Johnson, J. S. Lindsey, and D. F. Bocian. Soluble synthetic multiporphyrin arrays. 3. Static spectroscopic and electrochemical probes of electronic communication. *J. Am. Chem. Soc.*, 118:11194–11207, 1996.
- [82] J. Pawlik, L. Gherghel, S. Karabunarliev, and M. Baumgarten. Characterization of reduced porphyrinatozinc(II) complexes by EPR/ENDOR/TRIPLE and optical absorption spectroscopy. *Chem. Phys.*, 221:121–133, 1997.

- [83] J. Li, A. Ambroise, S. I. Yang, J. R. Diers, J. Seth, C. R. Wack, D. F. Bocian, D. Holten, and J. S. Lindsey. Template-directed Synthesis, excited-state photodynamics, and electronic communication in a hexameric wheel of porphyrins. *J. Am. Chem. Soc.*, 121:8927–8940, 1999.
- [84] P. Thamyongkit, A. Z. Muresan, J. R. Diers, D. Holten, D. F. Bocian, and J. S. Lindsey. Meso- ^{13}C -labeled porphyrins for studies of ground-state hole transfer in multiporphyrin arrays. *J. Org. Chem.*, 72:5207–5217, 2007.
- [85] J. R. Norris, M. E. Druyan, and J. J. Katz. Electron nuclear double resonance of bacteriochlorophyll free radical *in vitro* and *in vivo*. *J. Am. Chem. Soc.*, 834:1680–1682, 1973.
- [86] R. F. Kelley, M. J. Tauber, and M. R. Wasielewski. Linker-controlled energy and charge transfer within chlorophyll trefoils. *Angew. Chem. Int. Ed.*, 45:7979–7982, 2006.
- [87] S. Van Doorslaer, G. A. Sierra, and A. Schweiger. Dead time-dependent line distortions in absolute-value electron spin echo envelope modulation spectra. *J. Magn. Reson.*, 136:152–158, 1999.
- [88] TURBOMOLE v6.1, A development of University of Karlsruhe and Forschungszentrum Karlsruhe GmbH, 2009.
- [89] F. Weigend and R. Ahlrichs. Balanced basis sets of split valence, triple zeta valence and quadruple zeta valence quality for H to Rn: Design and assessment of accuracy. *Phys. Chem. Chem. Phys.*, 7:3297–3305, 2005.
- [90] F. Weigend. A fully direct RI-HF algorithm: Implementation, optimised auxiliary basis sets, demonstration of accuracy and efficiency. *Phys. Chem. Chem. Phys.*, 4:4285–4291, 2002.
- [91] F. Neese. The ORCA program system. *WIREs Comput. Mol. Sci.*, 2:73–78, 2012.
- [92] V. Barone. Structure, Magnetic Properties and Reactivities of Open-Shell Species from Density Functional and Self-Consistent Hybrid Methods. In D. P. Chong, editor, *Recent Advances in Density Functional Methods, Part I*. World Scientific Publ. Co., Singapore, 1996.
- [93] V. Barone. Characterization of the potential energy surface of the HO_2 molecular system by a density functional approach. *J. Chem. Phys.*, 101:10666–10676, 1994.
- [94] V. Barone. Inclusion of Hartree–Fock exchange in density functional methods. Hyperfine structure of second row atoms and hydrides. *J. Chem. Phys.*, 101:6834–6838, 1994.
- [95] S. Stoll and A. Schweiger. EasySpin: Simulating cw ESR Spectra. In M. A. Hemminga and L. J. Berliner, editors, *ESR spectroscopy in Membrane Biophysics*, pages 299–321. Springer, New York, 2006.
- [96] J. Fajer, D. C. Borg, A. Forman, D. Dolphin, and R. H. Felton. π -cation radicals and dications of metalloporphyrins. *J. Am. Chem. Soc.*, 92:3451–3459, 1970.
- [97] J. Fajer and M. S. Davis. Electron Spin Resonance of Porphyrin π Cations and Anions. In D. Dolphin, editor, *Porphyrins. Vol. IV*, chapter 4, pages 197–256. Academic Press, New York, 1979.

- [98] J. R. Diers, M. Taniguchi, D. Holten, J. S. Lindsey, and D. F. Bocian. Probing the rate of hole transfer in oxidized porphyrin dyads using thallium hyperfine clocks. *J. Am. Chem. Soc.*, 132:12121–12132, 2010.
- [99] Y. S. Lebedev and S. N. Dobryakov. Analysis of the EPR spectra of free radicals. *Zh. Strukt. Khim.*, 8:838–853, 1967.
- [100] J. Tang, S. N. Dikshit, and J. R. Norris. Electron spin resonance line shapes for one- and two-dimensional random walk processes. *J. Chem. Phys.*, 103:2873–2881, 1995.
- [101] W. R. Scheidt. Systematics of the Stereochemistry of Porphyrins and Metalloporphyrins. In K. M. Kadish, K. M. Smith, and R. Guilard, editors, *The Porphyrin Handbook: Inorganic, organometallic and coordination chemistry (Vol. 3)*, chapter 16. Academic Press, San Diego, 2000.
- [102] J. Harmer, S. Van Doorslaer, I. A. Gromov, and A. Schweiger. Corrin nitrogens and remote dimethylbenzimidazole nitrogen interactions in Cob(II)alamin studied with HYSCORE at X- and Q-band. *Chem. Phys. Lett.*, 358:8–16, 2002.
- [103] A. Petrenko, K. Redding, and L. D. Kispert. The influence of the structure of the radical cation dimer pair of aromatic molecules on the principal values of a g-tensor: DFT predictions. *Chem. Phys. Lett.*, 406:327–331, 2005.
- [104] A. J. Stone. g-factors of aromatic free radicals. *Mol. Phys.*, 6:509–515, 1963.
- [105] A. J. Stone. Gauge invariance of the g-tensor. *Proc. R. Soc. A*, 271:424–434, 1963.
- [106] A. J. Stone. g-tensors of aromatic hydrocarbons. *Mol. Phys.*, 7:311–316, 1964.
- [107] M. Rohrer, P. Gast, K. Möbius, and T. F. Prisner. Anisotropic motion of semiquinones in photosynthetic reaction centers of *Rhodobacter sphaeroides* R26 and in frozen isopropanol solution as measured by pulsed high-field EPR at 95 GHz. *Chem. Phys. Lett.*, 259:523–530, 1996.
- [108] A. Schnegg, M. Fuhs, M. Rohrer, W. Lubitz, T. F. Prisner, and K. Möbius. Molecular Dynamics of Q_A^- and Q_B^- in photosynthetic bacterial reaction centers studied by pulsed High-Field EPR at 95 GHz. *J. Phys. Chem. B*, 106:9454–9462, 2002.
- [109] K. Möbius and A. Savitsky. Applications of High-Field EPR on Selected Proteins and their Model Systems. In K. Möbius and A. Savitsky, editors, *High-Field EPR Spectroscopy on Proteins and their Model Systems*, chapter 5, pages 206–363. The Royal Society of Chemistry, Cambridge, 2009.
- [110] O. W. Howarth. Electron Spin Resonance spectra of monomeric and dimeric cation radicals. *J. Chem. Phys.*, 52:6258–6267, 1970.
- [111] T. M. Wilson, M. J. Tauber, and M. R. Wasielewski. Toward an n-type molecular wire: electron hopping within linearly linked perylenediimide oligomers. *J. Am. Chem. Soc.*, 131:8952–8957, 2009.

- [112] J. J. Piet, P. N. Taylor, H. L. Anderson, A. Osuka, and J. M. Warman. Excitonic interactions in the singlet and triplet excited states of covalently linked zinc porphyrin dimers. *J. Am. Chem. Soc.*, 122:1749–1757, 2000.
- [113] P. J. Angiolillo, H. T. Uyeda, T. V. Duncan, and M. J. Therien. Impact of electronic asymmetry on photoexcited triplet-state spin distributions in conjugated porphyrin oligomers probed via EPR spectroscopy. *J. Phys. Chem. B*, 108:11893–11903, 2004.
- [114] H. Levanon and J. R. Norris. The photoexcited triplet state and photosynthesis. *Chem. Rev.*, 78:185–198, 1978.
- [115] R. H. Clarke, D. R. Hobart, and W. R. Leenstra. The triplet state of the chlorophyll dimer. *J. Am. Chem. Soc.*, 101:2416–2423, 1979.
- [116] T. K. Chandrashekar. Optical and electron spin resonance study of cation and cation-crown ether induced dimerization of tetrakis (4-sulfonatophenyl) porphyrin. *J. Phys. Chem.*, 88:4326–4332, 1984.
- [117] A. J. Hoff, S. A. Dikanov, A. V. Astashkin, and Y. D. Tsvetkov. Electron Spin Echo Envelope Modulation spectroscopy (ESEEM) of the radical cations of ^{14}N and ^{15}N bacteriochlorophyll *a*. *Chem. Phys. Lett.*, 118:40–47, 1985.
- [118] D. E. Budil and M. C. Thurnauer. The chlorophyll triplet state as a probe of structure and function in photosynthesis. *Biochim. Biophys. Acta*, 1057:1–41, 1991.
- [119] P. Jägermann, M. Plato, B. Von Maltzan, and K. Möbius. Time-resolved EPR study of exciton hopping in porphyrin dimers in their photoexcited triplet state. *Mol. Phys.*, 78:1057–1074, 1993.
- [120] M. Huber, A. A. Doubinskii, C. W. M. Kay, and K. Möbius. ENDOR on the triplet state of the primary electron donor in the photosynthetic bacterium *Rhodobacter sphaeroides* - One step forward in a still unfinished story. *Appl. Magn. Reson.*, 13:473–485, 1997.
- [121] F. Lendzian, R. Bittl, and W. Lubitz. Pulsed ENDOR of the photoexcited triplet states of bacteriochlorophyll *a* and of the primary donor P865 in reaction centers of *Rhodobacter sphaeroides* R-26. *Photosynth. Res.*, 55:189–197, 1998.
- [122] J. Andréasson, H. Zetterqvist, J. Kajanus, J. Mårtensson, and B. Albinsson. Efficient non-radiative deactivation and conformational flexibility of *meso*-diaryloctaalkylporphyrins in the excited triplet state. *J. Phys. Chem. A*, 104:9307–9314, 2000.
- [123] F. Lendzian, R. Bittl, A. Telfer, and W. Lubitz. Hyperfine structure of the photoexcited triplet state $^3\text{P680}$ in plant PS II reaction centres as determined by pulse ENDOR spectroscopy. *Biochim. Biophys. Acta*, 1605:35–46, 2003.
- [124] M. Bennati, K. Németh, P. R. Surján, and M. Mehring. Zero-field-splitting and π -electron spin densities in the lowest excited triplet state of oligothiophenes. *J. Chem. Phys.*, 105:4441–4447, 1996.

- [125] M. Bennati, A. Grupp, M. Mehring, and P. Bäuerle. Pulsed EPR spectroscopy of the photoexcited triplet states of thiophene oligomers in frozen solution. *J. Phys. Chem.*, 100:2849–2853, 1996.
- [126] P. J. Angiolillo, V. S.-Y. Lin, J. M. Vanderkooi, and M. J. Therien. EPR spectroscopy and photo-physics of the lowest photoactivated triplet state of a series of highly conjugated porphinato Zn arrays. *J. Am. Chem. Soc.*, 117:12514–12527, 1995.
- [127] P. J. Angiolillo, K. Susumu, H. T. Uyeda, V. S.-Y. Lin, R. Shediach, and M. J. Therien. Trends in triplet excitation delocalization in highly conjugated porphinato zinc(II) arrays probed by EPR spectroscopy. *Synth. Met.*, 116:247–253, 2001.
- [128] P. J. Angiolillo, J. Rawson, P. R. Frail, and M. J. Therien. The evolution of spin distribution in the photoexcited triplet state of ethyne-elaborated porphyrins. *Chem. Commun.*, 49:9722–9724, 2013.
- [129] C. Riplinger, J. P. Y. Kao, G. M. Rosen, V. Kathirvelu, G. R. Eaton, S. S. Eaton, A. Kutateladze, and F. Neese. Interaction of radical pairs through-bond and through-space: scope and limitations of the point-dipole approximation in electron paramagnetic resonance spectroscopy. *J. Am. Chem. Soc.*, 131:10092–10106, 2009.
- [130] S. Siegel and H. S. Judeikis. A magnetophotoselection study of the polarizations of the absorption bands of some structurally related hydrocarbons and heterocyclic molecules. *J. Phys. Chem.*, 70:2205–2211, 1966.
- [131] M. C. Thurnauer and J. R. Norris. The ordering of the zero field triplet spin sublevels in the chlorophylls. A magnetophotoselection study. *Chem. Phys. Lett.*, 47:100–105, 1977.
- [132] D. J. Singel, W. A. J. A. van der Poel, J. Schmidt, J. H. van der Waals, and R. de Beer. Complete determination of ^{14}N hyperfine and quadrupole interactions in the metastable triplet state of free-base porphin via Electron Spin Echo Envelope Modulation. *J. Chem. Phys.*, 81:5453–5461, 1984.
- [133] T. Petrenko, S. Kossmann, and F. Neese. Efficient time-dependent density functional theory approximations for hybrid density functionals: analytical gradients and parallelization. *J. Chem. Phys.*, 134:054116, 2011.
- [134] O. Gonen and H. Levanon. Time-resolved EPR spectroscopy of electron spin polarized ZnTPP triplets oriented in a liquid crystal. *J. Phys. Chem.*, 89:1637–1643, 1985.
- [135] M. C. Thurnauer. ESR study of the photoexcited triplet state in photosynthetic bacteria. *Rev. Chem. Intermed.*, 3:197–230, 1979.
- [136] R. L. Ake and M. Gouterman. Porphyrins XIV. Theory for the luminescent state in VO, Co, Cu complexes. *Theor. Chim. Acta*, 42:20–42, 1969.
- [137] W. G. van Dorp, W. H. Schoemaker, M. Soma, and J. H. van der Waals. The lowest triplet state of free base porphin. *Mol. Phys.*, 30:1701–1721, 1975.

-
- [138] R. H. Clarke, R. E. Connors, T. J. Schaafsma, J. F. Kleibeuker, and R. J. Platenkamp. The triplet state of chlorophylls. *J. Am. Chem. Soc.*, 98:3674–3677, 1976.
- [139] F. Metz, S. Friedrich, and G. Hohlneicher. What is the leading mechanism for the nonradiative decay of the lowest triplet state of aromatic hydrocarbons? *Chem. Phys. Lett.*, 16:353–358, 1972.
- [140] M. K. Kuimova, M. Hoffmann, M. U. Winters, M. Eng, M. Balaz, I. P. Clark, H. A. Collins, S. M. Tavender, C. J. Wilson, B. Albinsson, H. L. Anderson, A. W. Parker, and D. Phillips. Determination of the triplet state energies of a series of conjugated porphyrin oligomers. *Photochem. Photobiol. Sci.*, 6:675–682, 2007.
- [141] J. Visser and E. J. J. Groenen. The fine-structure in the lowest triplet state of C₆₀ and C₇₀. *Chem. Phys. Lett.*, 356:43–48, 2002.
- [142] M. van Gastel. The effect of spin polarization on zero-field splitting parameters in paramagnetic π -electron molecules. *J. Chem. Phys.*, 131:124111, 2009.
- [143] M. van Gastel. Zero-field splitting of the lowest excited triplet states of C₆₀ and C₇₀ and benzene. *J. Phys. Chem. A*, 114:10864–10870, 2010.
- [144] O. Loboda, B. Minaev, O. Vahtras, B. Schimmelpfennig, H. Agren, K. Ruud, and D. Jonsson. *Ab initio* calculations of zero-field splitting parameters in linear polyacenes. *Chem. Phys.*, 286:127–137, 2003.
- [145] M. A. El-Sayed, D. S. Tinti, and E. M. Yee. Conservation of spin direction and production of spin alignment in Triplet-Triplet Energy Transfer. *J. Chem. Phys.*, 51:5721–5723, 1969.
- [146] M. A. El-Sayed. Optical pumping of the lowest triplet state and multiple resonance optical techniques in zero field. *J. Chem. Phys.*, 54:680–691, 1971.
- [147] K. Akiyama, S. Tero-Kubota, T. Ikoma, and Y. Ikegami. Spin polarization conservation during intramolecular Triplet-Triplet Energy Transfer studied by time-Resolved EPR spectroscopy. *J. Am. Chem. Soc.*, 116:5324–5327, 1994.
- [148] S. Perun, J. Tatchen, and C. M. Marian. Singlet and triplet excited states and intersystem crossing in free-base porphyrin: TDDFT and DFT/MRCI study. *Chem. Phys. Chem.*, 9:282–292, 2008.
- [149] D. Antheunis, J. Schmidt, and J. van der Waals. Spin-forbidden radiationless processes in iso-electronic molecules: Anthracene, acridine and phenazine. *Mol. Phys.*, 27:1521–1541, 1974.
- [150] M. C. Thurnauer and J. R. Norris. Magnetophotoselection applied to the triplet state observed by EPR in photosynthetic bacteria. *Biochem. Biophys. Res. Commun.*, 73:501–506, 1976.
- [151] H. A. Frank, J. Bolt, R. Friesner, and K. Sauer. Magnetophotoselection of the triplet state of reaction centers from *Rhodospseudomonas sphaeroides* R-26. *Biochim. Biophys. Acta*, 547:502–511, 1979.

- [152] M. Gouterman. Spectra of porphyrins. *J. Mol. Spectrosc.*, 6:138–163, 1961.
- [153] M. Gouterman, G. H. Wagnière, and L. C. Snyder. Spectra of porphyrins - Part II. Four Orbital Model. *J. Mol. Spectrosc.*, 11:108–127, 1963.
- [154] P. N. Taylor, J. Huuskonen, R. T. Aplin, H. L. Anderson, G. Rumbles, and E. Williams. Conjugated porphyrin oligomers from monomer to hexamer. *Chem. Commun.*, 8:909–910, 1998.
- [155] M. Drobizhev, Y. Stepanenko, Y. Dzenis, A. Karotki, A. Rebane, P. N. Taylor, and H. L. Anderson. Extremely strong Near-IR two-photon absorption in conjugated porphyrin dimers : Quantitative description with three-essential-states model. *J. Phys. Chem. B*, 109:7223–7236, 2005.
- [156] C. W. M. Kay. The electronic structure of the photoexcited triplet state of free-base tetraphenylporphyrin by time-resolved electron-nuclear double resonance and density functional theory. *J. Am. Chem. Soc.*, 125:13861–13867, 2003.
- [157] M. Ernzerhof. Hybrid Methods : Combining Density Functional and Wavefunction Theory. In D. Joubert, editor, *Density Functionals: Theory and Applications*, chapter 3, pages 60–90. Springer Verlag, 1998.
- [158] V. Polo, E. Kraka, and D. Cremer. Electron correlation and the self-interaction error of density functional theory. *Mol. Phys.*, 100:1771–1790, 2002.
- [159] M. K. Bowman and J. R. Norris. Electron spin-echo detection of nuclear quadrupole resonance in photoexcited triplets. *J. Chem. Phys.*, 77:731–734, 1982.
- [160] G. M. Muha. Exact solution of the NQR $I=1$ eigenvalue problem for an arbitrary asymmetry parameter and Zeeman field strength and orientation. *J. Chem. Phys.*, 73:4139–4140, 1980.
- [161] G. M. Muha. The Zeeman effect in spin=1 systems. *J. Magn. Reson.*, 49:431–443, 1982.
- [162] S. Van Doorslaer, G. Jeschke, B. Epel, D. Goldfarb, R.-A. Eichel, B. Kräutler, and A. Schweiger. Axial solvent coordination in "base-off" cob(II)alamin and related Co(II)-corrinates revealed by 2D-EPR. *J. Am. Chem. Soc.*, 125:5915–5927, 2003.
- [163] J. K. M. Sanders, N. Bampos, Z. Clyde-Watson, S. L. Darling, J. C. Hawley, H.-J. Kim, C. C. Mak, and S. J. Webb. Axial Coordination Chemistry of Metalloporphyrins. In K. M. Kadish, K. M. Smith, and R. Guilard, editors, *The Porphyrin Handbook: Inorganic, organometallic and coordination chemistry* (Vol. 3), chapter 15, pages 3–41. Academic Press, 2000.
- [164] M. U. Winters, J. Kärnbratt, H. E. Blades, C. J. Wilson, M. J. Frampton, H. L. Anderson, and B. Albinsson. Control of electron transfer in a conjugated porphyrin dimer by selective excitation of planar and perpendicular conformers. *Chem. Eur. J.*, 13:7385–7394, 2007.
- [165] S. Grimme and M. Parac. Substantial errors from time-dependent density functional theory for the calculation of excited states of large π systems. *Chem. Phys. Chem.*, 4:292–295, 2003.
- [166] J. Kärnbratt, M. Gilbert, J. K. Sprafke, H. L. Anderson, and B. Albinsson. Self-assembly of linear porphyrin oligomers into well-defined aggregates. *J. Phys. Chem. C*, 116:19630–19635, 2012.

- [167] J. Pipek and P. G. Mezey. A fast intrinsic localization procedure applicable for *ab initio* and semiempirical linear combination of atomic orbital wave functions. *J. Chem. Phys.*, 90:4916–4926, 1989.
- [168] C. W. M. Kay, G. Elger, and K. Möbius. The photoexcited triplet state of free-base porphycene : a time-resolved EPR and electron spin echo investigation. *Phys. Chem. Chem. Phys.*, 1:3999–4002, 1999.
- [169] J. E. Banham, C. M. Baker, S. Ceola, I. J. Day, G. H. Grant, E. J. J. Groenen, C. T. Rodgers, G. Jeschke, and C. R. Timmel. Distance measurements in the borderline region of applicability of CW EPR and DEER: a model study on a homologous series of spin-labelled peptides. *J. Magn. Reson.*, 191:202–218, 2008.
- [170] R. Ward, A. Bowman, E. Sozudogru, H. El-Mkami, T. Owen-Hughes, and D. G. Norman. EPR distance measurements in deuterated proteins. *J. Magn. Reson.*, 207:164–167, 2010.
- [171] L. J. Berliner, S. S. Eaton, and G. R. Eaton, editors. *Biological Magnetic Resonance, Volume 19: Distance Measurements in Biological Systems by EPR*. Kluwer Academic/Plenum Publishers, New York, 2000.
- [172] C. R. Timmel and J. Harmer, editors. *Structure and Bonding 152: Structural Information from Spin-Labels and Intrinsic Paramagnetic Centres in the Biosciences*. Springer Verlag, Berlin, Heidelberg, 2014.
- [173] S. A. Shelke and S. T. Sigurdsson. Site-Directed Nitroxide Spin Labeling of Biopolymers. In C. R. Timmel and J. Harmer, editors, *Structural Information from Spin-Labels Intrinsic Paramagnetic Centres in the Biosciences*, chapter 3, pages 121–162. Springer, Biological Magnetic Resonance, Berlin, Heidelberg, 2014.
- [174] G. W. Reginsson, N. C. Kunjir, S. T. Sigurdsson, and O. Schiemann. Trityl radicals: Spin labels for nanometer-distance measurements. *Chem. Eur. J.*, 18:13580–13584, 2012.
- [175] D. Goldfarb. Metal-Based Spin Labeling for Distance Determination. In C. R. Timmel and J. Harmer, editors, *Structural Information from Spin-Labels Intrinsic Paramagnetic Centres in the Biosciences*, chapter 4, pages 163–204. Springer, Structure & Bonding, Berlin, Heidelberg, 2014.
- [176] A. M. Bowen, C. E. Tait, C. R. Timmel, and J. Harmer. Orientation Selective DEER Using Rigid Spin Labels, Cofactors, Metals, and Clusters. In C. R. Timmel and J. Harmer, editors, *Structural Information from Spin-Labels Intrinsic Paramagnetic Centres in the Biosciences*. Springer, Structure & Bonding, 2014.
- [177] A. M. Raitsimring, C. Gunanathan, A. Potapov, I. Efremenko, J. M. L. Martin, D. Milstein, and D. Goldfarb. Gd^{3+} complexes as potential spin labels for high field pulsed EPR distance measurements. *J. Am. Chem. Soc.*, 129:14138–14139, 2007.

- [178] P. Lueders, G. Jeschke, and M. Yulikov. Double Electron-Electron Resonance measured between Gd^{3+} ions and nitroxide radicals. *J. Phys. Chem. Lett.*, 2:604–609, 2011.
- [179] M. D. Rabenstein and Y. K. Shin. Determination of the distance between two spin labels attached to a macromolecule. *Proc. Natl. Acad. Sci.*, 92:8239–8243, 1995.
- [180] A. Abragam. *The Principles of Nuclear Magnetism*. Oxford University Press, Oxford, 1961.
- [181] J. H. Van Vleck. The dipolar broadening of magnetic resonance lines in crystals. *Phys. Rev.*, 74: 1168–1183, 1948.
- [182] H.-J. Steinhoff, N. Radzwill, W. Thevis, V. Lenz, D. Brandenburg, A. Antson, G. Dodson, and A. Wollmer. Determination of interspin distances between spin labels attached to insulin: comparison of electron paramagnetic resonance data with the X-ray structure. *Biophys. J.*, 73: 3287–3298, 1997.
- [183] G. Jeschke. Determination of the nanostructure of polymer materials by Electron Paramagnetic Resonance spectroscopy. *Macromol. Rapid Commun.*, 23:227–246, 2002.
- [184] P. P. Borbat and J. H. Freed. Pulse Dipolar Electron Spin Resonance: Distance Measurements. In C. R. Timmel and J. Harmer, editors, *Structural Information from Spin-Labels Intrinsic Paramagnetic Centres in the Biosciences*, chapter 1, pages 1–82. Springer, Structure & Bonding, Berlin, Heidelberg, 2014.
- [185] G. Jeschke, M. Pannier, A. Godt, and H. W. Spiess. Dipolar spectroscopy and spin alignment in electron paramagnetic resonance. *Chem. Phys. Lett.*, 331:243–252, 2000.
- [186] G. Jeschke, V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. E. Banham, C. R. Timmel, D. Hilger, and H. Jung. DeerAnalysis2006 - a comprehensive software package for analyzing pulsed ELDOR data. *Appl. Magn. Reson.*, 30:473–498, 2006.
- [187] A. D. Milov, K. M. Salikhov, and M. D. Shirov. Application of the double resonance method to electron spin echo in a study of the spatial distribution of paramagnetic centers in solids. *Phys. Solid State*, 23:565–569, 1981.
- [188] A. D. Milov, A. B. Ponomarev, and Y. D. Tsvetkov. Electron-electron double resonance in electron spin echo: Model biradical systems and the sensitized photolysis of decalin. *Chem. Phys. Lett.*, 110:67–72, 1984.
- [189] R. G. Larsen and D. J. Singel. Double electron–electron resonance spin–echo modulation: Spectroscopic measurement of electron spin pair separations in orientationally disordered solids. *J. Chem. Phys.*, 98:5134–5146, 1993.
- [190] M. Pannier, S. Veit, A. Godt, G. Jeschke, and H.-W. Spiess. Dead-time free measurement of dipole-dipole interactions between electron spins. *J. Magn. Reson.*, 142:331–340, 2000.

-
- [191] G. Jeschke. Interpretation of Dipolar EPR Data in Terms of Protein Structure. In C. R. Timmel and J. Harmer, editors, *Structural Information from Spin-Labels Intrinsic Paramagnetic Centres in the Biosciences*, chapter 2, pages 83–120. Springer, Structure & Bonding, Berlin, Heidelberg, 2014.
- [192] G. Jeschke and Y. Polyhach. Distance measurements on spin-labelled biomacromolecules by pulsed electron paramagnetic resonance. *Phys. Chem. Chem. Phys.*, 9:1895–1910, 2007.
- [193] J. E. Lovett, B. W. Lovett, and J. Harmer. DEER-Stitch: Combining three- and four-pulse DEER measurements for high sensitivity, deadtime free data. *J. Magn. Reson.*, 223:98–106, 2012.
- [194] A. G. Maryasov and Y. D. Tsvetkov. Formation of the pulsed electron-electron double resonance signal in the case of a finite amplitude of microwave fields. *Appl. Magn. Reson.*, 18:583–605, 2000.
- [195] G. Jeschke, A. Koch, U. Jonas, and A. Godt. Direct conversion of EPR dipolar time evolution data to distance distributions. *J. Magn. Reson.*, 155:72–82, 2002.
- [196] G. Jeschke, G. Panek, A. Godt, A. Bender, and H. Paulsen. Data analysis procedures for pulse ELDOR measurements of broad distance distributions. *Appl. Magn. Reson.*, 26:223–244, 2004.
- [197] M. K. Bowman, A. G. Maryasov, N. Kim, and V. J. DeRose. Visualization of distance distribution from pulsed double electron-electron resonance data. *Appl. Magn. Reson.*, 26:23–39, 2004.
- [198] Y.-W. Chiang, P. P. Borbat, and J. H. Freed. The determination of pair distance distributions by pulsed ESR using Tikhonov regularization. *J. Magn. Reson.*, 172:279–295, 2005.
- [199] Y. W. Chiang, P. P. Borbat, and J. H. Freed. Maximum entropy: A complement to Tikhonov regularization for determination of pair distance distributions by pulsed ESR. *J. Magn. Reson.*, 177:184–196, 2005.
- [200] G. Jeschke. Conformational dynamics and distribution of nitroxide spin labels. *Prog. Nucl. Magn. Reson. Spectrosc.*, 72:42–60, 2013.
- [201] E. Bordignon and H.-J. Steinhoff. Membrane protein structure and dynamics studied by site-directed spin labeling ESR. In M. A. Hemminga and L. J. Berliner, editors, *ESR spectroscopy in Membrane Biophysics*, chapter 5, pages 129–164. Springer Science, New York, 2007.
- [202] H. S. Mchaourab, M. A. Lietzow, K. Hideg, and W. L. Hubbell. Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry*, 35:7692–7704, 1996.
- [203] J. P. Barnes, Z. Liang, H. S. Mchaourab, J. H. Freed, and W. L. Hubbell. A multifrequency electron spin resonance study of T4 lysozyme dynamics. *Biophys. J.*, 76:3298–3306, 1999.
- [204] T. M. Casey, Z. Liu, J. M. Esquiaqui, N. L. Pirman, E. Milshteyn, and G. E. Fanucci. Continuous wave W- and D-band EPR spectroscopy offer "sweet-spots" for characterizing conformational changes and dynamics in intrinsically disordered proteins. *Biochem. Biophys. Res. Commun.*, 450:723–728, 2014.

- [205] J. H. Freed. Theory of slow tumbling ESR spectra for nitroxides. In L. J. Berliner, editor, *Spin Labeling: Theory and Applications*, chapter 3, pages 53–132. Academic Press, New York, 2001.
- [206] S. Saxena and J. H. Freed. Two-dimensional electron spin resonance and slow motions. *J. Phys. Chem. A*, 101:7998–8008, 1997.
- [207] P. P. Borbat, A. J. Costa-Filho, K. A. Earle, J. K. Moscicki, and J. H. Freed. Electron spin resonance in studies of membranes and proteins. *Science*, 291:266–269, 2001.
- [208] Z. Liang, Y. Lou, J. H. Freed, L. Columbus, and W. L. Hubbell. A multifrequency Electron Spin Resonance study of T4 lysozyme dynamics using the slowly relaxing local structure model. *J. Phys. Chem. B*, 108:17649–17659, 2004.
- [209] W. L. Hubbell, D. S. Cafiso, and C. Altenbach. Identifying conformational changes with site-directed spin labeling. *Nat. Struct. Biol.*, 7:735–739, 2000.
- [210] W. L. Hubbell, H. S. Mchaourab, C. Altenbach, and M. A. Lietzow. Watching proteins move using site-directed spin labeling. *Structure*, 4:779–783, 1996.
- [211] J. M. Isas, R. Langen, H. T. Haigler, and W. L. Hubbell. Structure and dynamics of a helical hairpin and loop region in annexin 12: a site-directed spin labeling study. *Biochemistry*, 41: 1464–1473, 2002.
- [212] W. Doster. The protein-solvent glass transition. *Biochim. Biophys. Acta*, 1804:3–14, 2010.
- [213] E. J. Hustedt, R. A. Stein, L. Sethaphong, S. Brandon, Z. Zhou, and S. C. Desensi. Dipolar coupling between nitroxide spin labels: the development and application of a tether-in-a-cone model. *Biophys. J.*, 90:340–356, 2006.
- [214] Y. Polyhach, E. Bordignon, and G. Jeschke. Rotamer libraries of spin labelled cysteines for protein studies. *Phys. Chem. Chem. Phys.*, 13:2356–2366, 2011.
- [215] G. Hagelueken, R. Ward, J. H. Naismith, and O. Schiemann. MtsslWizard: *In silico* spin-labeling and generation of distance distributions in PyMOL. *Appl. Magn. Reson.*, 42:377–391, 2012.
- [216] G. Jeschke and H.-W. Spiess. Distance measurements in solid-state NMR and EPR spectroscopy. *Lect. Notes Phys.*, 63:21–63, 2006.
- [217] B. E. Bode, D. Margraf, J. Plackmeyer, G. Dürner, T. F. Prisner, and O. Schiemann. Counting the monomers in nanometer-sized oligomers by pulsed electron-electron double resonance. *J. Am. Chem. Soc.*, 129:6736–6745, 2007.
- [218] G. Jeschke, M. Sajid, M. Schulte, and A. Godt. Three-spin correlations in double electron-electron resonance. *Phys. Chem. Chem. Phys.*, 11:6580–6591, 2009.
- [219] D. Hilger, H. Jung, E. Padan, C. Wegener, K.-P. Vogel, H.-J. Steinhoff, and G. Jeschke. Assessing oligomerization of membrane proteins by four-pulse DEER: pH-dependent dimerization of NhaA Na⁺/H⁺ antiporter of *E. coli*. *Biophys. J.*, 89:1328–1338, 2005.

- [220] M. J. N. Junk, H.-W. Spiess, and D. Hinderberger. DEER in biological multispin-systems: a case study on the fatty acid binding to human serum albumin. *J. Magn. Reson.*, 210:210–217, 2011.
- [221] G. Jeschke. DEER distance measurements on proteins. *Annu. Rev. Phys. Chem.*, 63:419–446, 2012.
- [222] T. von Hagens, Y. Polyhach, M. Sajid, A. Godt, and G. Jeschke. Suppression of ghost distances in multiple-spin double electron-electron resonance. *Phys. Chem. Chem. Phys.*, 15:5854–5866, 2013.
- [223] A. Giannoulis, R. Ward, E. Branigan, J. H. Naismith, and B. E. Bode. PELDOR in rotationally symmetric homo-oligomers. *Mol. Phys.*, 111:2845–2854, 2013.
- [224] D. Margraf, B. E. Bode, A. Marko, O. Schiemann, and T. F. Prisner. Conformational flexibility of nitroxide biradicals determined by X-band PELDOR experiments. *Mol. Phys.*, 105:2153–2160, 2007.
- [225] A. Marko, D. Margraf, P. Cekan, S. T. Sigurdsson, O. Schiemann, and T. F. Prisner. Analytical method to determine the orientation of rigid spin labels in DNA. *Phys. Rev. E*, 81:1–9, 2010.
- [226] Y. Polyhach, A. Godt, C. Bauer, and G. Jeschke. Spin pair geometry revealed by high-field DEER in the presence of conformational distributions. *J. Magn. Reson.*, 185:118–129, 2007.
- [227] J. E. Lovett, A. M. Bowen, C. R. Timmel, M. W. Jones, J. R. Dilworth, D. Caprotti, S. G. Bell, L.-L. Wong, and J. Harmer. Structural information from orientationally selective DEER spectroscopy. *Phys. Chem. Chem. Phys.*, 11:6840–6848, 2009.
- [228] A. Marko, D. Margraf, H. Yu, Y. Mu, G. Stock, and T. F. Prisner. Molecular orientation studies by pulsed electron-electron double resonance experiments. *J. Chem. Phys.*, 130:064102, 2009.
- [229] A. Savitsky, A. A. Dubinskii, H. Zimmermann, W. Lubitz, and K. Möbius. High-field dipolar electron paramagnetic resonance (EPR) spectroscopy of nitroxide biradicals for determining three-dimensional structures of biomacromolecules in disordered solids. *J. Phys. Chem. B*, 115:11950–11963, 2011.
- [230] J. A. B. Abdalla, A. M. Bowen, S. G. Bell, L.-L. Wong, C. R. Timmel, and J. Harmer. Characterisation of the paramagnetic $[2\text{Fe-2S}]^+$ centre in palustrisredoxin-B (PuxB) from *Rhodospseudomonas palustris* CGA009: g-matrix determination and spin coupling analysis. *Phys. Chem. Chem. Phys.*, 14:6526–6537, 2012.
- [231] T. Palmer and B. C. Berks. The twin-arginine translocation (Tat) protein export pathway. *Nat. Rev. Microbiol.*, 10:483–496, 2012.
- [232] U. Gohlke, L. Pullan, C. A. McDevitt, I. Porcelli, E. de Leeuw, T. Palmer, H. R. Saibil, and B. C. Berks. The TatA component of the twin-arginine protein transport system forms channel complexes of variable diameter. *Proc. Natl. Acad. Sci.*, 102:10482–10486, 2005.

- [233] F. Rodriguez, S. L. Rouse, C. E. Tait, J. Harmer, A. De Riso, C. R. Timmel, M. S. P. Sansom, B. C. Berks, and J. R. Schnell. Structural model for the protein-translocating element of the twin-arginine transport system. *Proc. Natl. Acad. Sci.*, 110:E1092–1101, 2013.
- [234] Y. Hu, E. Zhao, H. Li, B. Xia, and C. Jin. Solution NMR structure of the TatA component of the twin-arginine protein transport system from gram-positive bacterium *Bacillus subtilis*. *J. Am. Chem. Soc.*, 132:15942–15944, 2010.
- [235] N. P. Greene, I. Porcelli, G. Buchanan, M. G. Hicks, S. M. Schermann, T. Palmer, and B. C. Berks. Cysteine scanning mutagenesis and disulfide mapping studies of the TatA component of the bacterial twin arginine translocase. *J. Biol. Chem.*, 282:23937–23945, 2007.
- [236] M. C. Leake, N. P. Greene, R. M. Godun, T. Granjon, G. Buchanan, S. Chen, R. M. Berry, T. Palmer, and B. C. Berks. Variable stoichiometry of the TatA component of the twin-arginine protein transport system observed by *in vivo* single-molecule imaging. *Proc. Natl. Acad. Sci.*, 105: 15376–15381, 2008.
- [237] G. F. White, S. M. Schermann, J. Bradley, A. Roberts, N. P. Greene, B. C. Berks, and A. J. Thomson. Subunit organization in the TatA complex of the twin arginine protein translocase: a site-directed EPR spin labeling study. *J. Biol. Chem.*, 285:2294–2301, 2010.
- [238] C. S. Chan, M. R. Zlomislic, D. P. Tieleman, and R. J. Turner. The TatA subunit of *Escherichia coli* twin-arginine translocase has an N-in topology. *Biochemistry*, 46:7396–7404, 2007.
- [239] C. S. Chan, E. F. Haney, H. J. Vogel, and R. J. Turner. Towards understanding the Tat translocation mechanism through structural and biophysical studies of the amphipathic region of TatA from *Escherichia coli*. *Biochim. Biophys. Acta*, 1808:2289–2296, 2011.
- [240] B. E. Bode, R. Dastvan, and T. F. Prisner. Pulsed electron-electron double resonance (PELDOR) distance measurements in detergent micelles. *J. Magn. Reson.*, 211:11–17, 2011.
- [241] K. Gouffi, F. Gérard, C.-L. Santini, and L.-F. Wu. Dual topology of the *Escherichia coli* TatA protein. *J. Biol. Chem.*, 279:11608–11615, 2004.
- [242] M. R. Fleissner, M. D. Bridges, E. K. Brooks, D. Cascio, T. Kálai, K. Hideg, and W. L. Hubbell. Structure and dynamics of a conformationally constrained nitroxide side chain and applications in EPR spectroscopy. *Proc. Natl. Acad. Sci.*, 108:16241–16246, 2011.
- [243] J. E. Banham. *Exploring Long Range Structure in Chemical and Biological Systems Using Electron Paramagnetic Resonance*. phdthesis, University of Oxford, 2006.
- [244] A. M. Bowen. *Magnetic Resonance Studies of Proteins and Model Systems*. phdthesis, University of Oxford, 2013.
- [245] A. M. Bowen, N. Hoskins, E. O. D. Johnson, S. G. Bell, F. Mercuri, C. R. Timmel, J. Harmer, and L.-L. Wong. A model of a ferredoxin-P450 complex in frozen solution from orientation-dependent DEER. *Manuscript in preparation*, 2015.

- [246] F. P. Guengerich. Enzymatic oxidation of xenobiotic chemicals. *Crit. Rev. Biochem. Mol. Biol.*, 25: 97–153, 1990.
- [247] D. Werck-Reichhart and R. Feyereisen. Protein family review Cytochromes P450 : a success story. *Genome Biol.*, 1:1–9, 2000.
- [248] F. P. Guengerich. Cytochrome P450 and chemical toxicology. *Chem. Res. Toxicol.*, 21:70–83, 2008.
- [249] W. Yang, S. G. Bell, H. Wang, W. Zhou, N. Hoskins, A. Dale, M. Bartlam, L.-L. Wong, and Z. Rao. Molecular characterization of a class I P450 electron transfer system from *Novosphingobium aromaticivorans* DSM12444. *J. Biol. Chem.*, 285:27372–27384, 2010.
- [250] I. F. Sevrioukova, H. Li, H. Zhang, J. A. Peterson, and T. L. Poulos. Structure of a cytochrome P450–redox partner electron-transfer complex. *Proc. Natl. Acad. Sci.*, 96:1863–1868, 1999.
- [251] N. Strushkevich, F. MacKenzie, T. Cherkasova, I. Grabovec, S. Usanov, and H.-W. Park. Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proc. Natl. Acad. Sci.*, 108:10139–10143, 2011.
- [252] S. Tripathi, H. Li, and T. L. Poulos. Structural basis for effector control and redox partner recognition in cytochrome P450. *Science*, 340:1227–1230, 2013.
- [253] Y. Hiruma, M. A. S. Hass, Y. Kikui, W. M. Liu, B. Ölmez, S. P. Skinner, A. Blok, A. Kloosterman, H. Koteishi, F. Löhr, H. Schwalbe, M. Nojiri, and M. Ubbink. The structure of the cytochrome P450cam-putidaredoxin complex determined by paramagnetic NMR spectroscopy and crystallography. *J. Mol. Biol.*, 425:4353–4365, 2013.
- [254] T. C. Pochapsky, T. A. Lyons, and S. Kazanis. A structure-based model for cytochrome P450 cam-putidaredoxin interactions. *Biochimie*, 78:723–733, 1996.
- [255] H. Shimada, S. Nagano, H. Hori, and Y. Ishimura. Putidaredoxin-cytochrome P450cam interaction. *J. Inorg. Biochem.*, 83:255–260, 2001.
- [256] M. Unno, J. F. Christian, T. Sjodin, D. E. Benson, I. D. G. Macdonald, S. G. Sligar, and P. M. Champion. Complex formation of cytochrome P450cam with putidaredoxin. *J. Biol. Chem.*, 277:2547–2553, 2001.
- [257] S. Nagano, H. Shimada, A. Tarumi, T. Hishiki, Y. Kimata-Arigo, T. Egawa, M. Suematsu, S.-Y. Park, S.-i. Adachi, Y. Shiro, and Y. Ishimura. Infrared spectroscopic and mutational studies on putidaredoxin-induced conformational changes in ferrous CO-P450cam. *Biochemistry*, 42: 14507–14514, 2003.
- [258] T. Tosha, S. Yoshioka, S. Takahashi, K. Ishimori, H. Shimada, and I. Morishima. NMR study on the structural changes of cytochrome P450cam upon the complex formation with putidaredoxin. *J. Biol. Chem.*, 278:39809–39821, 2003.

- [259] W. Zhang, S. S. Pochapsky, T. C. Pochapsky, and N. U. Jain. Solution NMR structure of putidaredoxin-cytochrome P450cam complex via a combined residual dipolar coupling-spin labeling approach suggests a role for Trp106 of putidaredoxin in complex formation. *J. Mol. Biol.*, 384:349–363, 2008.
- [260] W. K. Myers, Y.-T. Lee, R. D. Britt, and D. B. Goodin. The conformation of P450cam in complex with putidaredoxin is dependent on oxidation state. *J. Am. Chem. Soc.*, 135:11732–11735, 2013.
- [261] S. G. Bell and L.-L. Wong. P450 enzymes from the bacterium *Novosphingobium aromaticivorans*. *Biochem. Biophys. Res. Commun.*, 360:666–672, 2007.
- [262] S. G. Bell, F. Xu, E. O. D. Johnson, I. M. Forward, M. Bartlam, Z. Rao, and L.-L. Wong. Protein recognition in ferredoxin-P450 electron transfer in the class I CYP199A2 system from *Rhodopseudomonas palustris*. *J. Biol. Inorg. Chem.*, 15:315–328, 2010.
- [263] N. A. Baker, D. Sept, S. Joseph, M. J. Holst, and J. A. McCammon. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci.*, 98:10037–10041, 2001.
- [264] L. Noodleman, C. Y. Y. Peng, D. A. A. Case, and J.-M. Mouesca. Orbital interactions, electron delocalization and spin coupling in iron-sulfur clusters. *Coord. Chem. Rev.*, 144:199–244, 1995.
- [265] J. Fritz, R. Anderson, J. Fee, G. Palmer, R. H. Sands, J. C. Tsibris, I. C. Gunsalus, W. H. Orme-Johnson, and H. Beinert. The iron electron-nuclear double resonance (ENDOR) of two-iron ferredoxins from spinach, parsley, pig adrenal cortex and *Pseudomonas putida*. *Biochim. Biophys. Acta*, 253:110–133, 1971.
- [266] Y. Polyhach and G. Jeschke. Prediction of favourable sites for spin labelling of proteins. *Spectroscopy*, 24:651–659, 2010.
- [267] D. Schneidman-Duhovny, Y. Inbar, R. Nussinov, and H. J. Wolfson. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res.*, 33:W363–W367, 2005.
- [268] N. Andrusier, R. Nussinov, and H. J. Wolfson. FireDock: fast interaction refinement in molecular docking. *Proteins: Struct., Funct., Bioinf.*, 69:139–159, 2007.
- [269] E. Mashiach, D. Schneidman-Duhovny, N. Andrusier, R. Nussinov, and H. J. Wolfson. FireDock: a web server for fast interaction refinement in molecular docking. *Nucleic Acids Res.*, 36:W229–W232, 2008.
- [270] D. Batabyal and T. L. Poulos. Crystal structures and functional characterization of wild-type CYP101D1 and its active site mutants. *Biochemistry*, 52:8898–8906, 2013.
- [271] S. Stoll, Y.-T. Lee, and M. Zhang. Double electron–electron resonance shows cytochrome P450cam undergoes a conformational change in solution upon binding substrate. *Proc. Natl. Acad. Sci.*, 109:12888–12893, 2012.
- [272] D. F. V. Lewis and P. Hlavica. Interactions between redox partners in various cytochrome P450 systems: Functional and structural aspects. *Biochim. Biophys. Acta*, 1460:353–374, 2000.

- [273] R. A. Marcus and N. Sutin. Electron transfers in chemistry and biology. *Biochim. Biophys. Acta*, 811:265–322, 1985.
- [274] J. N. Onuchic, D. N. Beratan, J. R. Winkler, and H. B. Gray. Pathway analysis of protein electron-transfer reactions. *Annu. Rev. Biophys. Biomol. Struct.*, 21:349–377, 1992.
- [275] H. B. Gray and J. R. Winkler. Long-range electron transfer. *Proc. Natl. Acad. Sci.*, 102:3534–3539, 2005.
- [276] I. V. Kurnikov. HARLEM molecular modeling package, 2000.
- [277] S. G. Bell, A. Dale, N. H. Rees, and L.-L. Wong. A cytochrome P450 class I electron transfer system from *Novosphingobium aromaticivorans*. *Appl. Microbiol. Biotechnol.*, 86:163–175, 2010.
- [278] M. Ubbink. Dynamics in transient complexes of redox proteins. *Biochem. Soc. Trans.*, 40:415–418, 2012.
- [279] T.-S. Lin. Electron spin echo spectroscopy of organic triplets. *Chem. Rev.*, 84:1–15, 1984.
- [280] H. L. Yu, T.-S. Lin, S. I. Weissman, and D. J. Sloop. Time resolved studies of pentacene triplets by electron spin echo spectroscopy. *J. Chem. Phys.*, 80:102, 1984.
- [281] M. Di Valentin, S. Ceola, G. Agostini, G. M. Giacometti, A. Angerhofer, O. Crescenzi, V. Barone, and D. Carbonera. Pulse ENDOR and density functional theory on the peridinin triplet state involved in the photo-protective mechanism in the peridinin-chlorophyll *a*-protein from *Amphidinium carterae*. *Biochim. Biophys. Acta*, 1777:295–307, 2008.
- [282] H. Seidel, M. Mehring, and D. Stehlik. Room-temperature kinetics of the photoexcited triplet state of acridine in fluorene crystals as obtained from electron spin echo studies. *Chem. Phys. Lett.*, 104:552–559, 1984.
- [283] S. D. Köhler, S. Höfel, and M. Drescher. Triplet state kinetics of anthracene studied by pulsed electron paramagnetic resonance. *Mol. Phys.*, 111:1–6, 2013.
- [284] M. Bennati, A. Grupp, and M. Mehring. Pulsed EPR on the photoexcited triplet state of C₆₀ fullerene. *Chem. Phys. Lett.*, 200:440–444, 1992.
- [285] M. K. Bosch, I. I. Proskuryakov, P. Gast, and A. J. Hoff. Time-resolved EPR study of the primary donor triplet in D1-D2-cytb559 complexes of photosystem II: temperature dependence of spin-lattice relaxation. *J. Phys. Chem.*, 100:2384–2390, 1996.
- [286] L. Pekkariinen and H. Linschitz. Studies on metastable states of porphyrins. II. Spectra and decay kinetics of tetraphenylporphine, zinc tetraphenylporphine and bacteriochlorophyll. *J. Am. Chem. Soc.*, 82:2407–2411, 1960.
- [287] J. Rodriguez, C. Kirmaier, and D. Holten. Optical properties of metalloporphyrin excited states. *J. Am. Chem. Soc.*, 111:6500–6506, 1989.

- [288] D. A. Antheunis, J. Schmidt, and J. H. van der Waals. The dynamics of populating and depopulating of a phosphorescent triplet state as studied by microwave induced delayed phosphorescence. *Chem. Phys. Lett.*, 6:255–258, 1970.
- [289] J. Schmidt, D. A. Antheunis, and J. H. van der Waals. The dynamics of populating and depopulating the phosphorescent triplet state as studied by microwave induced delayed phosphorescence. *Mol. Phys.*, 22:1–17, 1971.
- [290] I. Y. Chan, W. G. van Dorp, T. J. Schaafsma, and J. H. van der Waals. The lowest triplet state of Zn porphin II. Investigation of its dynamics by microwave induced delayed phosphorescence. *Mol. Phys.*, 22:753–760, 1971.
- [291] W. G. Van Dorp, T. J. Schaafsma, M. Soma, and J. H. van der Waals. Investigation of the lowest triplet state of free base porphin by microwave induced changes in its fluorescence. *Chem. Phys. Lett.*, 21:221–225, 1973.