



Spin chemistry for biology: fluorescence detection of magnetic field effects on flavin photoreactions

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A thesis submitted for the degree of
Doctor of Philosophy

Jesus College
University of Oxford

Hilary Term 2016

Abstract

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Magnetic fields as weak as the Earth's can change the yields and/or kinetics of radical pair reactions even though the interaction energies involved are orders of magnitude smaller than $k_B T$ at room temperature. Proposed as the source of the light-dependent magnetic compass in migratory birds, Magnetic Field Effects (MFEs) on chemical reactions can be understood by the radical pair mechanism (Chapter 1) and are thought to operate in flavin-containing protein photoreceptors in the retina. Numerous studies on flavin-based model systems of biological importance have shown MFEs under physiological conditions. In many instances, these effects are small and are only likely to be of any importance to the postulated chemical compass if the primary effects of the magnetic fields on the geminate radical pairs are further enhanced via yet unknown amplification mechanisms.

In this thesis, a novel technique is introduced which measures MFEs on the prompt fluorescence of continuously photoirradiated flavin/electron donor model systems (Chapter 2). By exploiting the sensitivity of this method it is possible to profile the effects of the magnetic interaction characteristics within the radical pair as well as those of the embedding media. In Chapter 3, the flavin-ascorbic acid system and rare examples of Low Field Effects relevant for magnetosensitivity to weak magnetic fields under biologically relevant conditions are explored. The possibility for the chemical amplification of MFEs is demonstrated for cryptochromes and related model systems through experiment and simulation (Chapter 4–5). Under conditions of continuous photoexcitation, any change in the kinetics of radicals formed downstream to the geminate radical pair can significantly enhance the prompt field effect over the timescale of milliseconds and longer. Given the efficiency of amplification and the simplicity of its implementation, it is not inconceivable for nature to have adopted these effects for improved magnetoreception.

Acknowledgements

There are many, many people to thank for helping me along the way over the last few years. Thank you Professor Christiane Timmel for accepting me to New College to study chemistry and into the Timmel Group to study spin chemistry. You have inspired me over the last eight years and I will always look back fondly on my time in Oxford. Thank you for going above and beyond on all occasions, and for being the very best supervisor that anyone could hope to have — it has been a real privilege to be a part of New College and the fantastic Timmel Group.

I'd like to thank Professor Stuart Mackenzie for also taking me under his wing these past few years and the immense support that I've received during my Part II, doctorate and for the next steps. Thank you Professor Peter Hore for sharing the love of spin chemistry. "The supervisors" have provided the perfect environment for me and others to thrive in science. Great thanks to the SABMiller plc team (Dr Francis Bealin-Kelly, Dr Ian Cantrell, Professor Barry Axcell, Mangethe Zwane, Professor Katherine Smart, Dr David Jenkins, Dr Frieda Dehrmann and Dr Sue James) for financial support and academic guidance.

Next I'd like to acknowledge Dr Daniel Kattnig for being a patient proof-reader and guide — teaching me the ways of radical pairs — and for having made it all so enjoyable! Thank you Dr Kevin Henbest for your expertise on all things lasers, the endless ideas and for showing me around the labs when I first popped my head through the Timmel Group door — your infectious enthusiasm for science was apparent from Day 1. Dr Kiminori Maeda I must thank for guiding my initial foray into research and for taking me around Tokyo following AWEST (a brilliant time meeting Gundam and eating teppanyaki). Thank you Dr William Myers for help on all things EPR and consistently destroying me at squash (apart from that one time which I hold onto dearly). Dr Lauren Jarocha and Dr Christian Kerpel I'd like to thank for my delicious sheep birthday cake and being helpful, wonderful colleagues bringing good wine, BBQs, and much fun and laughter to the group.

I've spent my time in the Timmel Group with excellent DPhil students. Cheers to Dean Sheppard (honorary member) for being a great colleague/friend; I promise that I will get those photos I took in India to you one day! The brilliant group of Dr Alice Bowen, Dr Kelly-Anne Ferguson, Dr Jon Storey, Dr Cat Gunn ("FMN?"), Dr Claudia Tait and soon-to-be-Drs Sabine Richert, Tilo Zollitsch, Katie Buckton and Marcin Konowalczyk: great to have spent my DPhil in your lovely company and thank you for the endless support, ranging from wrapping crackers in old posters to help with science puzzles of related and unrelated nature. Special thanks to Alice for support on the final push and for her kind gift of protein. Also Claudia, thank you for being a fantastic neighbour and generally putting up with me! Further thank you's to Sabine and Katie for thorough

proof-reading and insertion of articles. A big thanks to Dr Jing Li for the introduction to life in 23-19.

I've also spent my time in the Timmel Group with fantastic Part II students: Tiago Marinheiro, Michael Teckman, Karol Zuchowski, Tim Newman, Ewelina Gregolinska, Gabriel Moise, James Carey and Olivia Foster Vander Elst. Special thanks to Tiago and Olivia for invaluable support on the SABMiller plc project. Congratulations/com-miserations James on Norwich staying up/going down. Good luck Gabriel for the DPhil, more happy hours of paper airplane-fun to come...

A very special thanks to Neville Baker and his electronics sorcery for key assistance with the magnetic field coils. I am enormously indebted to the expertise of the PTCL workshops.

I have to say that I'm very lucky to have been able to share the DPhil adventure with great friends who have been a great support — Eleanor Berry, Bobby Brooks, Sam Calderwood, Caitlin Clunie-O'Connor, Phil Derry, Adam Gammack, Megan Masters, Lottie Rapson, Paul Trethowan and Iwan Walters.

Going back a little, I'd like to thank Dr Jon Thomas and the Gŵyr science team: Paula Davies, Ann Ivey, Mary Moses and David Wheeler. Thanks also to Dafydd Jenkins for the maths. You all played important roles in shaping my interest in science.

My new brothers and sisters: Sean, Liam, Mary and Elina, thank you for being so welcoming these past years. Thank you Brian for the Shrek tickets and sorry for being useless. Diolch Astra for the Greggs pasties, cheese and thoughtful cards. Cheers BriBri for help with the car and the Champagne. Thanks to Mary, Wendy and John for your warmth and delicious salads.

Huge thanks for the love from my family across the world — from Wales to Thailand — Khun Yai (Happy 90th!), Pa Tom, Loong Charn, P'Tarn, P'Toey, Loong Dui, Pa Phun, P'June, P'Keng, Na Tum, P'Tong, Na Jim, Richard, Sian, Edward, Elin, Dave, Huw, Sian, Rhys and Steffan.

My brother, best friend and best man — Arwyn — thank you for the Stag in Berlin and sorry for being rubbish at contact (I blame the DPhil!).

Thank you Mam, you ignited a love of learning in me from a very young age and for that I am forever grateful. You have loved, guided and supported me all the way through my education and I certainly wouldn't have achieved this without you. Your drive, work ethic and humility inspire me on a day-to-day basis, and will continue to do so as I enter the next chapter. Thank you for everything you have done and continue to do for me.

Diolch Dad am y cymorth aruthrol ym mhob agwedd o fy mywyd. Rwy'n mor falch o dy gyflawniadau a chymeriad, ac yn edrych ymlaen i flwyddyn nesa' yn Caergrawnt.

Gobeithio fy mod i wedi wneud i chi teimlo'n falch hefyd wrth i mi ceisio dilyn dy esiampl pob dydd — hyd yn oed y barf presennol.

Diolch i Anna am fod yn Anna. You have been an amazing support throughout the DPhil and far beyond. Nobody could wish to have a more supportive partner — from tolerating disruptive sleeps due to middle-of-the-night ideas (see Equation 2.8), to driving my next step in science. You have inspired me to do something special, brilliant in life. I love you with all my heart and I cannot wait to marry you.

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Abbreviations

4-AP	4-Aminopyridine
AscH ₂	Ascorbic acid
BSA	Bovine Serum Albumin
D	(Electron) Donor
DFT	Density Functional Theory
EPR	Electron Paramagnetic Resonance
F	Flavin
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GMP	Guanosine Monophosphate
HEWL	Hen Egg White Lysozyme
ISC	Intersystem Crossing
LFE	Low Field Effect
MARY	Magnetically Altered Reaction Yield
MFE	Magnetic Field Effect
MQ	Maquette
PF	Prompt Fluorescence
RF	Riboflavin
RNase A	Ribonuclease A
RP	Radical Pair
RPM	Radical Pair Mechanism
SCRp	Spin-Correlated Radical Pair
TrpH (or W)	Tryptophan

To my family

Chapter 1

Introduction

Evidence of magnetoreception, the ability to detect magnetic fields, is widespread across the animal kingdom [1]. In some cases, the magnetosense enables the animals to detect the Earth’s weak (50 μ T) magnetic field and to learn the magnetic topography of the areas in which they live. The radical pair hypothesis has emerged as one of the main candidates for the underlying mechanism in compass orientation whereby magnetosensitivity is proposed to stem from the field-dependent reactivity of radical pairs (RPs) [2]. Magnetic Field Effects (MFEs) in these systems are fundamentally quantum mechanical and occur under conditions where the magnetic interactions are orders of magnitude weaker than the thermal energy per molecule. This remarkable phenomenon can be understood by the well-established radical pair mechanism (RPM) [3, 4] as presented in the following chapter.

A vast literature on MFEs is also available beyond the scope of magnetoreception as these effects have wide-ranging impact in regard to charge separation efficiencies and revealing reaction dynamics in donor acceptor systems [4–8]. Whilst the areas of research are diverse, they share the same fundamental spin chemistry.

This chapter provides a brief introduction to magnetoreception and outlines the theoretical basis for MFEs as explored in this thesis.

1.1 Magnetoreception

Animal magnetoreception has fascinated scientists and wider audiences for many decades [9, 10]. One of the most intensely studied organisms in regard to this intriguing phenomenon is the European robin, for which two principal mechanisms have been proposed based on magnetite [11] and radical pairs [12], respectively. In the magnetite hypothesis it is postulated that magnetic field information is detected by magnetoreceptors consisting of magnetite crystals found within certain regions of the beak. The magnetic crystal interactions with the external field are thought to induce physical deformations which modulate a neural response [11]. On the other hand, the radical pair hypothesis considers the ability to stem from magnetically sensitive photochemical reactions in cryptochrome proteins located in the retina [13]. Cryptochromes contain the flavin adenine dinucleotide (FAD) chromophore, photoexcitation of which leads to cascaded electron transfer along a triad of tryptophan (TrpH) residues to produce a $(\text{FAD}^{\bullet-} \text{TrpH}^{\bullet+})$ radical pair. The radical pair has a field-dependent concentration and causes a conformational change in the protein, thereby possibly initiating signals in the visual nervous system [14]. Effectively, the animals are thought to be able to *see* the magnetic field [13].

Support for the radical pair hypothesis has accumulated from many decades of behavioural studies. The Emlen funnel was invented in 1966, where birds are placed in cones lined with paper to measure their response to applied magnetic fields by analysing the recorded scratches [15]. In support of an inherent magnetosense, statistically significant correlation of the birds' average orientation with the applied magnetic field direction has been shown on a number of occasions [9, 10, 16, 17]. Furthermore, studies of the European robin and other migratory birds revealed their magnetoreception to be sensitive to the inclination and not the polarity of the field [18]. Thus, inverting the horizontal component of the field has the same effect as inverting the vertical component, sending the bird in the opposite direction to that found without inversion. A reversal of both components, which would mean an inversion of the polarity, sees the bird's original orientation restored.

The birds' ability to detect magnetic fields was also shown to be dependent on the wavelength of ambient light. Birds subjected to blue (443 nm) or green (565 nm) light showed good magnetic orientation, but became disoriented under yellow (590 nm) or red

light (633 nm) [19, 20]. Thus, the implication is that light with energy above a certain threshold (*e.g.* to generate RPs) is necessary for magnetoreception.

A recent, somewhat puzzling attribute to emerge is that anthropogenic electromagnetic noise in the broadband frequency range of 50 kHz to 5 MHz with nanotesla field intensities disrupts magnetic orientation [21]. For sensitivity to such exceptionally weak fields, the radical pair lifetime would have to be orders of magnitude longer than previously perceived possible. Although the full mechanistic details are unclear for the radical pair hypothesis in explaining this phenomenon, it is noted that magnetic particles of sufficient size to act as a compass are too large to orient in magnetic fields fluctuating as quickly as 1 MHz [12].

An inclination-, light- and radiofrequency-dependent compass sense exhibited by migratory birds favours the radical pair hypothesis over magnetite. In the following sections, the basis for magnetosensitivity in chemical reactions is discussed.

1.2 Electron spins in magnetic fields

By the fundamental principles of quantum mechanics, an electron has a spin angular momentum quantum number, $s = \frac{1}{2}$, and possible projection of spin angular momentum quantum numbers, $m_s = +\frac{1}{2}$ (spin-up, α state) and $-\frac{1}{2}$ (spin-down, β state). The magnitude of the spin angular momentum vector, $\vec{s}$, is related to s by $\|\vec{s}\| = \hbar\sqrt{s(s+1)}$. Further, s_z , the projection component of the spin angular momentum vector on to the quantisation axis is given by $s_z = m_s\hbar$. The two spin states of an electron are summarised in Table 1.1.

TABLE 1.1: Definition of the two spin states of an electron.

State	$ s, m_s\rangle$
$ \alpha\rangle$	$ \frac{1}{2}, +\frac{1}{2}\rangle$
$ \beta\rangle$	$ \frac{1}{2}, -\frac{1}{2}\rangle$

In the presence of a magnetic field, states with different m_s values are energetically split by the Zeeman interaction due to their different projections of spin angular momentum on to the external magnetic field. Classically, the energy of a magnetic moment, $\vec{m} = (m_x, m_y, m_z)$, in a magnetic field, $\vec{B} = (B_x, B_y, B_z) = (0, 0, B_0)$, is given by:

$$E = -\vec{\mathbf{m}} \cdot \vec{\mathbf{B}} = -m_z B_0. \quad (1.1)$$

For an electron spin, the magnetic moment, $\vec{\mathbf{m}}$, is related to the spin angular momentum, $\vec{\mathbf{S}}$, as:

$$\vec{\mathbf{m}} = -\frac{g_e \mu_B \vec{\mathbf{S}}}{\hbar} \quad (1.2)$$

where $g_e = 2.00232$ is the dimensionless g -value for a free electron and μ_B is the Bohr magneton.

Analogously, the quantum mechanical Hamiltonian operator for the Zeeman interaction is expressed as:

$$\hat{H}_{zee} = -\hat{\vec{\mathbf{m}}} \cdot \vec{\mathbf{B}} = \frac{g_e \mu_B}{\hbar} \hat{\vec{\mathbf{S}}} \cdot \vec{\mathbf{B}} \quad (1.3)$$

where $\hat{\vec{\mathbf{m}}}$ and $\hat{\vec{\mathbf{S}}}$ are the magnetic moment and spin angular momentum operators, respectively. Considering the (conventional) assignment of the field direction above and resulting quantisation axis, Equation 1.3 simplifies to:

$$\hat{H}_{zee} = \frac{g_e \mu_B B_0}{\hbar} \hat{s}_z \quad (1.4)$$

with $\hat{s}_z$ being the operator for the projection of spin angular momentum on to the quantisation axis (and, here, field direction). Thus, the eigenvalue for the state characterised by m_s has the corresponding Zeeman energy:

$$E_{zee} = g_e \mu_B m_s B_0. \quad (1.5)$$

For radicals, g_e is replaced by g as a result of spin-orbit coupling and will depend on the electronic structure of the radical. Thus, electrons in different local environments will differ in g -values¹.

¹In reality, the spin-orbit coupling interaction depends on the angle of the magnetic field relative to the molecular frame such that g is anisotropic. However, the anisotropy can be largely ignored for the systems considered in this thesis as: 1) for carbon-centred radicals, the anisotropy and deviation from g_e are small; 2) the anisotropy is averaged by molecular tumbling in solution.

The energy gap, ΔE_{zee} , between the $m_s = +\frac{1}{2}$ and $-\frac{1}{2}$ states is known as the Zeeman splitting and is probed by traditional continuous-wave Electron Paramagnetic Resonance (EPR).

Magnetic nuclei possess non-zero spin quantum numbers ($I \neq 0$) and also exhibit analogous Zeeman interactions with external fields. However, the field-induced separation between the spin states is generally orders of magnitude smaller than for electron spins and can be neglected for the magnetic field-dependent behaviour of radical pairs encountered in this thesis.

1.3 Radical pairs in magnetic fields

Radical pairs (RPs) contain two unpaired electron spins and are typically assigned as being correlated or uncorrelated according to their relative spin states [3]. For spin-correlated radical pairs (SCRPs), the electron spins are mutually oriented in a certain way as found following reactions which obey total spin conservation. In contrast, the spins of uncorrelated RPs are oriented in a random manner. Uncorrelated RPs, for example, result from the random encounter of two radicals in solution.

Singlet and triplet states of RPs arise from mutually anti-parallel and parallel orientation of electron spins. Formally, the spin states can be derived using the Clebsch-Gordan coupling series for two electron spins ($s = \frac{1}{2}$). The possible values of the total spin angular momentum quantum number are $S = 0, 1$ — corresponding to the singlet (S) and triplet (T) states, respectively. Further, the associated values for the projection of the total spin angular momentum quantum number, M_S , are as follows: for $S = 0$; $M_S = 0$ (state S) and for $S = 1$; $M_S = +1, 0, -1$ (states T_+, T_0, T_-). The coupled and uncoupled basis for describing the singlet and triplet spin states are related in Table 1.2.

TABLE 1.2: Definitions of the four spin states of a RP in the coupled basis.

State (coupled)	State (uncoupled)	$ S, M_S\rangle$
$ S\rangle$	$\frac{1}{\sqrt{2}}(\alpha_A\beta_B\rangle - \beta_A\alpha_B\rangle)$	$ 0, 0\rangle$
$ T_+\rangle$	$ \alpha_A\alpha_B\rangle$	$ 1, 1\rangle$
$ T_0\rangle$	$\frac{1}{\sqrt{2}}(\alpha_A\beta_B\rangle + \beta_A\alpha_B\rangle)$	$ 1, 0\rangle$
$ T_-\rangle$	$ \beta_A\beta_B\rangle$	$ 1, -1\rangle$

In an analogous manner to the spin states of individual electrons with different m_s (Equation 1.5), the triplet substates with different M_S are split by the Zeeman interaction: $E_{zee} = g\mu_B B_0 M_S$.

1.4 Radical pair mechanism

For chemical reactions proceeding via SCRPs intermediates, magnetic fields can alter their rates and yields by the radical pair mechanism [3, 4]. Chemical magnetosensitivity by this scheme requires the following events: 1) formation of the radical pair with correlated electron spins, often in a pure singlet (S) or triplet (T) state; 2) field-dependent, coherent evolution of the radical pair between the S and T spin states (*i.e.* S-T spin mixing); 3) spin-selective reactivity such that the S and T states form different products (or the same at different rates).

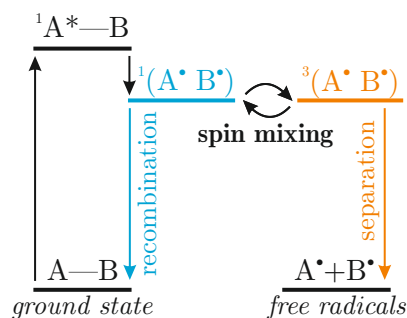


FIGURE 1.1: Scheme of the radical pair mechanism for the in-cage recombination of $^1(\text{A}\bullet\text{B}\bullet)$ following molecular dissociation. The efficiency of singlet recombination reduces as a result of spin mixing.

One of the simplest reactions to follow the radical pair mechanism is depicted in Figure 1.1. The excited singlet state $^1\text{A}^*\text{—B}$ reacts, under spin conservation, to generate the singlet geminate (or ‘cage’) radical pair, $^1(\text{A}\bullet\text{B}\bullet)$ thus, satisfying criterion 1) above. The ‘cage’ is considered often as the region of effective recombination of two radicals forming a pair. In this region, the probability of the reaction with species outside of the cage is small in comparison to their own geminate recombination. The RP species can escape from the geminate region and re-encounter radicals from the same origin or freely diffuse and encounter radicals from a different geminate cage to form F-pairs. However, it is probably most instructive to consider the ‘cage’ as the state in which the spins are

correlated without (necessarily) having to stipulate a specific environment. If the spins lose their correlation, they have escaped the ‘cage’ regardless of their proximity.

Neither the singlet nor triplet states are RP eigenstates in the absence of strong interactions between the unpaired electrons (as discussed further in the next section). Therefore, a spin-correlated RP is described by a superposition of eigenfunctions and undergoes time evolution due to coherences between the singlet and triplet states [22]. As prescribed by criterion 2) above, the evolution and efficiency of RP spin mixing is magnetic field dependent due to the energetic splitting of the spin substates by the Zeeman interaction. Thus the effect of a strong magnetic field is to energetically isolate the T_+, T_- manifold from S, T_0 , thereby reducing the extent of spin interconversion versus zero field.

The elementary step of RP recombination occurs with overall electron spin conservation. In the majority of systems, the radical recombination energy is insufficient to generate an excited (singlet or triplet) product state. Consequently, RP recombination occurs exclusively via the singlet geminate RP to yield the singlet ground state. The spin-selective recombination reaction fulfills requirement 3) of the RPM.

By this scheme, variation of the external magnetic field strength influences the extent of spin mixing and results in a Magnetic Field Effect (MFE) on the yield of RP recombination versus escape from the geminate cage. The extent of spin mixing in the geminate pair is determined by interactions between spins and with the external magnetic field as considered in further detail in the next section.

1.5 Spin-spin interactions in radical pairs

1.5.1 Hyperfine interactions

Nuclei with non-zero spin quantum number ($I \neq 0$) possess magnetic dipoles which interact with electron spins in radicals. This is known as the hyperfine interaction and is critical to understanding the spin evolution of radical pairs.

Most generally, the interaction is expressed by the following Hamiltonian:

$$\hat{H}_{hf} = \sum_e \sum_n \hat{\mathbf{S}}_e^T \cdot \mathbf{A}_{(e,n)} \cdot \hat{\mathbf{S}}_n \quad (1.6)$$

where e is an index over the electron spins in the RP; n is an index over the magnetic nuclei; $\hat{\mathbf{S}}_e$ and $\hat{\mathbf{S}}_n$ are spin angular momentum operators for electron e and nucleus n , respectively; and $\mathbf{A}_{(e,n)}$ is the hyperfine tensor for coupling between electron e and nucleus n .

For the systems studied in this thesis, fast molecular tumbling causes the anisotropy to average to zero. Consequently, the reduced Hamiltonian for the isotropic interaction becomes:

$$\hat{H}_{hf} = \sum_e \sum_n a_{e,n} \hat{\mathbf{S}}_e \cdot \hat{\mathbf{S}}_n \quad (1.7)$$

where $a_{e,n}$ is a scalar hyperfine coupling parameter.

1.5.2 Exchange interactions

As a direct consequence of the Pauli exclusion principle, an exchange interaction arises when the electronic wavefunctions of the two unpaired electrons in a RP overlap. It is a spin- and distance-dependent interaction, decreasing exponentially with increasing electron separation.

For radical pairs in solution, the exchange interaction is approximated as being isotropic, again, due to fast molecular tumbling. $\hat{H}_{ex}$, the appropriate Hamiltonian is defined as follows:

$$\hat{H}_{ex} = J(r) \left(2\hat{\mathbf{S}}_A \cdot \hat{\mathbf{S}}_B + \frac{1}{2} \right) \quad (1.8)$$

where $J(r)$ is the exchange interaction parameter and is a function of the radical separation, r ; $\hat{\mathbf{S}}_A$ and $\hat{\mathbf{S}}_B$ are the spin angular momentum operators for radicals A and B, respectively. The singlet (S) and triplet (T_+ , T_0 , T_-) states are eigenstates of $\hat{H}_{ex}$. Thus, at the instant of RP creation, the two radicals are sufficiently close that the mutual exchange interaction locks the pair into its initial spin state. Only when the radicals have

separated such that the exchange interaction is comparable to the hyperfine and Zeeman interactions (typically 1.0 nm), can spin evolution of the RP begin. For the magnetic field to affect the reactivity of an intermolecular RP, the radicals must re-encounter and react spin-selectively so that the field-dependent spin mixing is translated to a field-dependent product yield.

1.5.3 Dipolar interactions

The dipolar interaction involves electron spins interacting via their magnetic dipoles. $\hat{H}_{dip}$, the Hamiltonian for this anisotropic interaction is:

$$\hat{H}_{dip} = \hat{\mathbf{S}}^T \cdot \mathbf{D} \cdot \hat{\mathbf{S}} \quad (1.9)$$

where $\mathbf{D}$ is the dipolar tensor and $\hat{\mathbf{S}}$ is the total spin angular momentum operator, $\hat{\mathbf{S}} = \hat{\mathbf{S}}_A + \hat{\mathbf{S}}_B$. $\mathbf{D}$ is a traceless tensor and is expressed in the eigenframe (x, y, z) :

$$\mathbf{D} = \begin{bmatrix} D_x & 0 & 0 \\ 0 & D_y & 0 \\ 0 & 0 & D_z \end{bmatrix}. \quad (1.10)$$

Conventionally, the dipolar interaction tensor is parameterised in terms of two constants: $D = \frac{3}{2}D_z$; and $E = \frac{1}{2}(D_x - D_y)$. The corresponding Hamiltonian, $\hat{H}_{dip}$, becomes:

$$\hat{H}_{dip} = D(\hat{S}_Z^2 - \frac{1}{3}S(S+1)\hbar^2) + E(\hat{S}_X^2 - \hat{S}_Y^2) \quad (1.11)$$

where $\hat{S}_Z$ is the operator corresponding to the z -component of total spin angular momentum; analogously, $\hat{S}_X$ and $\hat{S}_Y$ yield the x - and y - components.

For RPs in solution, fast molecular tumbling causes the anisotropic dipolar interaction to average to zero. However, the instantaneous interaction is non-negligible and is one cause of spin relaxation (as discussed below). Dipolar interactions are inversely proportional to the cubed distance ($\propto \frac{1}{r^3}$) between the magnetic dipoles. Thus, they are an important consideration at longer RP separations than for the exchange interaction (which exhibits an exponential distance dependence).

1.6 Spin mixing by the vector model

To understand the nature of spin mixing qualitatively, it is instructive to consider the classical vector model for RPs [3]. In an external magnetic field, B_0 , the spin angular momentum of an unpaired electron will precess about the field direction at the Larmor frequency:

$$\omega_L = \frac{g\mu_B B_0}{\hbar}. \quad (1.12)$$

In the vector model, $\vec{S}_A$ and $\vec{S}_B$ are the vector representations for the spin angular momenta of electrons A and B , respectively. $\vec{S}_A$ and $\vec{S}_B$ for the singlet RP ($S = 0$) precess such that their vector sum is always zero. However, the vector representations for the triplet RP ($S = 1$) must sum to give a total spin angular momentum magnitude of $\hbar\sqrt{2}$. Figure 1.2 depicts the vector model for the singlet and triplet states, whereby the electron spins are denoted by arrows. By this scheme, which is applicable for high field strengths, it appears that spin precession dephasing leads to transitions between S and T_0 states. However, S- T_+ and S- T_- interconversion requires a change in one of the radical components with regard to its spin projection on to the quantisation axis — a ‘spin-flip.’

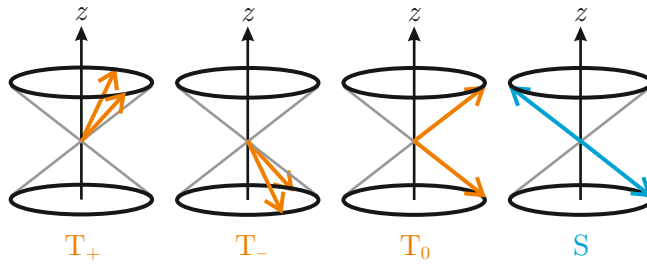


FIGURE 1.2: Depiction of the T_+ , T_- , T_0 and S radical pair spin states by the vector model. The spin angular momentum of an individual electron spin is represented by an arrow and precesses about the external magnetic field direction, z , at the Larmor frequency.

Generally, spin mixing in a RP is induced by: 1) the Δg mechanism, 2) the hyperfine mechanism, 3) the relaxation mechanism, or any combination of the three [3].

1.6.1 Δg Mechanism

Spin mixing by the Δg mechanism occurs when the two radicals comprising the RP are characterised by different g -values. As a consequence, the Larmor precession frequencies are different for the two electron spins. This results in periodic transitions between the S and T₀ configurations. The higher the external field, the higher the frequency of transitions as governed by the difference in the Larmor precession frequencies:

$$\Delta\omega_L = \omega_A - \omega_B = \frac{1}{\hbar}(g_A - g_B)\mu_B B_0. \quad (1.13)$$

Here, ω_A , g_A and ω_B , g_B are the Larmor precession frequencies and g -values for radicals A and B , respectively.

It is worth noting that for effective S-T₀ mixing, transitions must occur within τ , the geminate RP lifetime; *i.e.* $\Delta\omega_L \cdot \tau \geq 1$. For typical carbon-centred RPs as studied in this thesis, $\Delta g \approx 0.001$ and $\tau \approx 10$ – 100 ns. To satisfy the prescribed condition for appreciable spin mixing, magnetic field strengths in excess of 10^4 mT are required, far higher than those which are relevant to later chapters motivated by magnetoreception in geomagnetic fields. Consequently, the Δg mechanism is ignored.

1.6.2 Hyperfine mechanism

In the presence of a magnetic field which is stronger than the hyperfine couplings in the RP, the electron A , electron B and nuclear spins precess about the applied field direction. The magnetic momenta of the nuclei behave as a perturbation to the local fields experienced by the electrons, hence influencing their Larmor precession frequencies.

Consider the one proton RP system where electron spin A is coupled to one proton (^1H , with spin quantum number, $I = \frac{1}{2}$) and the other, B , has no magnetic nuclei. The hyperfine interaction between electron spin A and the proton is isotropic, and is characterised by the constant a . The Larmor precession frequencies for electron spins A and B are [3]:

$$\omega_A = \frac{1}{\hbar}g_A\mu_B B_0 \pm \frac{a}{2} \quad (1.14)$$

$$\omega_B = \frac{1}{\hbar} g_B \mu_B B_0. \quad (1.15)$$

The $\pm \frac{a}{2}$ term in Equation 1.14 depends on the nuclear spin orientation. Thus, in analogy to the Δg mechanism, the S-T₀ mixing frequency is evaluated as $\Delta\omega_L = \frac{a}{2}$.

For magnetic field intensities which are weaker than the hyperfine interactions (including zero field cases), the electron spins precess about the resultant of the external and hyperfine fields. More extensive spin mixing is possible versus high fields as precession about the resultant field can allow changes in the relative spin projection of the two radicals, *i.e.* S-T_± transitions in addition to S-T₀. The high-field scenario discussed above corresponds to spin mixing by the hyperfine mechanism whereby the resultant and external fields effectively coincide.

1.6.3 Relaxation mechanism

Relaxation of spins in external magnetic fields drives incoherent mechanisms of S-T interconversion. Spin-lattice relaxation is the phenomenon by which the thermal equilibrium value for the projection of the spin angular momentum onto the external field direction is attained. These ‘spin-flip’ transitions enable S-T_± mixing. On the other hand, spin-spin relaxation involves the destruction of spin coherences and polarisation perpendicular to the applied field direction, giving rise to S-T₀ interconversion. At low fields the distinction is invalid.

Spin relaxation is caused by random modulations of anisotropic interactions. Using the point-dipole approximation, the relative rates for spin-lattice relaxation ($1/T_1$) and spin-spin relaxation ($1/T_2$) due to dipole-dipole interactions between two electron spins are described at high fields by [23]:

$$\frac{1}{T_1} = \left(\frac{g\mu_B}{\hbar} \right)^2 \langle B^2 \rangle J(\omega_0) \quad (1.16a)$$

$$\frac{1}{T_2} = \frac{1}{2} \left(\frac{g\mu_B}{\hbar} \right)^2 \langle B^2 \rangle J(\omega_0) + \frac{1}{2} \left(\frac{g\mu_B}{\hbar} \right)^2 \langle B^2 \rangle J(0) \quad (1.16b)$$

where $\langle B^2 \rangle$ is the mean-square value of the local fluctuating fields; and the spectral density function, $J(\omega)$, is proportional to the probability of finding a component of the induced local field corresponding to a frequency of ω . $J(\omega)$ is given by:

$$J(\omega) = \frac{2\tau_c}{1 + \omega^2\tau_c^2} \quad (1.17)$$

with τ_c being the time taken for the root-mean-square deflection of molecules from their original orientation to reach 1 radian. From Equations 1.16a and b, the spin mixing rate is seen to decrease with increasing field strength. The same qualitative field- and τ_c -dependence is observed for relaxation induced by dipolar interactions between electrons and nuclei. Furthermore, it is also found for relaxation which originates from the g -tensor anisotropy due to the Zeeman effect.

Typical values of organic radical relaxation rates are found to be in the range of 10^5 s^{-1} to 10^6 s^{-1} for solutions with a dynamic viscosity of $1 \times 10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$. Hence, considering the typical 10–100 ns geminate pair lifetime, relaxation-induced mixing appears ineffective.

From the considerations above, the hyperfine mechanism appears to be the most relevant means of spin mixing for the RP systems examined in this thesis². Although the intuitive vector model is able to give a physical representation of the transition mechanism, for further insight, a quantum-mechanical approach is employed in the next section. Details such as the intriguing Low Field Effect emerge, whereby spin mixing can be more efficient at low magnetic field intensities versus zero field.

1.7 Spin mixing by quantum mechanics

Spin evolution as required by the radical pair mechanism originates from the non-stationary nature of the singlet and triplet states in the absence of strong exchange interactions. The non-stationary states are described by the superposition of several eigenfunctions with different singlet and triplet character, and are subject to time evolution [22].

²However, spin relaxation must not be entirely ignored. In Chapter 2, singlet-triplet and triplet-triplet dephasing processes are considered where spin relaxation is able to affect the efficiency of spin mixing by the hyperfine mechanism.

Consider once more the simple one proton RP comprised of electrons A and B , with electron A coupled to a proton (^1H , spin quantum number $I = \frac{1}{2}$ and spin projection $m_I = \pm\frac{1}{2}$) via an isotropic hyperfine interaction (constant, a). The pertinent spin Hamiltonian describing the system is:

$$\hat{H} = \hat{H}_{zee} + \hat{H}_{hf} = \omega_A \hat{S}_{Az} + \omega_B \hat{S}_{Bz} + a(\hat{\mathbf{S}}_A \cdot \hat{\mathbf{S}}_n), \quad (1.18)$$

where the first and second terms account for the electron Zeeman and hyperfine interactions, respectively. Previous theoretical treatments by Brocklehurst [22] and Timmel *et al.* [24] find that coherences between non-degenerate pairs of states created in a superposition will oscillate at a frequency corresponding to their energy difference. The active coherences for the one proton RP can be seen by examination of the initial density matrix for the RP created in a pure singlet state:

$$\hat{\rho}(0) = \frac{1}{2} |\text{S}\alpha_I\rangle \langle \text{S}\alpha_I| + \frac{1}{2} |\text{S}\beta_I\rangle \langle \text{S}\beta_I|, \quad (1.19)$$

when re-expressed in the eigenbasis of the Hamiltonian in Equation 1.18.

1.7.1 Zero field

Closely following the approach outlined in [24], spin evolution is first considered in the absence of any magnetic field ($B_0 = 0$). The pertinent spin Hamiltonian for the system is reduced and contains the hyperfine term only ($\hat{H}_{hf}$); the initial density matrix is:

$$\hat{\rho}(0) = \begin{matrix} & |1\rangle & |2\rangle & |3\rangle & |4\rangle & |5\rangle & |6\rangle & |7\rangle & |8\rangle \\ \begin{matrix} \langle 1| \\ \langle 2| \\ \langle 3| \\ \langle 4| \\ \langle 5| \\ \langle 6| \\ \langle 7| \\ \langle 8| \end{matrix} & \begin{pmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & \frac{1}{4} & -\kappa & 0 & 0 & 0 & -\kappa & 0 \\ 0 & -\kappa & \frac{1}{8} & 0 & 0 & 0 & \frac{1}{8} & 0 \\ 0 & 0 & 0 & \frac{1}{4} & -\kappa & 0 & 0 & \kappa \\ 0 & 0 & 0 & -\kappa & \frac{1}{8} & 0 & 0 & -\frac{1}{8} \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & -\kappa & \frac{1}{8} & 0 & 0 & 0 & \frac{1}{8} & 0 \\ 0 & 0 & 0 & \kappa & -\frac{1}{8} & 0 & 0 & \frac{1}{8} \end{pmatrix} \end{matrix} \quad (1.20)$$

where $\kappa = \frac{1}{4\sqrt{2}}$. The eigenvalues for the system are $\frac{1}{4}a$ and $-\frac{3}{4}a$; the corresponding eigenfunctions given in the S-T basis are as follows:

$$\epsilon_{(1-6)} = \frac{1}{4}a \quad (1.21a)$$

$$|1\rangle = |T_+\alpha_I\rangle \quad (1.21a)$$

$$|2\rangle = \frac{1}{\sqrt{2}} |T_+\beta_I\rangle + \frac{1}{2} |T_0\alpha_I\rangle - \frac{1}{2} |S\alpha_I\rangle \quad (1.21b)$$

$$|3\rangle = \frac{1}{\sqrt{2}} |T_0\alpha_I\rangle + \frac{1}{\sqrt{2}} |S\alpha_I\rangle \quad (1.21c)$$

$$|4\rangle = \frac{1}{\sqrt{2}} |T_0\beta_I\rangle - \frac{1}{\sqrt{2}} |S\beta_I\rangle \quad (1.21d)$$

$$|5\rangle = \frac{1}{\sqrt{2}} |T_-\alpha_I\rangle + \frac{1}{2} |T_0\beta_I\rangle + \frac{1}{2} |S\beta_I\rangle \quad (1.21e)$$

$$|6\rangle = |T_-\beta_I\rangle \quad (1.21f)$$

$$\epsilon_{(7-8)} = -\frac{3}{4}a$$

$$|7\rangle = -\frac{1}{\sqrt{2}} |T_+\beta_I\rangle + \frac{1}{2} |T_0\alpha_I\rangle - \frac{1}{2} |S\alpha_I\rangle \quad (1.21g)$$

$$|8\rangle = \frac{1}{\sqrt{2}} |T_-\alpha_I\rangle - \frac{1}{2} |T_0\beta_I\rangle - \frac{1}{2} |S\beta_I\rangle. \quad (1.21h)$$

where α_I and β_I correspond to $m_I = +\frac{1}{2}$ and $-\frac{1}{2}$, respectively. The diagonal of the density matrix in Equation 1.20 represents the initial populations, whilst the off-diagonal elements indicate the coherently linked states as: $|2\rangle \leftrightarrow |3\rangle$, $|4\rangle \leftrightarrow |5\rangle$, $|2\rangle \leftrightarrow |7\rangle$, $|3\rangle \leftrightarrow |7\rangle$, $|4\rangle \leftrightarrow |8\rangle$ and $|5\rangle \leftrightarrow |8\rangle$. However, the first two coherences (orange) are inactive in terms of spin mixing contribution as the states are degenerate. The latter four coherences (blue) connect states separated in energy by a and undergo periodic oscillations of the form $\cos(at)$ with time, t . By evaluating the eigenfunctions of the Hamiltonian in the S-T basis it appears that the S state of the RP can interconvert with all three triplet substates (T_+ , T_0 , T_-) at zero field.

1.7.2 High magnetic field and the ‘normal’ MFE

For the limiting case of a high magnetic field, whereby $\omega_A, \omega_B \gg a$, the reduced Hamiltonian for the one proton RP can be approximated as consisting of the Zeeman terms only.

Under this condition, the eigenstates (and stationary states) of the system are simply: $|T_+m_I\rangle$, $|T_-m_I\rangle$; and the combinations: $\frac{1}{\sqrt{2}}(|T_0m_I\rangle + |Sm_I\rangle)$, $\frac{1}{\sqrt{2}}(|T_0m_I\rangle - |Sm_I\rangle)$. Thus, it is apparent that S- T_0 mixing is possible but the pathways for singlet-state mixing with T_+ and T_- are absent.

From the basic considerations above it can be deduced that the efficiency of spin mixing is reduced for a high magnetic field versus zero field. The so-called ‘normal’ MFEs studied in this thesis are concerned with fields which are intermediate to the two limiting cases, with the hyperfine mechanism becoming less efficient with Zeeman splitting of the S, T_0 and T_+ , T_- manifolds.

1.7.3 Low magnetic field and the LFE

Under certain conditions at low magnetic field intensities, the Low Field Effect (LFE) can be observed whereby increased spin mixing is observed versus zero field [22, 24]. This behaviour contrasts with the ‘normal’ MFE and leads to an opposite phase for the response of the system. So-called ‘low’ magnetic fields are those which are weaker or comparable to the hyperfine interactions (*i.e.* in the context of the Hamiltonian in Equation 1.18, $a \gtrsim \omega_A, \omega_B$). The phenomenon arises due to lifted degeneracies of the zero-field eigenstates at ‘low’ magnetic fields, thus increasing the number of oscillatory, coherently linked states and the extent of spin mixing.

Timmel et al. presented a detailed discussion of the effect’s origin using the one proton RP, whereby a weak magnetic field is considered to be a weak perturbation to the system at zero field [24]. Assuming identical g -values for the two radicals ($\omega_A = \omega_B = \omega_0$), the eigenvalues of the system are schematically depicted in Figure 1.3. The eigenstates for the weak field scenario can each be associated with those in the eigenbasis at zero field: the difference only being in the coefficients of the $|\text{RP state}, m_I\rangle$ components in the S-T basis *e.g.* $|2\rangle \rightarrow |2'\rangle = c_{a2'}|T_+\beta_I\rangle + c_{b2'}|T_0\alpha_I\rangle - c_{c2'}|S\alpha_I\rangle$.

Coherences $|2\rangle \leftrightarrow |3\rangle$ and $|4\rangle \leftrightarrow |5\rangle$ become active in the presence of a weak field as the states which were degenerate at zero field are split by approximately $\omega_0/2$ if $a \gg \omega_0$. Thus, the activated coherences result in additional spin state fluctuations and more extensive spin mixing versus zero field as the original $\cos(at)$ oscillations persist. The

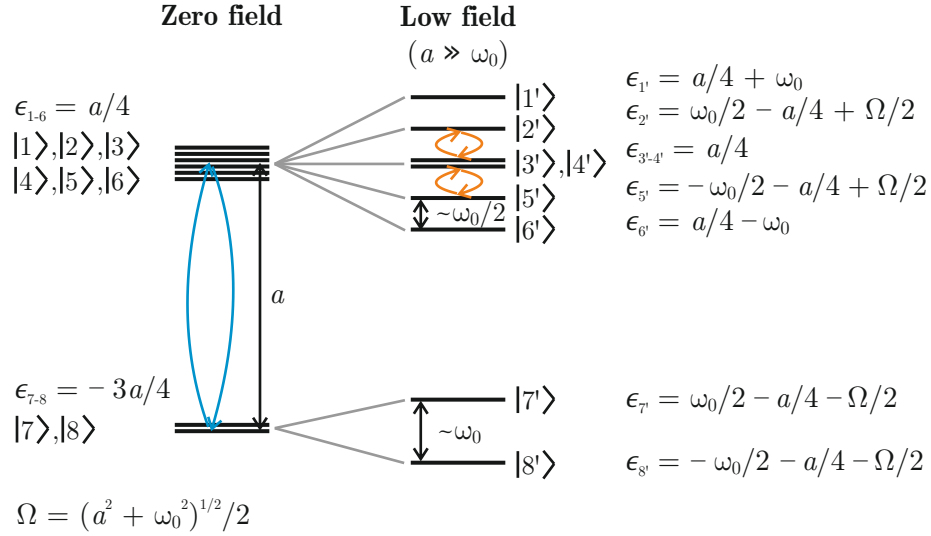


FIGURE 1.3: The field dependence of the eigenvalues for a one proton radical pair in the limit of $a \gg \omega_0$.

additional interconversion pathways oscillate with a lower frequency of $\approx \omega_0/2$, leading to the requirement of longer-lived RPs for establishing LFEs versus ‘normal’ MFEs.

Furthermore, LFEs also require that the RP does not live so long that spin relaxation becomes dominant. Theoretical and experimental studies have revealed that the LFE is expected to be large if all hyperfine interactions occur exclusively in one of the radicals comprising the pair [25]. As a consequence, deuteration of one of the RP partners is often accompanied by an increase in the size of the LFE [25]. Additionally, the effect is critically dependent on the relative sign of the involved hyperfine interactions and may also be enlarged by degenerate electron exchange of one of the radicals with its diamagnetic precursors [26].

Importantly, the LFE gives rise to the possibility of increased sensitivity to weak magnetic fields relevant to magnetoreception. Further discussion of the phenomenon can be found in [24].

1.8 Flavins

Flavins play a fundamental role in biology. Their different redox states (oxidised, semiquinone and reduced) enable catalytic function in a variety of one-electron and

two-electron processes [27]. Blue-light photoreceptor flavoproteins mediate many functions including phototropism by phototropin, regulation of transcription in bacterial (BLUF) proteins, light-induced DNA repair by photolyase and entrainment of the circadian clock in *Drosophila melanogaster* by cryptochromes [28–30]. As aforementioned, flavin-containing cryptochromes are candidate magnetoreceptors by means of a light-initiated, magnetosensitive radical pair reaction [13].

All flavin derivatives contain an isoalloxazine moiety but differ in the nature of the ribityl chain, and thus their hydrophilicity and reactivity. Commonly encountered flavins in biology are riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), see Figure 1.4.

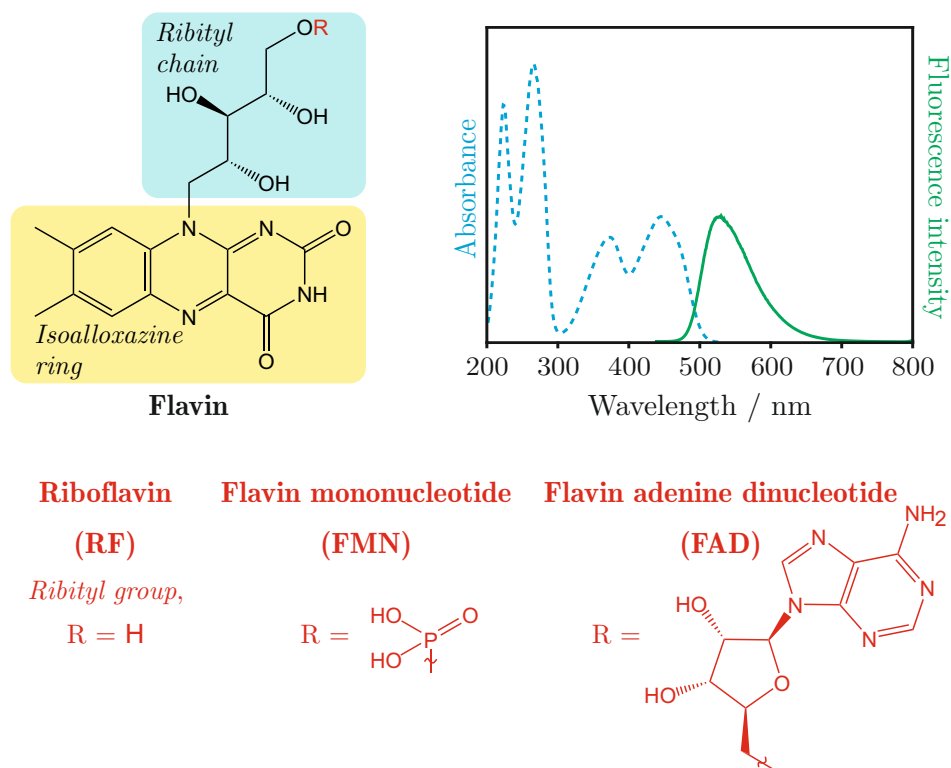


FIGURE 1.4: Flavin chemical structure and ribityl groups (red) for riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Top right: typical flavin absorbance (dotted blue) and fluorescence (green) spectral profiles as measured for FMN, pH 7 phosphate buffer (298 K).

1.8.1 Photochemistry of flavins

The absorption profile for FMN (pH 7, phosphate buffer) is given in Figure 1.4. It displays two broad peaks centred at 375 nm and 446 nm which correspond to $S_0 \rightarrow$

$S_1, \pi \rightarrow \pi^*$ and $S_0 \rightarrow S_2, n \rightarrow \pi^*$ transitions for the isoalloxazine group, respectively [31, 32]. The absorption bands at wavelengths lower than 300 nm are assigned to $S_0 \rightarrow S_3$ and $S_0 \rightarrow S_4$ (but the changes in the orbital bonding type are ambiguous). Broadly identical profiles can be found for other flavins as variation in the ribityl chain causes small perturbations in the recorded spectra.

Following photoexcitation, subsequent processes occur via S_1 due to rapid internal conversion from higher order states. For FMN (pH 7, phosphate buffer, 298 K), S_1 has a lifetime of 4.6 ns and displays a bright, green fluorescence peaking at 545 nm with a quantum yield of 0.26 [33, 34]. Again, near-identical fluorescence spectra are exhibited by other flavin derivatives.

Another significant pathway undertaken by the first singlet excited state is intersystem crossing to the triplet ($^3\text{FMN}^*$, T_1) at a rate of $k_{\text{ISC}} = 10^8$ /s at pH 7 [35].

Population of the energetic S_1 , 2.79 eV, and T_1 , 2.04 eV, states drives electron transfer reactions to produce radical pairs by electron abstraction from suitable donor (D) species. The resultant flavin radical is commonly referred to as the semiquinone. Feasibility of radical pair formation can be assessed from the change in Gibbs free energy for photoinduced electron transfer (ΔG_{PET}):

$$\Delta G_{\text{PET}} = E_{\text{D}^{\bullet+}/\text{D}} - E_{\text{F}/\text{F}^{\bullet-}} + C - \Delta G_{0*} \quad (1.22)$$

where $E_{\text{D}^{\bullet+}/\text{D}}$ is the reduction potential for the donor cationic radical: $\text{D}^{\bullet+} + e^- \rightarrow \text{D}$; $E_{\text{F}/\text{F}^{\bullet-}}$ is the reduction potential for flavin, F: $\text{F} + e^- \rightarrow \text{F}^{\bullet-}$; C is the Coulombic energy term at the instant of RP creation³; and ΔG_{0*} is the energy of the flavin excited state with respect to the ground state. Thermodynamically favourable radical pairs by photoinduced electron transfer are characterised by negative ΔG_{PET} values.

For unitary charges, C is evaluated as -0.02 eV in water. The FMN reduction potential at pH 7 is $E_{\text{F}/\text{F}^{\bullet-}} = -0.313$ V [36, 37]. From Equation 1.22, singlet-born and triplet-born radical pairs require reduction potentials of $E_{\text{D}^{\bullet+}/\text{D}} < 2.50$ V and < 1.75 V, respectively — thereby highlighting the highly oxidising nature of FMN, which is also true of other flavins.

³where the internuclear separation can be reasonably estimated as 8.0 Å

1.8.2 MFEs in intermolecular flavin-based RPs

Traditionally, magnetic field-dependent flavin chemistry has been studied through transient absorption spectroscopy [38–44]. By virtue of the flavins’ characteristic absorption profiles for different redox and protonation states [45, 46], reaction details can be disentangled via $\Delta A(t)$, the time-dependent difference in absorption of the sample before and after photoexcitation. Further, subtraction profiles such as $\Delta\Delta A(t, B_0) = \Delta A(t, B_0) - \Delta A(t, 0)$ can be exploited to focus only on species whose concentrations are magnetically sensitive. Here, B_0 denotes measurements in the presence of a B_0 -strength magnetic field.

Flavin-mediated MFEs have been recorded for RPs arising from quenching reactions with tryptophan, indoles, adenine, micelles and several proteins [38–44]. As photoexcited flavin species undergo efficient intersystem crossing with a quantum yield of 0.6 (FMN, pH 7 [35]), triplet-born RPs are typically observed for bimolecular electron transfer reactions at relatively low quencher concentrations. All of these studies are conducted in the so-called ‘normal’ MFE regime, whereby the applied field leads to reduced spin mixing efficiency and thus reduced singlet character of the geminate radical pair versus zero field. Consequently, as geminate recombination occurs exclusively from the singlet state, increasing the applied field intensity is expected to cause an increase in the concentration of the radicals. The initial state of the RP in these systems can be verified by an increase in absorption ($\propto$ concentration) in the band corresponding to the flavin semiquinone ($F^{\bullet-}$, where $\lambda_{\max} = 510$ nm; $FH^{\bullet}$, broad band for 500 to 650 nm with indistinct maximum); this is known as a positive MFE. Concomitantly, a negative MFE (*i.e.* further decrease in the $\Delta A(t)$ difference absorption signal) is observed in the ground state bleaching region of typically 400–500 nm. The spectral properties (*i.e.* radical and ground state) of the counter-radical species will affect the overall appearance of these regions.

1.8.3 MFEs in flavin-containing cryptochromes

Cryptochromes and photolyases are single domain proteins sharing a highly conserved photolyase homology region (PHR) which consists mainly of α -helices and a single region of β -sheets towards the N-terminus of the protein [14]. In contrast to photolyases,

cryptochromes also have C-terminal (and occasionally N-terminal) extensions which enable interactions with protein partners and cellular functions.

Two structural features present in both photolyases and cryptochromes are the buried FAD cofactor and a chain of three tryptophan residues (assigned TrpH_A , TrpH_B and TrpH_C for proximal, medial and distal TrpH positions with respect to FAD). The so-called TrpH-triad connects the flavin with the surface of the protein via an electron transport chain. An average spacing of approximately 5 Å between the tryptophans enables stepwise ultrafast electron transfer, which for the photolyase from *Escherichia coli* is complete within 30 ps [47]. It is these tryptophans which are implicated in forming the radical pairs at the heart of magnetoreception [12].

Photoexcitation of FAD generates the singlet excited state, $^1\text{FAD}^*$, and triggers sequential electron transfer along the TrpH triad to form a semiquinone radical, $\text{FAD}^{\bullet-}$ or $\text{FADH}^\bullet$ after protonation, together with a radical derived from the distal tryptophan [48, 49]. Contrary to the free flavin systems discussed above, the radical pair is in an initial singlet state due to the highly efficient electron transfer arising from the short distance between the proximal tryptophan and flavin species. Anisotropic hyperfine couplings in the $(\text{FAD}^{\bullet-}\text{TrpH}^{\bullet+})$ radical pair are proposed to induce a field inclination-dependent interconversion efficiency between different spin states of the radical pair [50]. The resultant field anisotropy of the radical recombination rate could be exploited in a flavin-based semiquinone signalling state for magnetoreception [51].

Studies of an isolated cryptochrome *in vitro* prove that the $(\text{FAD}^{\bullet-}\text{TrpH}^{\bullet+})$ radical pair has kinetics sensitive to variation in the magnetic field intensity, but the effects of Earth-strength fields and directional responses have yet to be shown [49].

1.8.4 Chemical compass

The cryptochrome hypothesis for a so-called ‘chemical compass’ in magnetoreception remains controversial. In light of the spin chemistry discussed above, it is useful to summarise the required properties for a radical pair to function as a geomagnetic compass sensor [12]: 1) the radical pair must have anisotropic hyperfine interactions; 2) the effect of the mutual (*e.g.* exchange) interaction of the two radicals should be small; 3) the

radical pair must react spin-selectively and 4) not be too short-lived, having a sufficient coherence lifetime whereby 5) spin relaxation is relatively slow.

One of the main criticisms of the hypothesis is that pronounced anisotropic responses of a radical pair reaction to an Earth-strength magnetic field ($\approx 50\mu\text{T}$) at physiological temperatures seem unlikely. Experimentally determined MFEs of 1% have been reported for cryptochrome proteins in 1 mT fields under optimised, unphysiological conditions [49]. In combination with theoretical predictions of reaction yield anisotropies of less than 10%, the cryptochrome hypothesis requires remarkable sensitivity to relative concentration changes which might be no larger than 0.1% [12, 50]. It is unknown if these small effects are sufficiently large to form the basis of a viable geomagnetic compass and better understanding of the signal-transduction mechanism is required [52]. However, it does not seem improbable that nature would have exploited opportunities to enhance the fundamental response. One speculation is that magnetic nanoparticles containing magnetite could act to amplify the local magnetic field of the radical pair sensor [53–55].

In this thesis, a novel fluorescence-based method for probing the magnetic field dependence of radical pairs is presented. The high sensitivity of the method is exploited in unveiling mechanistic details of the photochemistry in flavin-based systems relevant to magnetoreception and other biological processes. In later chapters, the nature of the low field effect and possible mechanisms of chemical amplification are investigated.

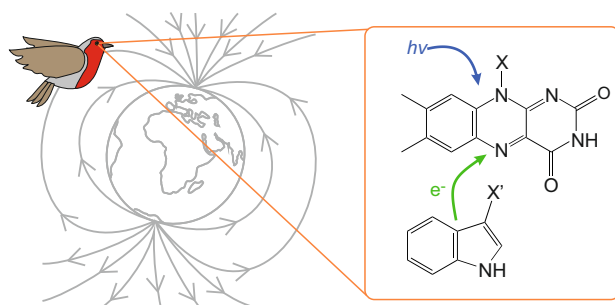


FIGURE 1.5: A number of migratory birds (including the European robin) are thought to rely on a chemical compass sense which stems from the magnetosensitivity of flavin-tryptophan radical pair photoreactions in their retina.

Chapter 2

Method for sensitive fluorescence-based detection of magnetic fields effects in photoreactions of flavins

Fluorescence is commonly exploited as a highly sensitive probe in many disciplines, its widespread applications ranging from determination of the nature of photophysical processes to cellular and molecular imaging [56]. In this chapter a novel fluorescence-based method for detecting magnetic field effects (MFEs) in photoinduced flavin-based radical pairs is introduced. The use of flavin fluorescence as the magnetosensitive probe enables the detection of MFEs in flavin samples of nanomolar concentration. As demonstrated, the exceptional sensitivity of this approach can be used to characterise the influence of external factors on the spin dynamics of the geminate pair and the MFE. Some of the initial measurements of the presented technique were conducted with Dr. Jing Li (Mackenzie group, University of Oxford) and content from this chapter has been published in [57].

2.1 Fluorescence and MFEs

Fluorescence detection of magnetic field effects in photoreactions has long been exploited for a number of systems, including: 1) geminate radical ion pairs derived from radiolytically excited aromatic hydrocarbons (*e.g.* fluorene, anthracene, naphthalene) in organic solvents (*e.g.* squalane, cyclohexane) which recombine to fluorescent excited states [58–64]; and 2) photoexcited pyrene and N,N-dimethylaniline/dicyanobenzene solutions for which the resulting geminate pairs undergo spin-selective recombination to produce field-dependent fluorescent exciplexes [65, 66]. All of these systems exhibit reactions that form luminescent products directly from the radical pair and are considered to be ‘delayed’ fluorescence variants of the Magnetically Altered Reaction Yield (MARY) technique. A non-‘delayed’ two-colour approach, involving a second excitation source to induce fluorescence in a reaction product, has been used to study the photochemistry of anthraquinone in micellar solutions [67].

Despite appreciable quantum yields¹, flavin fluorescence has not been used for MFE studies to date. However, it has been exploited in diverse areas such as: conformational studies [68], assignment of amino acid residues involved in electron transfer in flavoproteins [69, 70], determination of rotational correlation times [71, 72], and to study the energy metabolism in cells by imaging intracellular concentrations of flavin [73].

2.2 Basis of PF MARY

As discussed in the Introduction, following blue-light photoexcitation the flavin, F, forms the fluorescent singlet excited state ($^1F^*$) which undergoes intersystem crossing to the triplet state on the timescale of a few nanoseconds ($k_{ISC} = 10^8 \text{ s}^{-1}$ for FMN at pH 7 [35]). The reactive triplet state is prone to reduction by intermolecular electron transfer with suitable donor species (D). Due to the conservation of total spin angular momentum, the triplet geminate radical pair $^3(F^{\bullet-} D^{\bullet+})$ is produced. For intermolecular systems at relatively low quencher concentrations ($< \text{mM}$) as studied in this chapter, this is the predominant reaction pathway as the singlet-born RP contributes $< 10\%$ overall. The photocycle for the dominant triplet-born SCRPs is shown in Figure 2.1.

¹The fluorescence quantum yield for FMN (pH 7, phosphate buffer, 298 K) is 0.26 [34].

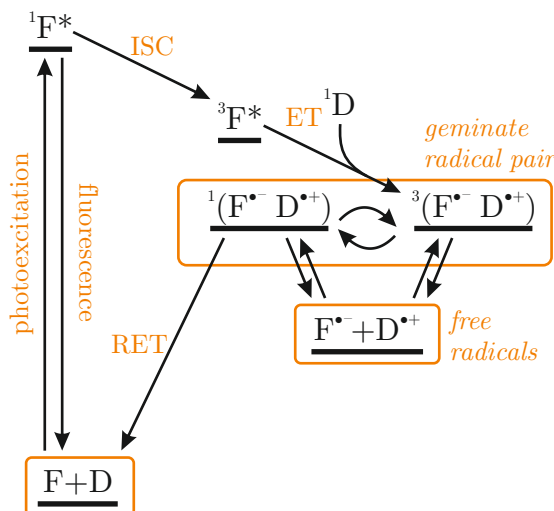


FIGURE 2.1: Photochemical reaction scheme for the formation of intermolecular radical pairs involving flavin (F) and a suitable electron donor (D). Following photoexcitation, the flavin excited state ($^1F^*$) undergoes rapid intersystem crossing (ISC) to form the triplet state ($^3F^*$), which is then susceptible to electron transfer (ET), resulting in the triplet-born spin-correlated radical pair. Magnetic field-dependent singlet-triplet mixing of the geminate pair is detected via the prompt fluorescence from $^1F^*$ under conditions of continuous illumination.

In the presence of a magnetic field of intensity larger than the effective hyperfine couplings, the extent of spin mixing between the singlet and triplet spin states of the geminate pair is reduced. For triplet-born radicals, the result is a reduced singlet character for the geminate pair. As geminate recombination occurs exclusively from the singlet state (via reverse electron transfer, RET), the concentration of free radicals increases with increasing applied magnetic field strength (*i.e.* the ‘normal’ MFE as defined in the Introduction). Previously, field-dependent radical pair concentrations have been monitored experimentally using absorption-based methods [38–44], whereas here the MFE is detected *via* the flavin ‘prompt’ fluorescence and is referred to as ‘prompt’ fluorescence (PF) MARY.

2.3 Experimental methods

2.3.1 Apparatus

The experimental arrangement used for PF MARY is depicted in Figure 2.2. Solutions of the flavin/donor model systems were flowed gravimetrically at a rate of ~ 0.25 ml/min through a sample cell (Starna Scientific, UV Quartz, 45/Q, 1 mm optical pathlength) so

as to minimise photodegradation. Static magnetic fields of up to 14 mT were generated by a pair of Helmholtz coils positioned around the sample cell, reaching a desired field value within 5 ms. A continuous wave diode laser (Power Technology, 405 nm, 350 mW; ≈ 1 mm beam diameter) served the dual purpose of photoinduced generation of the radicals and prompting the fluorescence from $^1F^*$. The sample fluorescence was recorded for varying strengths of the applied magnetic field. To ensure that the magnetic field was set to the desired value for each measurement, a delay time (T) of 50 ms was introduced after changing the field strength. The collected light was focused using an achromatic doubled pair into an optical fibre coupled to a spectrograph (Andor Technology, SR-303i) and charge-coupled device (CCD) camera (Andor Technology, Newton) arrangement for a spectrally-resolved detection scheme. An improved fluorescence collection efficiency was achieved by placing a concave mirror at the rear of the optical cell. A 420 nm long-pass filter was employed to block scattered light from the diode laser. All studies were conducted at room temperature (*ca.* 298 K) using field strengths set between 0–14 mT, averaging 100–300 times at each field value in a random order. A control program (originally written by Jonathan Storey) was modified and used for the experiments.

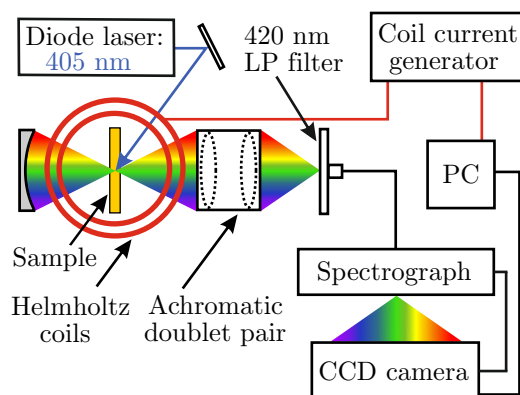


FIGURE 2.2: Experimental arrangement used for PF MARY, details in the text.

2.3.2 Materials

Flavin mononucleotide (FMN), riboflavin (RF), hen egg white lysozyme (HEWL), tryptophan (TrpH), indole, tyrosine, 4-aminopyridine (4-AP), guanosine monophosphate (GMP), adenosine triphosphate (ATP), α -lactalbumin, bovine serum albumin (BSA), human serum albumin (HSA), DNA (salmon testes, molecular weight = 1.3×10^6 Da), ribonuclease A (RNase A) and glycerol were purchased from Sigma Aldrich and used

without further purification. All solutions were prepared using ultrapure water (Milli-Q, $18.2 \text{ M}\Omega \cdot \text{cm}$).

2.4 Results and discussion

2.4.1 Flavin/HEWL

The field-dependent photochemistry of the radical pair derived from photoexcited flavin/HEWL solutions has previously been characterised in some detail, thereby making it an ideal candidate for proof-of-principle studies [43, 74, 75]. Furthermore, the system contains the same redox active residues as in cryptochromes as photoinduced electron transfer occurs between the flavin and a surface-accessible tryptophan residue on the lysozyme [76].

2.4.1.1 Two-state model

Under continuous illumination, photostationary concentrations of the ground state F and free radical $\text{F}^{\bullet-}$ (lifetime, $\tau = 1 - 10 \text{ ms}$ [43]) are far higher than those of the shorter-lived $^1\text{F}^*$ ($\tau = 4.6 \text{ ns}$ [34]), $^3\text{F}^*$ ($\tau = 1 - 10 \mu\text{s}$ [43]), and $^1,^3(\text{F}^{\bullet-} \text{D}^{\bullet+})$ ($\tau \approx 10 - 100 \text{ ns}$ [3]). Thus, to a reasonable approximation, a two-state system follows:

$$[\text{F}]_{\text{tot}} = [\text{F}] + [\text{F}^{\bullet-}]. \quad (2.1)$$

The relative proportions of F and $\text{F}^{\bullet-}$ depend on the effective rate constants of free radical formation ($\approx 10^3 \text{ s}^{-1}$, see Appendix 3) and decay ($10^9 - 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, diffusion controlled). A direct consequence of Equation 2.1 is that any magnetic field-dependence of $[\text{F}^{\bullet-}]$ translates directly to an MFE on $[\text{F}]$ and therefore on the fluorescence of $^1\text{F}^*$. If the magnetic field intensity is increased and spin mixing becomes less efficient, fewer radical pairs recombine via the singlet geminate pair and the free radical concentration increases. Therefore, a reduction in the ground state concentration and in the ‘prompt’ fluorescence from $^1\text{F}^*$, *i.e.* a negative MFE, is anticipated in the ‘normal’ MFE field regime for triplet-born radical pairs.

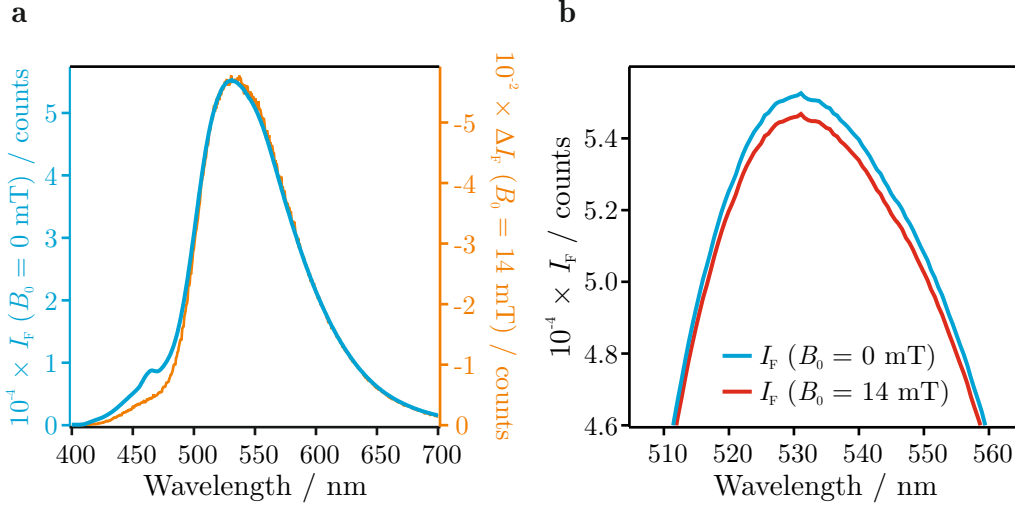


FIGURE 2.3: a) Fluorescence intensity profile, I_F , for 10 μM FMN/0.5 mM HEWL (aq) in the absence of an applied magnetic field, $B_0 = 0$ mT (blue, left-hand y-axis); and the fluorescence intensity subtraction profile (orange, right-hand y-axis with a negative scale), ΔI_F , at magnetic field strength, $B_0 = 14$ mT. b) Fluorescence intensity profiles obtained in the absence (blue) and presence (red) of a 14 mT magnetic field.

The fluorescence profile for 10 μM FMN/0.5 mM HEWL (aq) in the absence of an applied magnetic field is displayed in Figure 2.3a (blue). Spectrally, it is indistinguishable from aqueous samples containing FMN only (see Figure 1.4 [33]). A minor feature at 470 nm is ascribed to the Raman scattering of the 405 nm laser light by water. Figure 2.3b shows the sample's fluorescence intensity to decrease upon application of a 14 mT magnetic field (red). The orange trace in Figure 2.3a is the subtraction of the two profiles, $\Delta I_F(14 \text{ mT}) = I_F(14 \text{ mT}) - I_F(0)$, and its inverted form can be overlaid on the full fluorescence profile in the absence of magnetic field (blue). As expected, the overlaid traces match perfectly apart from the field-independent Raman band. The magnetosensitive fluorescence is exclusively assigned to $^1\text{FMN}^*$ and the negative MFE is reassuringly consistent with a triplet-born SCRP.

The MFE at magnetic field strength B_0 is here defined as:

$$\text{MFE}(B_0) = 100\% \cdot \Delta I_F(B_0)/I_F(0). \quad (2.2)$$

The field dependence for the photoilluminated FMN/HEWL solution is shown in Figure 2.4 (black triangles) where the MFE(B_0), averaged over the wavelength range 500–600 nm, is plotted as a function of the applied field strength, B_0 . As the extent of

spin mixing within the geminate pair decreases with increasing applied magnetic field strength, a Lorentzian-like profile is observed. This occurs at field intensities which are intermediate to the ‘zero’ and ‘high magnetic field’ regimes discussed in the Introduction. In agreement with all prior studies of the system, no Low Field Effect (*i.e.* inverted sign of the MFE) is observed in the low-field region. This implies that the spin correlation of the radical pair is too short-lived for a weak magnetic field to affect its spin dynamics significantly [43, 74, 75].

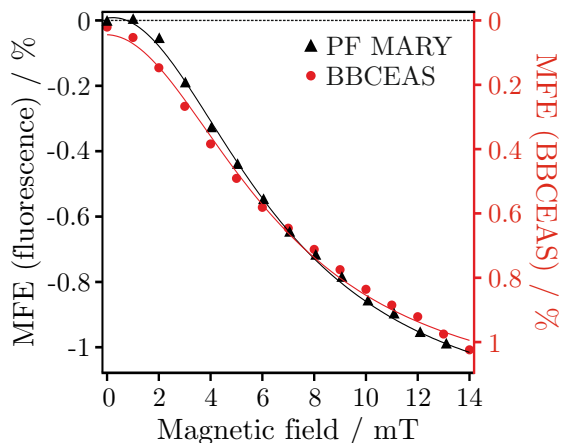


FIGURE 2.4: MFE profiles for 10 μ M FMN/0.5 mM HEWL (aq) as obtained by BBCEAS (red circles) from [75] and PF MARY (black triangles). The standard error bars for the PF MARY plots shown in Figure 2.4 and other figures in this thesis are smaller or equal to the marker size. The lines are Lorentzian fits to the data.

The MFE for the same system obtained by following the radical concentration using BroadBand Cavity Enhanced Absorption Spectroscopy (BBCEAS) [75] is also shown in Figure 2.4 (red circles). This technique combines the advantages of boosted sensitivity from optical cavity-based detection and pseudo-white light supercontinuum radiation to enable highly sensitive, simultaneous absorption measurement across the visible spectrum. In Figure 2.4, the MARY plot is averaged over the absorption band for the flavin radical, $F^{\bullet-}$, region (530–600 nm).

The MARY plots derived by prompt fluorescence and BBCEAS can be related by the two-state model in Equation 2.1 as follows:

$$\text{MFE}(B_0, \text{PF}) \propto \frac{[\text{F}(B_0)] - [\text{F}(0)]}{[\text{F}(0)]} = \frac{([\text{F}]_{\text{tot}} - [\text{F}^{\bullet-}(B_0)]) - ([\text{F}]_{\text{tot}} - [\text{F}^{\bullet-}(0)])}{[\text{F}(0)]} \quad (2.3a)$$

$$\text{MFE}(B_0, \text{BBCEAS}) \propto \frac{[\text{F}^{\bullet-}(B_0)] - [\text{F}^{\bullet-}(0)]}{[\text{F}^{\bullet-}(0)]} \quad (2.3b)$$

$$\frac{\text{MFE}(B_0, \text{PF})}{\text{MFE}(B_0, \text{BBCEAS})} = -\frac{[\text{F}^{\bullet-}(B_0)] - [\text{F}^{\bullet-}(0)]}{[\text{F}^{\bullet-}(B_0)] - [\text{F}^{\bullet-}(0)]} \cdot \frac{[\text{F}^{\bullet-}(0)]}{[\text{F}(0)]} = -\frac{[\text{F}^{\bullet-}(0)]}{[\text{F}(0)]}. \quad (2.3c)$$

Consequently, the identical magnitudes for the MFEs measured by the two methods are not generally expected and, under these photoexcitation conditions, suggest that the photostationary state has approximately equal populations of F and $\text{F}^{\bullet-}$.

2.4.1.2 MFE behaviour by the two-state model

Despite its approximate nature, the two-state model readily predicts many behavioural aspects of the MFE. From Equations 2.1 and 2.3, the maximum magnitude for the MFE detected by prompt fluorescence occurs when $[\text{F}(0)] \rightarrow 0$, and thus $[\text{F}^{\bullet-}(0)] \rightarrow [\text{F}]_{\text{total}}$.

From the two-state model, the (pseudo-)steady-state expressions for the photostationary flavin radical concentrations are:

$$\frac{d[\text{F}^{\bullet-}(B_0)]}{dt} = I_{\text{abs}}(B_0) \cdot \Phi_{\text{FR}}(B_0) - k_{\text{re}}[\text{F}^{\bullet-}(B_0)][\text{D}^{\bullet+}(B_0)] = 0 \quad (2.4a)$$

$$= I_{\text{abs}}(B_0) \cdot \Phi_{\text{FR}}(B_0) - k_{\text{re}}[\text{F}^{\bullet-}(B_0)]^2 = 0 \quad (2.4b)$$

where $I_{\text{abs}}(B_0)$ is the rate of $\text{F} \rightarrow {}^1\text{F}^*$ excitation at B_0 ; $\Phi_{\text{FR}}(B_0)$ is the magnetic field-dependent quantum yield for free radical (FR) formation; k_{re} is bimolecular rate constant for free radical recombination to the ground state species; and $[\text{F}^{\bullet-}(B_0)]$ and $[\text{D}^{\bullet+}(B_0)]$ are the free radical concentrations at B_0 . Here, the bimolecular rate of free radical removal is re-expressed as $k_{\text{re}}[\text{F}^{\bullet-}(B_0)]^2$ due to $[\text{F}^{\bullet-}(B_0)] = [\text{D}^{\bullet+}(B_0)]$.

In the limit of low sample absorbance, $A = \log_{10}(e) \cdot \alpha[\text{F}(B_0)]l \rightarrow 0$, $I_{\text{abs}}(B_0) \rightarrow I_0\alpha[\text{F}(B_0)]l$ where α is the extinction coefficient of F at the pump wavelength; l is

the pathlength; and I_0 is the rate of incident photons on the sample. Further, the photostationary flavin radical concentration can be evaluated as follows:

$$\frac{d[\mathbf{F}^{\bullet-}(B_0)]}{dt} = I_0\alpha l[\mathbf{F}(B_0)]\Phi_{\text{FR}}(B_0) - k_{\text{re}}[\mathbf{F}^{\bullet-}(B_0)]^2 = 0 \quad (2.5a)$$

$$= I_0\alpha l([\mathbf{F}]_{\text{tot}} - [\mathbf{F}^{\bullet-}(B_0)])\Phi_{\text{FR}}(B_0) - k_{\text{re}}[\mathbf{F}^{\bullet-}(B_0)]^2 = 0, \quad (2.5b)$$

thus,

$$k_{\text{re}}[\mathbf{F}^{\bullet-}(B_0)]^2 + I_0\alpha l\Phi_{\text{FR}}(B_0)[\mathbf{F}^{\bullet-}(B_0)] - I_0\alpha l\Phi_{\text{FR}}(B_0)[\mathbf{F}]_{\text{tot}} = 0, \quad (2.6)$$

and

$$[\mathbf{F}^{\bullet-}(B_0)] = \frac{-I_0\alpha l\Phi_{\text{FR}}(B_0) + \sqrt{(I_0\alpha l\Phi_{\text{FR}}(B_0))^2 + 4I_0\alpha l\Phi_{\text{FR}}(B_0)[\mathbf{F}]_{\text{tot}}k_{\text{re}}}}{2k_{\text{re}}} \quad (2.7a)$$

$$= \frac{I_0\alpha l\Phi_{\text{FR}}(B_0)}{2k_{\text{re}}} \left(\sqrt{1 + \frac{4[\mathbf{F}]_{\text{tot}}k_{\text{re}}}{I_0\alpha l\Phi_{\text{FR}}(B_0)}} - 1 \right). \quad (2.7b)$$

An expression for the MFE detected by prompt fluorescence is obtained by substitution of $[\mathbf{F}^{\bullet-}(B_0)]$ from Equation 2.7 into Equations 2.1 and 2.3a:

$$\text{MFE}(B_0, \text{PF}) = \frac{\Phi_{\text{FR}}(0) \left(\sqrt{1 + z/\Phi_{\text{FR}}(0)} - 1 \right) - \Phi_{\text{FR}}(B_0) \left(\sqrt{1 + z/\Phi_{\text{FR}}(B_0)} - 1 \right)}{z/2 - \Phi_{\text{FR}}(0) \left(\sqrt{1 + z/\Phi_{\text{FR}}(0)} - 1 \right)}, \quad (2.8)$$

where $z = (4[\mathbf{F}]_{\text{tot}}k_{\text{re}})/(I_0\alpha l)$. From this expression, the magnitude of $\text{MFE}(B_0, \text{PF})$ can be seen to increase with decreasing z . Further, in the limit of $z \rightarrow 0$, $\text{MFE}(B_0, \text{PF}) \rightarrow (\Phi_{\text{FR}}(0) - \Phi_{\text{FR}}(B_0))/\Phi_{\text{FR}}(B_0)$; whereas for $z \rightarrow \infty$, $\text{MFE}(B_0, \text{PF}) \rightarrow 0$. These limits can be determined using L'Hospital's Rule, see Appendix 3. In practical terms, z can be decreased by: i) increasing the pump power of photoexcitation (I_0); ii) lowering the total fluorophore concentration ($[\mathbf{F}]_{\text{tot}}$); iii) lowering k_{re} , the rate constant for recombination

of the free radicals (*e.g.* in more viscous solvents for predominantly diffusion-controlled reactions)².

2.4.1.3 Varying pump power and flavin concentration

The pump power dependence (1.8–350 mW) of the MFE for aqueous solutions of 10 μM FMN/0.4 mM HEWL at 12 mT is shown in Figure 2.5a (orange circles). Figure 2.5b (blue circles) depicts the MFE for samples of varying fluorophore concentration ($[\text{F}]_{\text{tot}} = 10\text{--}200\ \mu\text{M}$) and 0.4 mM HEWL (aq), with fixed pump power (350 mW). Both studies exhibit the qualitative trends predicted by the two-state model ($\|\text{MFE}(12\text{ mT}, \text{PF})\|$ increases with increasing I_0 and decreasing $[\text{F}]_{\text{tot}}$, note the negative y-scale of the graphs).

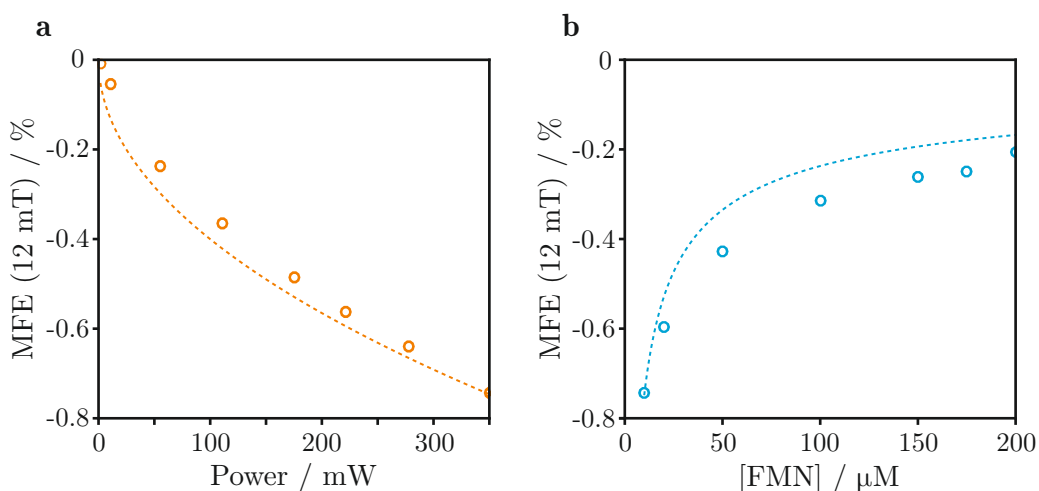


FIGURE 2.5: a) Pump power dependence (1.8–350 mW) of the PF MFE(12 mT) for aqueous solutions of 10 μM FMN/0.4 mM HEWL. b) Fluorophore concentration dependence of the PF MFE(12 mT) for aqueous solutions of 10–200 μM FMN/0.4 mM HEWL, with photoexcitation pump power fixed at 350 mW. The PF MFE values are averaged over the wavelength range 500–600 nm.

The dotted lines in Figure 2.5 indicate the fit to the data using Equation 2.8. Setting $(4k_{\text{re}})/(\alpha l) = 3.5 \times 10^7\ \text{s}^{-1}$ (from the parameter values stated in Appendix 3) and $\Phi_{\text{FR}}(0) = 0.33$ [32, 34, 35, 77], simultaneous fitting of the MFE dependence for I_0 and $[\text{F}]_{\text{tot}}$ yields $\Phi_{\text{FR}}(12\text{ mT}) = 0.36$. The estimated $\text{MFE}(12\text{ mT}) = 10\%$ for the quantum yield of free radicals somewhat exceeds that found by Miura et al. (4% [43]) for FMN/HEWL (aq).

²The two-state model for predicting the behaviour of prompt fluorescence MFEs relies on the assumption that $\text{F}^{\bullet-}$ and $\text{D}^{\bullet+}$ react exclusively with each other. This assumption is not generally applicable as found in Chapters 4 and 5, but is appropriate for prompt effects, χ_{p} , as distinguished later.

However, the two-state model's failure in this regard is perhaps not unexpected as flavin photochemistry is more complex than discussed so far. 1) Photoproducts such as lumichrome [78] result from the photodegradation of flavins. 2) Disproportionation of the neutral flavin radicals formed by protonation of $F^{\bullet-}$ produces ground state flavin, F, and its fully reduced form, FH_2 [79],



As both lumichrome and FH_2 could inherit appreciable MFEs, the two-state model is compromised. The formation of lumichrome was confirmed by a decrease in sample absorbance between 400–500 nm and an increase in the fluorescence below 475 nm, *c.f.*[78]. Further evidence was found by mass spectrometry (see Appendix 3). From Figure 2.6 it is apparent that the production of these species is much reduced if the sample is flowed (green dashed lines).

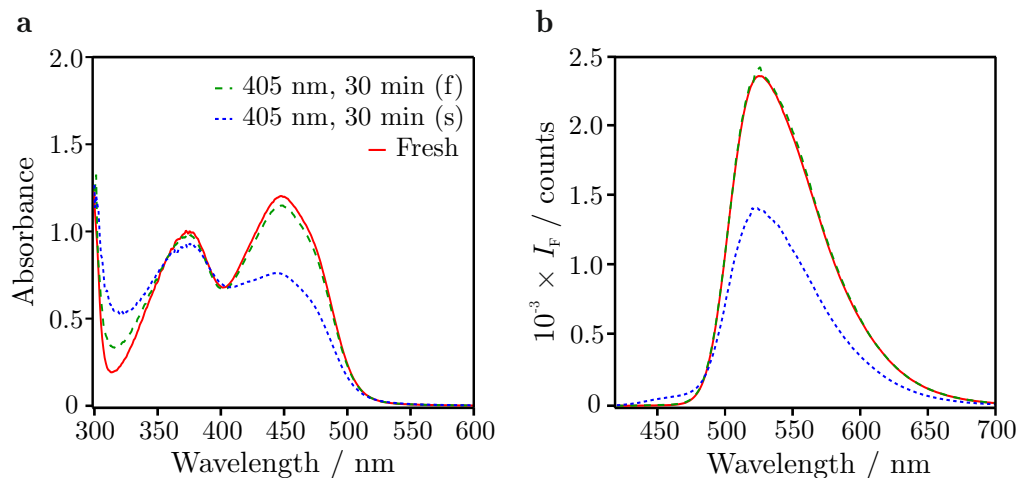


FIGURE 2.6: a) UV/vis and b) fluorescence profiles of 100 μ M FMN/0.5 mM HEWL in aqueous solution (solid red line, 'Fresh'). Following continuous photoexcitation by a 405 nm laser at 350 mW for 30 min, the spectra are virtually unaltered if the solution is continually refreshed as in the experiments conducted here (flow rate 0.5 ml min⁻¹, dashed green line, f) but significant changes are observed for a static sample (dotted blue line, s).

Further, a third reaction neglected by the two-state model is flavin triplet-triplet annihilation which gains greater significance with increasing $[^3F^*]$ [80].



The ‘ $F^{\bullet-} + F^{\bullet+}$ ’ ion pair is also formed by self-quenching reactions between $^3F^*$ and ground state F [81]. However, for the studies in this thesis, these second order $^3F^*$ processes can be largely ignored as the photostationary concentrations of $[^3F^*] \approx \text{nM}$ and $[F] \approx \mu\text{M}$ are much lower than $[D] \approx \text{mM}$. Radical pair formation is expected to be the predominant reaction pathway for the flavin triplet excited state.

2.4.1.4 $B_{1/2}$ and spin-dephasing

The relative slope of the MFE with respect to the applied magnetic field is unique to each radical pair and its individual spin dynamics. This effect can be characterised by the empirical parameter, $B_{1/2}$, defining the magnetic field at which the MFE reaches half its maximum magnitude (*i.e.* $|\text{MFE}_{\text{sat}}|$) by the hyperfine mechanism; it is typically obtained by Lorentzian fitting. As a first approximation, $B_{1/2}$ is considered by the simple Weller equation prediction [82]:

$$B_{1/2(\text{Weller})} = 2 \frac{B_1^2 + B_2^2}{B_1 + B_2} \quad (2.11)$$

where B_x is the effective hyperfine coupling constant for radical $x = 1, 2$:

$$B_x = \left(\sum_i a_{xi}^2 I_{xi}(I_{xi} + 1) \right)^{1/2} \quad (2.12)$$

with I_{ix} being the spin quantum number of nuclear spin i with hyperfine coupling constant a_{ix} in radical x . The estimated value of $B_{1/2}$ for the RP derived from FMN/TrpH(in HEWL) is 3 mT. This value is based on the $\text{FMNH}^\bullet/\text{Trp}^\bullet$ geminate pair, being the thermodynamically most stable form for aqueous solutions of 0.5 mM HEWL, pH 4.5, as $\text{p}K_a(\text{TrpH}^{\bullet+}) = 4.3$ [83] and $\text{p}K_a(\text{FMNH}^\bullet) = 8.5$ [84]. All other feasible RPs — *i.e.* combinations of $\text{FMNH}^\bullet/\text{FMN}^{\bullet-}$ and $\text{TrpH}^{\bullet+}/\text{Trp}^\bullet$ — display little deviations ($\pm 0.2 \text{ mT}$) for their Weller estimates as the presence/absence of a single proton hyperfine interaction in each radical is largely insignificant overall. Further details on the estimates, including the hyperfine parameters used for the calculations, can be found in Appendix 3.

Experimentally, a larger $B_{1/2}$ value of 6.5 ± 0.1 mT is found, consistent with previous findings implying significant spin-dephasing [43, 74, 75].

There are two main types of dephasing processes to consider: singlet-triplet dephasing (STD) and triplet-triplet dephasing (TTD) [85–90]. The former, STD, is generally considered to result from stochastic modulation of the electron exchange interaction, caused by re-encounter of the RP. The latter, TTD, arises due to the random modulation of anisotropic dipole-dipole interactions. Further, there are two such interactions for TTD: 1) the electron-electron dipolar interaction which fluctuates by the rotational diffusion of the RP as a whole and the re-encounter process (r^{-3} dependence for separation, r); 2) the hyperfine interaction which is modulated by the rotational diffusion of each radical component in the RP. The dipolar interactions can also induce STD.

Despite similar origins to the relaxation mechanisms discussed in the Introduction, it is noteworthy that these interactions do not affect singlet-triplet population transfer (*i.e.* spin mixing) directly. However, their effects are manifested in co-operation with the hyperfine mechanism. A broadening of the MFE field-dependence is observed, becoming more pronounced with increasing geminate pair lifetime and increasing rate of dephasing.

As highlighted by Maeda et al., in the uppermost limit of highly efficient dephasing, the overall spin mixing process appears indistinguishable from relaxation [90]. A useful guideline for three regimes of spin mixing has been proposed:

1. hyperfine mechanism ($t \lesssim 10$ ns, $B_0 \lesssim B_{1/2(\text{Weller})}$);
2. hyperfine mechanism combined with fast spin-dephasing (10 ns $< t < 300$ ns, $B_{1/2(\text{Weller})} < B_0 < 10B_{1/2(\text{Weller})}$);
3. relaxation mechanism ($t > 300$ ns, $B_0 > 10B_{1/2(\text{Weller})}$).

The significance of dephasing is enhanced in the FMN/HEWL system due to the slowly diffusing nature of the protein (diffusion coefficient, $D_{\text{HEWL}} = 1.2 \times 10^{-10}$ m² s⁻¹ [91]), leading to the high probability of multiple re-encounters that boosts STD and TTD. This is further reinforced by the Coulombic attraction of the negatively charged FMN and the positively charged protein (isoelectronic point $pI = 11.35$) which also increases the lifetime of the geminate pair.

If TTD (*i.e.* destruction of coherences by spin-lattice relaxation) is dominant, from Equation 1.16a it is instructive to also evaluate the rotational correlation time (τ_c) of the larger, more slowly tumbling component in the radical pair: here this is HEWL as $\tau_c(\text{FMN}) = 155 \text{ ps}$ [71] and $\tau_c(\text{HEWL}) = 7.2 \text{ ns}$ [92].

In field regime 2 (as defined above, $B_0 < 10B_{1/2(\text{Weller})}$ [90]), the slower the rotational and translational motion of the radicals, the more pronounced is spin-dephasing and the greater is the deviation of the experimental $B_{1/2}$ from the Weller formula.

There are no statistically significant deviations in the $B_{1/2}$ values obtained for photoilluminated solutions of FMN/HEWL upon varying the pump power (1.8–350 mW) and flavin concentration (10–200 μM), confirming that the magnetosensitivity in these systems stem from identical RP spin dynamics. This invariance is also implied by Equation 2.8 when the photostationary concentrations are such that $[\text{F}] \gg [\text{F}^{\bullet-}]$ and $\Phi_{\text{FR}}(B_0) \approx \Phi_{\text{FR}}(0)$. Under these conditions, Equation 2.8 is well-approximated by $\gamma \cdot (\Phi_{\text{FR}}(0) - \Phi_{\text{FR}}(B_0)) / \Phi_{\text{FR}}(0)$ where $\gamma = \frac{2\Phi_{\text{FR}}(0)}{z} (1 - \sqrt{1 + z/\Phi_{\text{FR}}(0)})$.

2.4.1.5 Altering the spin dynamics

In efforts to alter the spin dynamics of the RP in flavin/HEWL systems, the effects of changing the dielectric constant of the solvent have been investigated (Figure 2.7). As previously shown by Maeda et al. [43], the addition of salt (NaCl) screens the Coulombic attraction between the negatively charged flavin radical and the positively charged HEWL, resulting in a shorter geminate lifetime of the radical pair and concomitant decrease in the MFE (but no significant change in $B_{1/2}$, *cf.* filled and open red triangles in Figure 2.7). Furthermore, the field dependence for an aqueous solution of riboflavin (RF)/HEWL was measured (filled blue diamonds). RF and FMN are photochemically similar due to their common isoalloxazine moiety but principally differ in the absence of a charged phosphate group for RF (RF/HEWL: $B_{1/2} = 5.9 \pm 0.1 \text{ mT}$). Intuitively, this leads to a reduced Coulombic attraction for RF to HEWL and its PF MARY profile exhibits no salt dependence ($B_{1/2}(\text{NaCl}) = 6.0 \pm 0.1 \text{ mT}$, open blue diamonds).

Increasing the viscosity of the solution increases the contribution of spin-dephasing and broadens the MARY curves. To fix the dielectric constant (relative permittivity) of the samples, studies were conducted for aqueous solutions of sodium chloride (1.47 M [93])

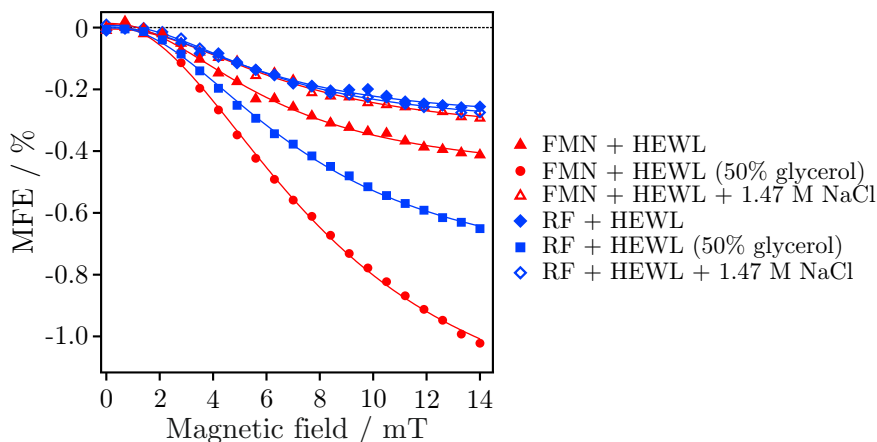


FIGURE 2.7: PF MARY profiles for 10 μ M FMN/0.2 mM HEWL and 10 μ M RF/0.2 mM HEWL in aqueous solutions with varying NaCl concentration and glycerol content (% by volume), averaged over the wavelength range 500–600 nm. The lines are Lorentzian fits to the data.

and 50% glycerol without NaCl: RF(NaCl $\rightarrow$ 50 % glycerol): $B_{1/2} = 6.0 \pm 0.1$ mT $\rightarrow$ 7.4 ± 0.1 mT (*cf.* open blue diamonds and filled blue squares in Figure 2.7); FMN(NaCl $\rightarrow$ 50% glycerol): $B_{1/2} = 6.7 \pm 0.1$ mT $\rightarrow$ 8.2 ± 0.1 mT (*cf.* open red triangles and filled red circles in Figure 2.7). Moreover, the size of the observed MFE also grows with solvent viscosity as expected from Equation 2.8 when considering k_{re} to be diffusion-controlled ($\propto 1/\eta$). A more extensive investigation of the solvent viscosity dependence for spin-dephasing and MFEs is discussed for flavin/tryptophan (free amino acid) RPs below.

2.4.2 Flavin/tryptophan

2.4.2.1 Altering the spin dynamics

The PF MARY profile for 10 μ M FMN/0.5 mM TrpH (pH 6.2, aq) is shown in Figure 2.8. Although a FMN-TrpH radical pair is also formed as above, a smaller MFE and narrower field-dependence profile is observed when HEWL is replaced by free TrpH (filled red triangles). The experimentally determined $B_{1/2}$ of 3.5 ± 0.1 mT is broader than the Weller prediction but less than for FMN/HEWL, suggesting reduced spin-dephasing contributions. This observation is easily rationalised on the basis of the free amino acid's higher diffusion coefficient ($D_{\text{TrpH}} = 6.5 \times 10^{-10}$ m² s⁻¹ [56]) and faster rotational correlation time ($\tau_c = 40$ ps [94]), overall leading to reduced STD and TTD.

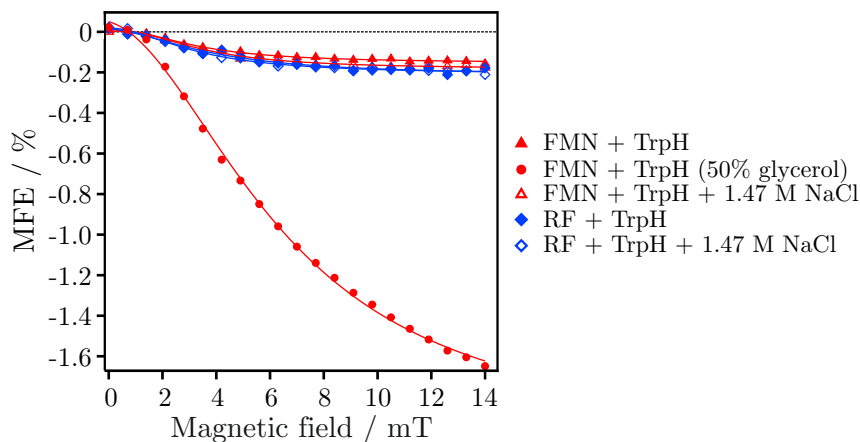


FIGURE 2.8: PF MARY profiles for 10 μ M FMN/0.5 mM TrpH and 10 μ M RF/0.5 mM TrpH in aqueous solutions with varying NaCl concentration and glycerol content (% by volume), averaged over the wavelength range 500–600 nm. The lines are Lorentzian fits to the data.

For the flavin/TrpH systems, varying the salt concentration and replacing FMN with riboflavin leads to no discernible change in the MARY profiles (see Figure 2.8, open red triangles and filled blue diamonds). This is not unexpected as the tryptophan radical exists in its neutral form at pH 6.2; thus, there can be no Coulombic attraction between the RP components. As above, in the presence of more viscous solvents (*i.e.* with higher glycerol content), both the $B_{1/2}$ ($3.5 \pm 0.1 \rightarrow 6.8 \pm 0.3$ mT, filled red circles) and the size of the MFE increase.

Figure 2.9a) shows the PF MARY profiles for 10 μ M FMN/0.5 mM TrpH in aqueous solutions of varying glycerol content. Figure 2.9b) depicts the measured $B_{1/2}$ values as a function of solvent viscosity. A trend of increasing $B_{1/2}$ with increasing glycerol concentration is found, consistent with increasing dephasing contribution to spin mixing (see spin mixing regime 2 [90], above). Furthermore, the origin of the increased contribution is two-fold, arising from the viscosity dependence of the dephasing mechanisms and the geminate pair lifetime.

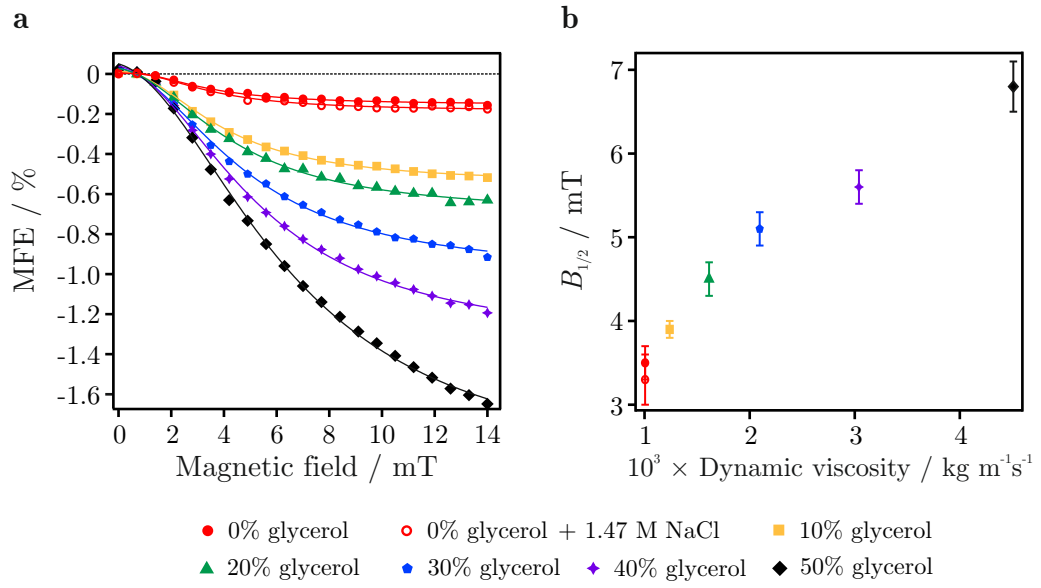


FIGURE 2.9: a) PF MARY profiles (averaged over the wavelength range 500–600 nm) for 10 μ M FMN/0.5 mM TrpH in aqueous solutions with different glycerol content (0–50 % by volume). The lines are Lorentzian fits to the data and the resultant $B_{1/2}$ values are plotted as a function of solvent viscosity in b) [95].

2.4.3 Other systems

The relative difference in the $B_{1/2}$ values obtained from MARY curves for the magnetosensitive photoreactions of RPs comprising ‘large’ (*e.g.* HEWL) and ‘small’ (*e.g.* free tryptophan amino acid) donor species is a general phenomenon.

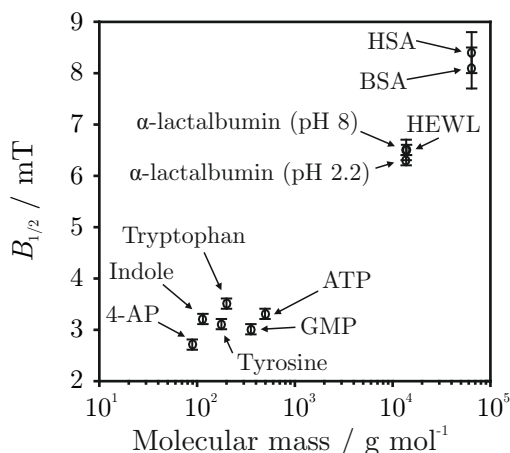


FIGURE 2.10: $B_{1/2}$ of PF MARY profiles as function of the molecular mass of the donor, D. The MARY curves were derived for aqueous solutions of 10 μ M FMN/donor (D), where D = 4-AP (1 mM), indole (1 mM), tyrosine (0.5 mM), tryptophan (0.5 mM), GMP (1.0 mM), ATP (1 mM), α -lactalbumin (pH 2.2 and pH 8), HEWL (0.5 mM), BSA (0.1 mM), HSA (0.1 mM).

Figure 2.10 shows the experimentally-determined $B_{1/2}$ value versus donor molecular mass for aqueous FMN/D solutions, where D = 4-aminopyridine (4-AP), indole, tyrosine, tryptophan, guanosine monophosphate (GMP), adenosine triphosphate (ATP), α -lactalbumin (pH 2.2 and pH 8), HEWL, bovine serum albumin (BSA), human serum albumin (HSA). A general trend of broader MFE field-dependence (*i.e.* increasing $B_{1/2}$) is observed with increasing the molecular mass of the donor species. As aforementioned, the broadening is indicative of an increased significance of dephasing in spin mixing for the RP. Thus the trend follows the notion that dephasing becomes ever more significant as increased donor size results in slower relative motion of the radicals, manifested in a lower diffusion constant, D , and longer rotational correlation time, τ_c , of the donor, and prolonged radical pair lifetime.

MFE measurements for FMN/ α -lactalbumin at different pH were conducted to study the radical pairs arising by electron transfer from the protein in the molten globule (pH 2.2, citric acid buffer) and native state (pH 8, tris buffer). However, there was no statistically significant difference in the resultant MARY curves despite anticipated differences in the

efficiency of electron transfer due to the $\sim 10\%$ longer rotational correlation time for the molten globule state ($9.4\text{ ns} \rightarrow 10.5\text{ ns}$) [96].

Furthermore, no significant difference was found in the MFE profiles for radical pairs derived from FMN with (likely) surface-accessible tryptophan residues on BSA and HSA. This is in agreement with work conducted by Mohtat et al. [97], but conflicts with other suggestions in the literature for appreciable variation in flavin binding ability between the two proteins [98].

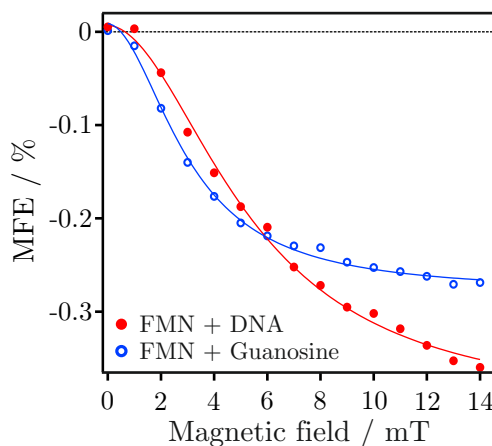


FIGURE 2.11: PF MARY profiles: $10\text{ }\mu\text{M}$ FMN/ 10 mg ml^{-1} ($\sim 7.7\text{ }\mu\text{M}$) DNA (filled red circles) and $10\text{ }\mu\text{M}$ FMN/ 1.0 mM guanosine GMP (open blue circles), averaged over the wavelength range $500\text{--}600\text{ nm}$. The lines are Lorentzian fits to the data.

A similar ‘large-small’ effect on the shape of the PF MARY curve is observed for the radical pair formed by photoinduced electron transfer from guanosine to FMN, Figure 2.11. The PF MARY curve broadens considerably when the free base guanosine ($B_{1/2} = 3.0 \pm 0.1\text{ mT}$, blue) is replaced by DNA ($B_{1/2} = 5.4 \pm 0.2\text{ mT}$, red). The DNA ($\sim 1.3 \times 10^6\text{ Da}$) used in the studies is significantly larger than all donors in Figure 2.10, yet the $B_{1/2}$ value for the associated MARY curve is far from being the largest. However, a number of studies by NMR provide evidence for considerable flexibility and faster motion than predicted for a molecule of that size [99]. The high flexibility is likely to be the principal cause for the departure from the general trend for increasing contributions of spin-dephasing and increasing $B_{1/2}$ with increasing donor molecular mass.

2.4.4 MFEs in nanomolar solutions

The high sensitivity of the fluorescence-based MFE detection is illustrated in Figure 2.12 for a solution of 1 nM FMN/0.5 mM HEWL (filled black circles). Also shown in the same figure are data obtained for (i) a solution of 1 nM FMN only (open green squares) and (ii) a 0.5 mM solution of the protein only (filled blue squares) as controls. An unambiguous MFE is detected in the FMN/HEWL solution with good signal-to-noise even under conditions that are beyond the sensitivity limits of existing transient absorption and cavity-based absorption measurements which require μM flavin concentrations.

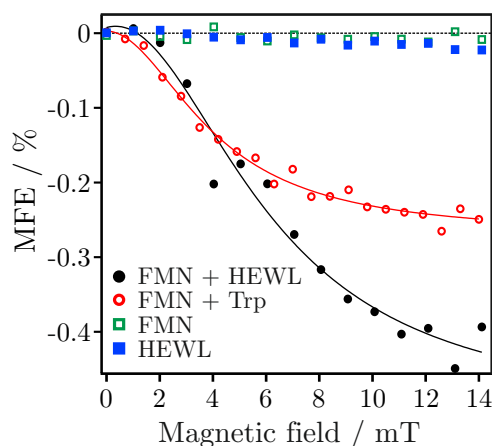


FIGURE 2.12: PF MARY profiles: 1 nM FMN/0.5 mM HEWL (filled black circles), 1 nM FMN/0.5 mM TrpH (open red circles), 1 nM FMN (open green squares) and 0.5 mM HEWL (filled blue squares), averaged over the wavelength range 500–600 nm. The red and black lines are Lorentzian fits to the data.

Notably, the size of the MFE is smaller for FMN/HEWL solutions of 1 nM photosensitiser concentration versus prior studies in the μM range (Figure 2.5b). As by Equation 2.8, if it is assumed that the flavin and donor radicals are exclusively removed by radical-radical recombination then a larger MFE is expected at lower FMN concentrations. One possible reason for the discrepancy is that fluorescent impurities in the protein become increasingly significant as the concentration of the flavin fluorophore is reduced. As seen in Figure 2.12, these impurities do not exhibit a measurable MFE but contribute to the denominator in Equation 2.2.

Furthermore, the unexpected reduction in the amplitude of the MFE can also be explained by (relatively) slow termination reactions for the radicals as discussed extensively in Chapter 4.

2.4.5 Beyond $^1\text{F}^*$ fluorescence

As highlighted in Figure 2.6, continuous illumination of static samples generate additional photoproducts to the reaction scheme presented in Figure 2.1. Appreciable reduced flavin and lumichrome concentrations may be accumulated which affect the viability of the two-state model. As the additional photoproducts also have field-dependent photostationary concentrations, any fluorescence derived from the photoexcitation of these species will also exhibit MFEs.

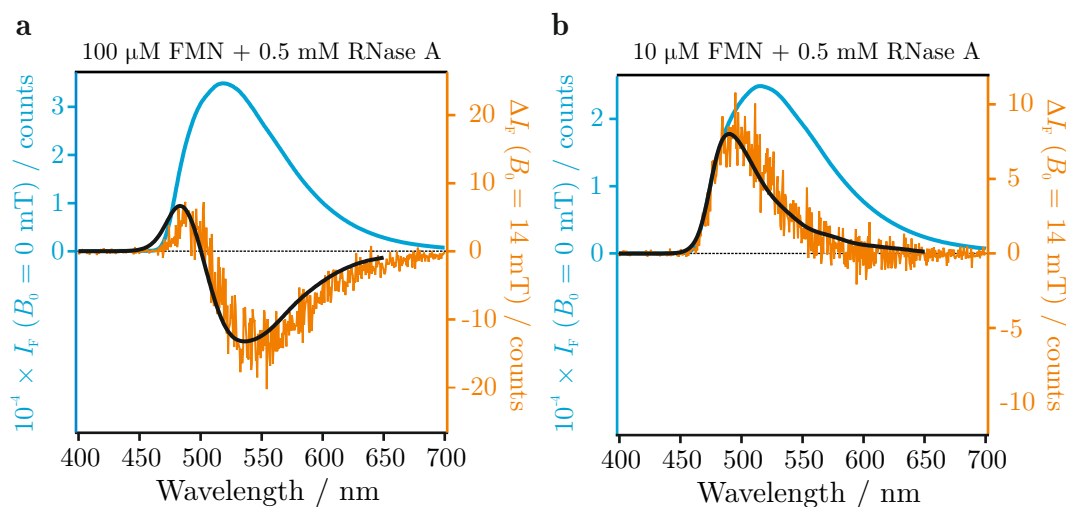


FIGURE 2.13: a) 100 μM FMN/0.5 mM RNase A, b) 10 μM FMN/0.5 mM RNase A. Fluorescence intensity profile, I_F , for aqueous solutions of a) and b) (blue, left hand y -axis) in the absence of an applied magnetic field, $B_0 = 0 \text{ mT}$; and the fluorescence intensity subtraction profile (orange, right hand y -axis), ΔI_F , at magnetic field strength, $B_0 = 14 \text{ mT}$. Fits by composite profiles as discussed in the text are indicated by solid black lines.

One of the most striking examples for non- $^1\text{F}^*$ field-dependent fluorescence is found for the FMN/RNase A photocycle whereby RPs are formed by electron transfer from surface-exposed tyrosine residues on the surface of the protein [100]. The average fluorescence profiles (blue) for 100 μM FMN/0.5 mM RNase A and 10 μM FMN/0.5 mM RNase A are given in Figure 2.13a and b, respectively. Both emission plots are blue-shifted with respect to the flowed samples in Figure 2.6: the shift being larger for the 10 μM sample. Also shown in the figure are the fluorescence subtraction profiles recorded at 14 mT (orange). The subtraction plot for 100 μM FMN contains both positive (~ 450 – 500 nm) and negative (~ 500 – 700 nm) components which can be simulated via linear combination of FMN and FMNH₂ fluorescence profiles (obtained from Figure 2.6 and [101], respectively). Analogously, the entirely positive MFE fluorescence

subtraction plot derived from the 10 μM sample can also be replicated by a composite profile. Both fits are given by solid black lines in Figure 2.13. The positive MFE component appears to derive from the fluorescence of the fully reduced flavin formed by disproportionation (Equation 2.9). A positive sign can be rationalised by the location of the reduced flavin downstream to the geminate radical pair in the photocycle, thus the reduced flavin concentration directly follows that of the flavin radical. As well as oxidation by oxygen, reduced flavin undergoes a reverse comproportionation reaction which closes the photocycle as follows:



Thus, the higher FMN concentration sample exhibits an overall lower $^1\text{FH}_2^*$ contribution due to the much increased efficiency of the comproportionation reaction.

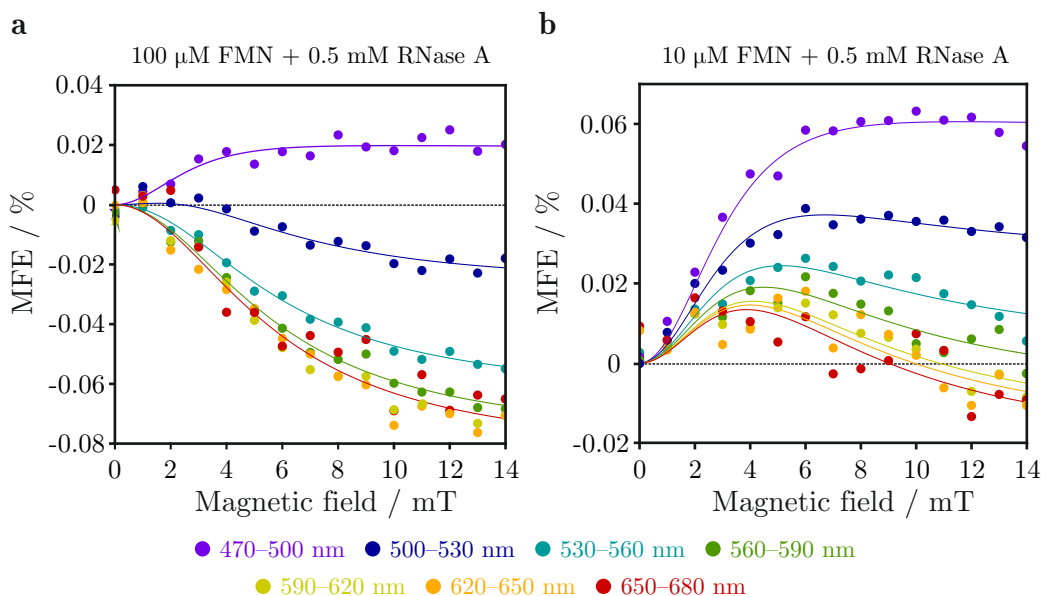


FIGURE 2.14: a) 100 μM FMN/0.5 mM RNase A, b) 10 μM FMN/0.5 mM RNase A. PF MARY profiles averaged over different wavelength ranges for aqueous solutions of a) and b). The solid lines are composite Lorentzian fits to the data.

The wavelength dependencies of the resultant PF MARY plots for the two samples is given in Figure 2.14. For both systems, on going from 680 nm $\rightarrow$ 470 nm, the MARY curves become progressively more positive in keeping with a shift in majority contribution from $^1\text{F}^*$ to $^1\text{FH}_2^*$. On first inspection, some of the MARY profiles could be misinterpreted as exhibiting LFEs. That the relative shapes of the profiles change in

addition to the sign implies the presence of multiple geminate pairs with different spin dynamics. A decomposition of the MARY profiles by a procedure analogous to a target fit reveals that the lineshapes actually correspond to a superposition of two different MARY profiles. The datasets were simultaneously fitted to a composite lineshape of two Lorentzian profiles of opposite sign with different $B_{1/2}$ values, yielding: $B_{1/2}(1) = 5.5 \text{ mT}$ and $3.3 \text{ mT} \leq B_{1/2}(2) < 5.5 \text{ mT}$. It is not inconceivable that different spin dynamics could result for RPs derived from different surface-accessible tyrosine residues on the RNase A [100].

2.4.6 No LFEs

In this chapter, MFEs have been characterised for bimolecular flavin-based radical pairs arising from quenching with HEWL, tryptophan, indole, tyrosine, 4-AP, GMP, ATP, α -lactalbumin, BSA, HSA, DNA and RNase A — none of which exhibiting a LFE. This implies that the spin correlation in these radical pair systems is universally too short-lived to affect their spin dynamics significantly at weak fields [24]. An extensive investigation into LFEs exhibited by the flavin/ascorbic acid system is presented in the next chapter. The LFE is promoted by the near-negligible hyperfine coupling associated with the ascorbyl radical and slow relaxation for radical pairs containing relatively ‘small’ donor species.

2.5 Conclusion and outlook

A new detection method for investigating MFEs in flavin-based systems has been presented in this chapter. Due to the high sensitivity of the approach, field effects have been detected for samples of concentration as low as 1 nM flavin. This technique involves monitoring magnetic field-dependent changes in the prompt fluorescence of continuously irradiated systems. With the prompt fluorescence shown to reflect the magnetosensitivity of the radical pair, the method was used to characterise the influence of external factors on the spin dynamics of the geminate pair and the size of the MFE in intermolecular reactions of flavins. The merits of the technique have led to its implementation by

others [102, 103]. In later chapters, the technique is used to unveil novel LFEs, mechanistic insights into the chemical amplification of MFEs and field-dependent photochemistry in cryptochromes.

Chapter 3

Low Field Effects in flavin-ascorbic acid charge recombination reactions

It is worth emphasising that the interaction energy for an electron spin in the presence of the Earth's weak magnetic field ($\sim 50 \mu\text{T}$) is approximately six orders of magnitude smaller than the thermal energy ($k_{\text{B}}T$) at 298 K, and 10–100 times weaker than the strength of a typical chemical bond [12]. From a thermodynamic viewpoint it would appear entirely inconceivable that such low field strengths can affect the outcome of chemical reactions. As discussed in Chapter 1, the magnetosensitivity of reactions involving radical pair intermediates can be explained by the radical pair mechanism. The intermediates are formed in non-equilibrium states for which the effects of varying weak magnetic fields are no longer negligible, promoting/retarding some reaction pathways over others. In the ‘low’ field regime¹, Low Field Effects (LFEs) can be observed whereby the response of the system to an applied magnetic field is of an opposite phase to ‘normal’ magnetic field effects found at higher field intensities. These effects offer a means of boosted sensitivity to weak magnetic fields versus ‘normal’ MFEs and thus are of particular interest to the radical pair hypothesis for magnetoreception. The origin of the LFE can be explained in the framework of the RPM (see Chapter 1), but experimental

¹‘Low’ refers to magnetic field strengths that are comparable or weaker than the hyperfine interactions in a radical pair.

examples are rare — especially for flavin-based radical pair reactions under biologically relevant conditions.

In this chapter, Low Field Effects in the flavin-ascorbic acid system are examined. Despite the intermolecular nature of the recombination reaction, the relative magnitude of the LFEs exceed those of all other known donor/acceptor systems. Furthermore, direct control over the spin state for the initial radical pair configuration is demonstrated by modifying the efficiency of electron transfer quenching. Compound magnetic field effects involving at least two chemically distinct radical complexes are observed. By altering their relative contributions, a steep response to low fields can be engineered. Work presented in this chapter has been conducted in collaboration with Dr. Daniel Kattinig and Dr. Kevin Henbest.

3.1 Low Field Effects

Sensitivity of chemical reactions to magnetic fields of the order of 50 μT has so far only been demonstrated for a custom-designed triad under cryogenic conditions [104]. However, effects induced by fields of the order of several Gauss are well-documented, in particular for donor-acceptor systems forming exciplexes in organic solvents [25]. LFEs have also been observed in the electroluminescence of organic-LEDs, thereby influencing the efficiency of these devices [5]. To date, only a few systems have been shown to exhibit a LFE in aqueous environments. A relatively small LFE has been detected by *in vitro* studies of cryptochrome and closely related photolyase proteins [49]. For the former, the LFE occurs at 1 mT and amounts to 3% of the saturated effect. In both proteins, the pertinent radical pair is comprised of a semi-reduced flavin cofactor and an oxidised tryptophan residue. Beardmore et al. demonstrated a LFE for the intramolecular radical pair resulting from photoexcited flavin adenine dinucleotide (FAD) at pH 2.2 [103]. The size of the LFE is $\lesssim 10\%$ relative to the saturated effect.

Although many bimolecular donor-acceptor systems are expected to give rise to a LFE in homogeneous aqueous solution, these effects are in general even harder to observe than their intramolecular counterparts. This is predominantly attributed to small radical re-encounter probabilities and typical hyperfine parameters that even under conditions

of optimal kinetics are (theoretically) predicted to give rise to small LFEs [25]. Consequently, despite extensive efforts, LFEs for intermolecular radical pairs in aqueous solution have remained a rarity.

3.2 Ascorbic acid

Radical pairs involving ascorbic acid, AscH_2 , have been tentatively implicated as part of the cryptochrome-based magnetoreceptor at the heart of animal navigation [105]. Theoretical calculations have suggested that a radical pair containing a cryptochrome-bound FAD radical partnered to another species ($\text{Z}^\bullet$) with no hyperfine couplings can be two order of magnitude more sensitive to the inclination of a magnetic field than the typically considered FAD-tryptophan pair. Ascorbic acid is a commonly encountered reductant in biology, capable of reducing photoexcited flavins and tryptophan radicals to form the ascorbyl radical (pictured, Figure 3.1) with near-negligible hyperfine interactions: $\sim 200 \mu\text{T}$ for H4; $10\text{--}20 \mu\text{T}$ for H5 and $2 \times \text{H6}$ [105]. The $(\text{FAD}^{\bullet-} \text{Asc}^{\bullet-})$ radical pair is expected to have a compass sensitivity of approximately 50 times that of $(\text{FAD}^{\bullet-} \text{TrpH}^{\bullet+})$, and half that of the hypothetical $(\text{FAD}^{\bullet-} \text{Z}^\bullet)$ [105].

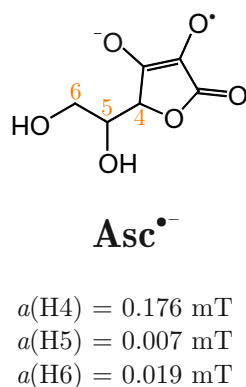


FIGURE 3.1: Chemical structure of the ascorbyl radical, $\text{Asc}^{\bullet-}$, with hyperfine coupling constants indicated.

Furthermore, theoretical studies have also found that LFEs are expected to be large if all hyperfine interactions occur exclusively in one of the two radicals comprising the geminate pair [25]. For this reason, deuteration of one of the radical pair partners is often accompanied by an increase in the size of the LFE [25]. Thus, not only might the $(\text{FAD}^{\bullet-} \text{Asc}^{\bullet-})$ pair offer more optimised anisotropic sensitivity to magnetic fields for a

flavin-based cryptochrome magnetoreceptor, but also enhanced responses to weak fields relevant to the geomagnetic field of the Earth.

3.3 Experimental methods

Materials: Flavin mononucleotide (FMN) and ascorbic acid (AscH_2) samples were prepared in formate (pH 3, ionic strength $I_c = 0.1$ M) and phosphate (pH 7, $I_c = 0.1$ M) buffers unless otherwise specified. Ultrapure Millipore water was used ($18.2 \text{ M}\Omega \cdot \text{cm}$). The contribution from the ascorbic acid/ascorbate equilibrium ($\text{p}K_a = 4.25$ [106]) and its associated ionic charges were considered in regulating the ionic strength of the solutions. Chemicals were purchased from Sigma Aldrich and used without further purification.

Magnetic Field Effects: For the magnetic field effect studies, the experimental arrangement introduced in Chapter 2 was used. As before, the investigated samples were continuously photoexcited using a 405 nm/350 mW diode laser and changes for the induced prompt fluorescence were recorded, $\Delta I_F(B_0)$, as a function of the applied magnetic field, B_0 . In order to highlight any stray asymmetry in the experiments, the magnetic field was swept between equal and opposite extremes of ± 12 mT. A delay time of 50 ms was set between changing the magnetic field strength and measuring the fluorescence, for which the exposure time was 20 ms. MFEs are reported as the relative change in the fluorescence intensity with respect to the zero field value: $\text{MFE}(B_0) = 100\% \times \Delta I_F(B_0)/I_F(B_0 = 0)$. For obtaining MARY curves geared towards resolving LFEs, the field step size was 0.10 mT for $\|B_0\| < 2.70$ mT and 1.15 mT for $2.70 \text{ mT} < \|B_0\| < 11.90$ mT. Further experimental details can be found in Chapter 2.

Stern Volmer analysis: For the Stern Volmer plots, a Varian Cary fluorescence spectrometer was employed and the fluorescence concentration was set to 1 μM . The solutions were kept at a fixed ionic strength of 0.1 M by appropriate addition of NaCl.

Electron Paramagnetic Resonance (EPR): EPR spectra were recorded using a Bruker Elexsys 680 spectrometer with a high-sensitivity 4119 HS-W1 resonator at room temperature (ca. 298 K). Samples containing semiquinone radical were prepared in a N_2 glove box at pH 7 (phosphate buffer) by comproportionation of the fully reduced

form (FMNH₂) and FMN in a 1:1 mixture of 2.6 mM concentration determined by UV-Vis [46]. The fully reduced FMNH₂ was obtained by passing the solution through a Jones reductor (Zn amalgam prepared by reacting granular Zn with 2% v/v HgCl₂, aq).

Transient absorption spectroscopy: Transient absorption profiles were obtained using a home-built arrangement. Samples were held in a quartz cuvette (Hellma, 104.002F QS; 10 mm path length, internal dimension $2 \times 10 \times 45$ mm) and radical pairs were produced by flash photolysis using a dye laser (Sirah Cobra) pumped by a Nd:YAG laser (Continuum Surelite 1). The output of the dye laser was ~ 5 mJ at 450 nm and operated at 1 Hz. A 300 W xenon arc lamp was employed as a probe light in an orthogonal orientation to the laser. Water and long-pass filters were used to remove unwanted infrared and other wavelength components in an effort to protect the samples from degradation. Transient absorption profiles for different wavelengths were acquired by the use of a monochromator (Oriel 77250) and photomultiplier tube (Hamamatsu R928) coupled to an oscilloscope (LeCroy Waverunner LT342L).

3.4 Results and discussion

3.4.1 T-pathway MFEs

FMN/AscH₂ photochemistry follows the general photochemical scheme in Figure 3.2a, which contains S- and T-pathways for the formation of singlet- and triplet-born radical pairs, respectively. Absorption and fluorescence profiles for FMN are given in Figure 1.4. The fluorescence is derived from the flavin singlet excited state and can serve as a sensitive indicator of the ground state concentration. In Chapter 2 it was demonstrated that under conditions of continuous photoexcitation, the photostationary state depends on the efficiency of charge recombination within the geminate pair which can be impacted by variation in the external magnetic field intensity. In this way, MFEs on radical pair dynamics can be detected via fluorescence with exceptional sensitivity.

Figure 3.2b shows typical MARY curves for FMN/ascorbic acid at pH 3 and 7 with quencher concentrations on the order of a few mM. At low quencher (D) concentrations, the majority of the radical ion pairs are formed in the electronic triplet state following intersystem crossing of the fluorophore (T-pathway in Figure 3.2a). For a triplet-born

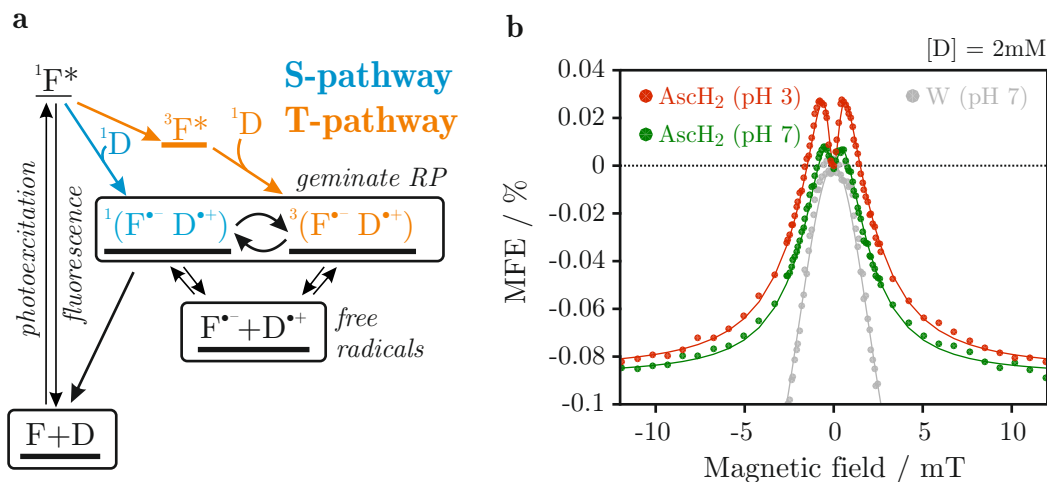


FIGURE 3.2: a) Reaction scheme for photoinduced radical pair formation between photosensitiser F and electron donor D. Depending on the efficiency of the electron quenching reaction, the radical pair is formed either directly from the $^1F^*$ singlet excited state (S-pathway, blue) or following intersystem crossing and via the triplet $^1F^*$ (T-pathway, orange). Due to the conservation of spin angular momentum, the S- and T-pathways produce the radical pair in an initial singlet and triplet state, respectively. The interconversion of the singlet and triplet states of the pair is driven by hyperfine interactions and is modulated by the Zeeman interaction with an external magnetic field. As radical pair recombination occurs exclusively via the singlet state, the field-dependent spin mixing manifests itself in field-dependent photocycle populations for samples subjected to continuous illumination. b) MARY plots for 10 μ M FMN/2 mM AsCH₂ at pH 3 (formate buffer, red) and pH 7 (phosphate buffer, green) obtained by fluorescence detection. The MARY plot for 10 μ M FMN/2 mM tryptophan (W) at pH 7 (phosphate buffer, grey) is also overlaid as a reference and shows no (distinguishable) LFE. Measurements were conducted using the experimental arrangement for detecting PF MFEs as introduced in Chapter 2 (405 nm, 350 mW).

radical ion pair, the effect of a saturating magnetic field ($\|B_0\| = 12$ mT, high with respect to the hyperfine interaction) is to reduce the efficiency of its conversion to the singlet, thereby enhancing the depletion of the ground state under conditions of continuous irradiation. As a result, the fluorescence intensity is reduced and the MFE is negative. In the low field region, LFEs are observed which are unusually large at both pH conditions. The LFE amplitudes at pH 3 and 7 amount to $\sim 30\%$ and $\sim 9\%$ of the saturated MFE for $[AsCH_2] = 2$ mM. The position of the LFE maximum at pH 7 is 0.45 mT and approximately 0.6 mT at pH 3, denoting narrower field profiles than for prior observations in flavin-based systems [49, 103]. Furthermore, it appears that the LFE feature is narrower than for most known intermolecular organic-based systems, *e.g.* exciplexes, photoinitiators, radiolysis products. To contrast, the MARY profile for FMN/tryptophan (aq, phosphate buffer, pH 7) is also overlaid in Figure 3.2b, the system showing no discernible LFE. That the field dependence of the FMN/AsCH₂ system

differs at pH 3 and 7 is indicative of different geminate pair species.

3.4.2 T-pathway photochemistry

As discussed in Chapter 1, flavins undergo a broad range of chemistry and are commonly found in a number of redox/excited states under illumination. Absorption-based techniques are routinely employed in mechanistic studies as different flavin states can be differentiated by their unique absorption profiles.

Thus, in order to shed light on the underlying photochemistry for the T-pathway radical pairs formed by FMN/AscH₂ at different pH, transient absorption (TA) experiments were conducted. The spectra cover a wavelength range of 320–700 nm, with a μ s temporal resolution.

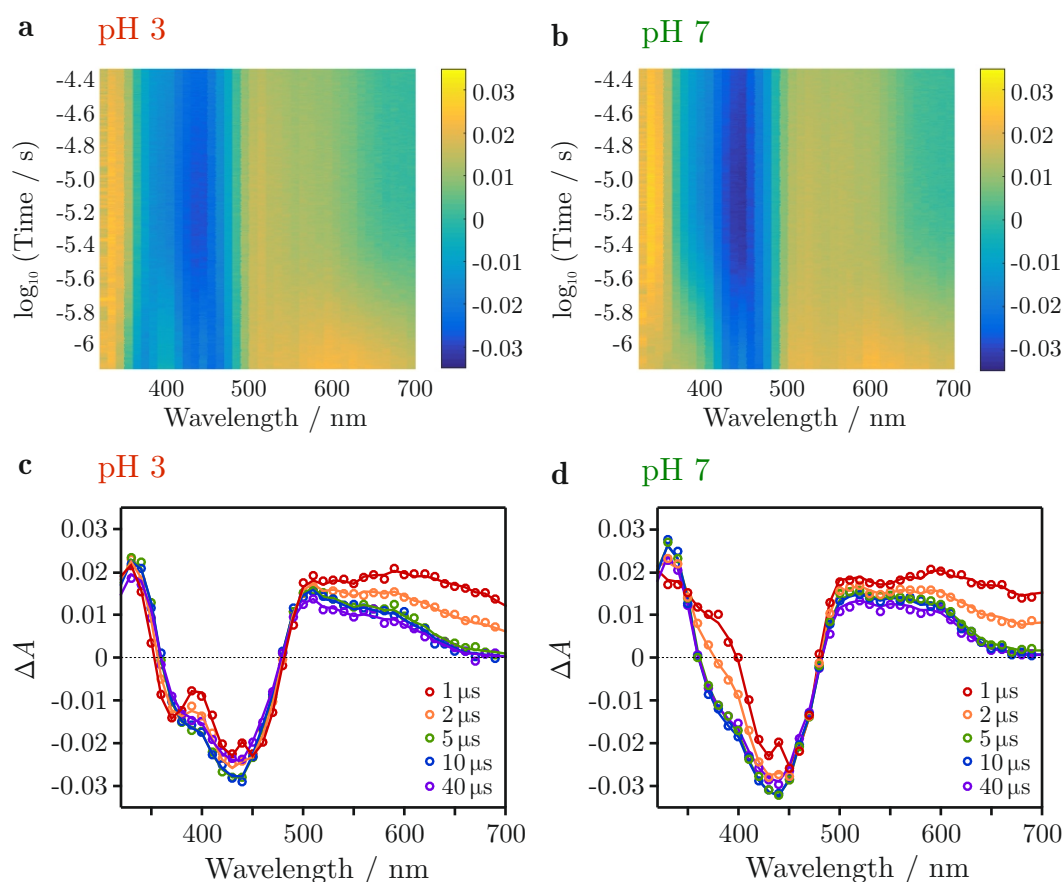


FIGURE 3.3: Density plots of the transient absorption profiles obtained for a) 0.1 mM FMN/0.5 mM AscH₂ (aq, formate buffer, pH 3); and b) 0.1 mM FMN/0.5 mM AscH₂ (aq, phosphate buffer, pH 7). TA profiles at 1, 2, 5, 10 and 40 μ s are depicted by circles and solid lines denote the fits derived from the target analysis: c) pH 3; d) pH 7.

Figure 3.3a and b depict the density plots of the transient absorption profiles, $\Delta A(\lambda, t)$, derived for the systems. Figures 3.3c and d contain the $\Delta A(\lambda)$ spectra for the time points: $t = 1, 2, 5, 10, 40 \mu\text{s}$. By inspection, the most notable differences between the two appears in the narrower ground state bleaching feature ($\sim 430 \text{ nm}$) at early times (1–5 μs) for pH 7. Due to the broad and overlapping nature of the absorption profiles associated with different species, deconvolution is critical.

Global (target) analysis of the transient absorption data was performed. The goal of the analysis is to factorise the data for each present species in terms of its spectral and temporal components. Consider the transient absorption data ($\mathbf{A}$) of the form:

$$\mathbf{A} = \begin{pmatrix} \Delta A(\lambda_1, t_1) & \Delta A(\lambda_1, t_2) & \cdots & \Delta A(\lambda_1, t_j) \\ \Delta A(\lambda_2, t_1) & \Delta A(\lambda_2, t_2) & \cdots & \Delta A(\lambda_2, t_j) \\ \vdots & \vdots & \ddots & \vdots \\ \Delta A(\lambda_i, t_1) & \Delta A(\lambda_i, t_2) & \cdots & \Delta A(\lambda_i, t_j) \end{pmatrix}. \quad (3.1)$$

If the photochemistry for the studied reactions involve n species, then:

$$\mathbf{A} = \mathbf{DC} = \begin{pmatrix} d_1(\lambda_1) & d_2(\lambda_1) & \cdots & d_n(\lambda_1) \\ d_1(\lambda_2) & d_2(\lambda_2) & \cdots & d_n(\lambda_2) \\ \vdots & \vdots & \ddots & \vdots \\ d_1(\lambda_i) & d_2(\lambda_i) & \cdots & d_n(\lambda_i) \end{pmatrix} \begin{pmatrix} c_1(t_1) & c_1(t_2) & \cdots & c_1(t_j) \\ c_2(t_1) & c_2(t_2) & \cdots & c_2(t_j) \\ \vdots & \vdots & \ddots & \vdots \\ c_n(t_1) & c_n(t_2) & \cdots & c_n(t_j) \end{pmatrix} \quad (3.2)$$

where the columns of $\mathbf{D}$ are the species associated difference spectra (SADS) and the rows of $\mathbf{C}$ are the time-dependent concentrations of the species involved in the photoreaction. Here, the term ‘species’ encompasses both the flavin and ascorbic acid states, whereby different species are associated with different combinations. The SADS for x is given by $\epsilon_x(\lambda) - \epsilon_{\text{GS}}(\lambda)$, where $\epsilon_x(\lambda)$ and $\epsilon_{\text{GS}}(\lambda)$ are the extinction coefficients for species x and the ground state, respectively.

Data were fitted using models containing one-, two- and three-excited state species as depicted in Figure 3.4. For each model, the best fit was obtained by least squares minimisation of χ^2 :

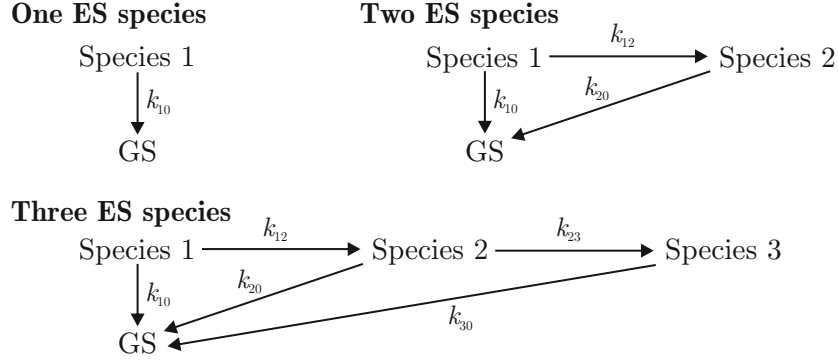


FIGURE 3.4: Kinetic models for the target analysis. Species 1 in each contains the flavin triplet excited state.

$$\chi^2 = \sum_{i,j} (A_{i,j} - A_{\Gamma i,j})^2 \quad (3.3)$$

where $\mathbf{A}_{\Gamma} = \mathbf{D}_{\Gamma} \mathbf{C}_{\Gamma}$, and the Γ -subscript denotes the fit matrices. $A_{i,j}$ are the matrix elements for the data ($\mathbf{A}$). To obtain $\mathbf{C}_{\Gamma}$, the kinetics of the system were numerically calculated using MATLAB's ode45s ordinary differential equation solver. If $\mathbf{C}_{\Gamma}$ is known or assumed, $\mathbf{D}_{\Gamma}$ can be derived as follows²:

$$\mathbf{A} = \mathbf{D}_{\tau} \mathbf{C}_{\tau} \quad (3.4a)$$

$$\mathbf{A} \mathbf{C}_{\tau}^{-1} = \mathbf{D}_{\tau} \mathbf{C}_{\tau} \mathbf{C}_{\tau}^{-1} = \mathbf{D}_{\tau} \quad (3.4b)$$

Following Fita et al. [107], $\mathbf{D}_{\tau}$ and $\mathbf{C}_{\tau}$ can be used to reconstruct $\mathbf{A}_{\tau}$:

$$\mathbf{A}_{\tau} = \mathbf{D}_{\tau} \mathbf{C}_{\tau}. \quad (3.5)$$

$\mathbf{A}_{\tau}$ is optimised by minimisation of χ^2 in Equation 3.3. A genetic algorithm is employed for the best fit ('ga' in MATLAB). In short, the algorithm starts by generating a large population of random sets of rate constants to determine $\mathbf{C}_{\tau}$ and $\mathbf{A}_{\tau}$ by the matrix reconstruction procedure above. This population then breeds into successive generations using a 'survival of the fittest' approach.

²Note that the concentrations of the species in $\mathbf{C}_{\tau}$ for the following analysis are given in terms of fractional contributions to the total system. Consequently, the units of $\mathbf{D}_{\tau}$ are $\text{fraction}^{-1} \text{cm}^{-1}$.

From F-tests on the results of the target analysis, the photochemistry of FMN/AscH₂ at pH 3 and 7 require two and three excited state species, respectively. The results of the target analysis using these models are given in Figure 3.5. More complicated schemes offer no statistically significant improvement in the fits of the data; thus they cannot be justified.

Under acidic conditions (pH 3), the TA spectra reveal quenching of the protonated $^3\text{FMNH}^{+*}$ to form $\text{FMNH}^\bullet$. Whereas at pH 7, $^3\text{FMN}^*$ is reduced to $\text{FMNH}^\bullet$. The target analysis using three excited state species reveals that this process at pH 7 involves the $\text{FMN}^{\bullet-}$, which is protonated to $\text{FMNH}^\bullet$ on a timescale of 1 μs . At pH 3, the analogous analysis did not reveal the anionic intermediate for the available time resolution. These observations are in agreement with prior studies by Heelis et al. [108].

Figure 3.5a and b contain the SADS from the analysis at pH 3 and 7, respectively. The SADS predominantly reflect the spectra for the flavin excited state species. Differences in the SADS for the flavin triplet excited state for the two samples are most noticeable in the wavelength range of $\lambda < 400\text{ nm}$. These are characteristic regions which allow the assignment of the protonation states from reference spectra obtained for flavin only samples (see Appendix 3). The presence of $^3\text{FMNH}^{+*}$ at pH 3 and $^3\text{FMN}^*$ at pH 7 is in agreement with a $\text{p}K_a(^3\text{FMNH}^{+*})$ of 4.4 [109]. SADS determined for the flavin radical-containing species exhibit the expected spectral features for $\text{F}^{\bullet-}$ and $\text{FH}^\bullet$ [108]. The time-dependent profiles for the concentration of the species are given in Figures 3.5c and d. Rate constants pertaining to these profiles are indicated by the photochemical schemes in Figures 3.5e and f.

MFE studies at 20 mT and $\lambda = 530\text{ nm}$ using the TA apparatus could not detect an effect on the free flavin radical concentration, suggesting that it must be smaller than $< 1\%^3$. However, the TA data support the notion of different geminate RP species for FMN/ascorbic acid photoreactions depending on the pH of the solution. At pH 3, the geminate pair species appears to be comprised of $(\text{FMNH}^\bullet\text{ascorbyl}^\bullet)$; whereas at pH 7, the data suggests that the geminate pair consists of $(\text{FMN}^{\bullet-}\text{ascorbyl}^\bullet)$. With little known about the spectral properties for the different ascorbyl radical protonation states, its assignment within the geminate pair is ambiguous.

³MFEs detected by transient absorption spectroscopy on the free radical concentration are defined as: $100\% \times (\Phi_{\text{FR}}(B_0) - \Phi_{\text{FR}}(B_0 = 0)) / \Phi_{\text{FR}}(B_0 = 0)$. In Chapter 2, a model for comparing MFEs detected by prompt fluorescence and TA is presented.

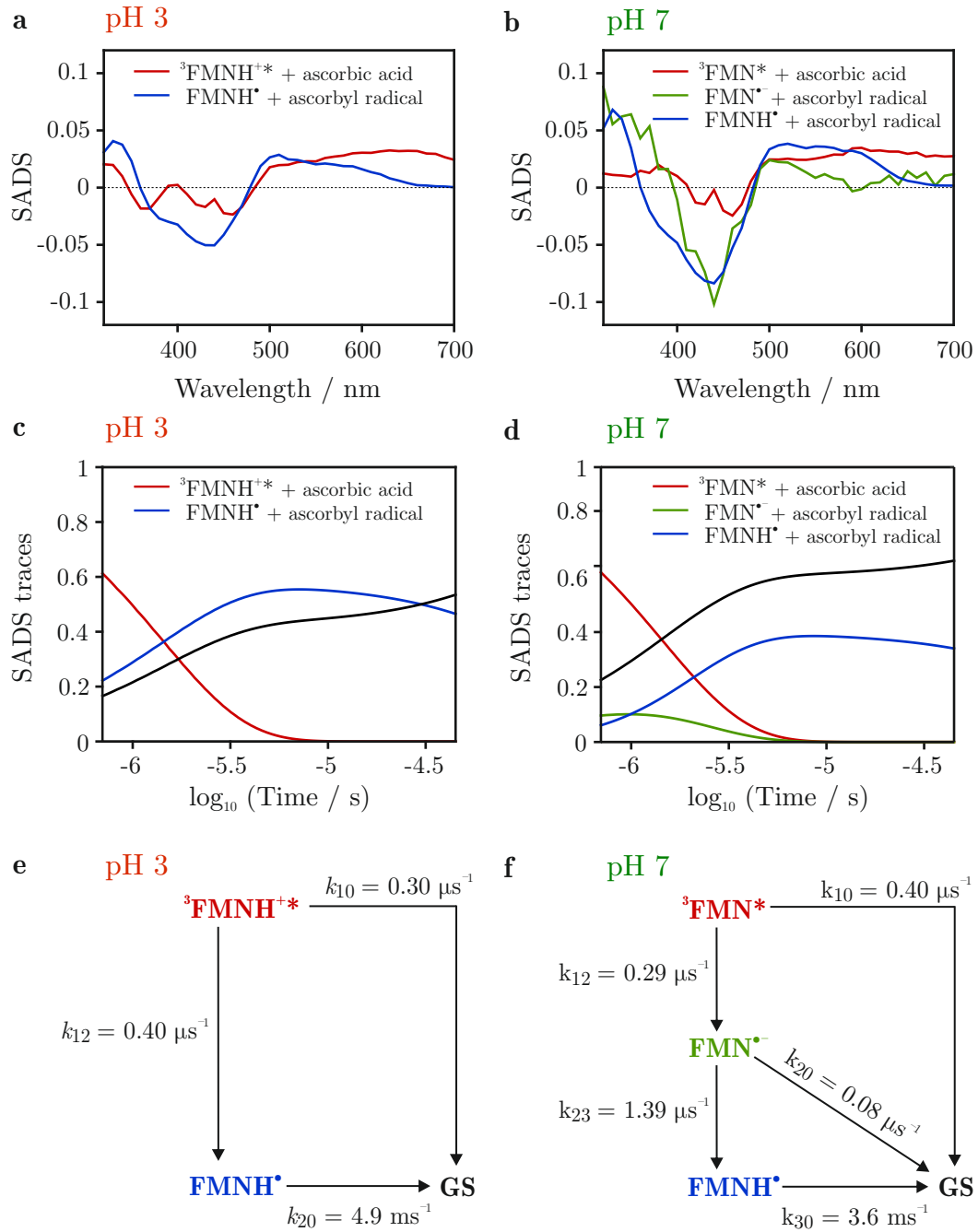


FIGURE 3.5: The Species Associated Difference Spectra (SADS): a) pH 3, b) pH 7 and corresponding time profiles: c) pH 3, d) pH 7 from the target analysis of the data in Figure 3.3. e) and f) contain the photochemical model and fitted rate constants for the pH 3 and 7 data, respectively.

3.4.3 From T-pathway to S-pathway MFEs

The dependence of the MFE on quencher concentration (2–300 mM) was examined at pH 3 (Figure 3.6a) and pH 7 (Figure 3.6c). Upon increasing the ascorbic acid concentration, the saturated MFE is gradually transformed from being negative to positive. This is in agreement with increased competition of singlet quenching versus intersystem crossing for increasing quencher concentration.

Stern Volmer constants determined for the two conditions were found to be: $14.0 \pm 0.4 \text{ M}^{-1}$ at pH 3; and $30.9 \pm 0.9 \text{ M}^{-1}$ at pH 7 (Figure 3.6b). Thus, assuming a $^1\text{FMN}^*$ lifetime of 4.6 ns in the absence of quencher, the bimolecular quenching rate constants are as follows: $3.04 (\pm 0.09) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (pH 3) and $6.72 (\pm 0.19) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (pH 7). From these values it is possible to estimate the probability that the radical pair is initiated in the singlet state, P_{0S} (see Appendix 3 for further details). For a concentration of 2 mM ascorbic acid, the probabilities are found to be $P_{0S}(\text{pH } 3) \approx 5\%$ and $P_{0S}(\text{pH } 7) \approx 11\%$, increasing to $P_{0S}(\text{pH } 3) \approx 87\%$ and $P_{0S}(\text{pH } 7) \approx 94\%$ with 300 mM quencher concentration. Remarkably, the initial spin configuration is switched from being (near)-entirely triplet born to singlet born. It should be highlighted that the MARY plots obtained in the high quencher concentration/singlet-born RP limit also display LFEs at pH 7 but they are absent at pH 3. Further measurements at pH 4 resembled those at pH 3 and are summarised in Appendix 3.

With the aim of a more systematic evaluation of the LFE and the shape of the MARY curves, four characteristic parameters were identified: i) $B_{1/2}$, the field strength corresponding to half the saturated MFE; ii) A_{LFE} , the amplitude of the LFE relative to the saturated MFE; iii) B_{LFE} , the field strength giving rise to the maximal LFE amplitude; and iv) B_{zero} , the field strength where the MFE changes sign upon transitioning from the low-field to the high-field region. These parameters are depicted for the typical MARY curve presented in Figure 3.7. Data were fitted using a double Lorentzian function. The MFE parameters were then obtained from these fits so as not to be limited by the field resolution of the measurements.

Varying the ascorbic acid concentration from 2–25 mM at pH 7 ($I_c = 0.1 \text{ M}$) finds the four aforementioned parameters largely unchanged. Furthermore, upon normalisation of the saturated MFE, the MARY curves are perfectly overlaid as seen in Figure 3.6d, *i.e.* not only do the parameters but also the relative shape of the profiles agree in general. This

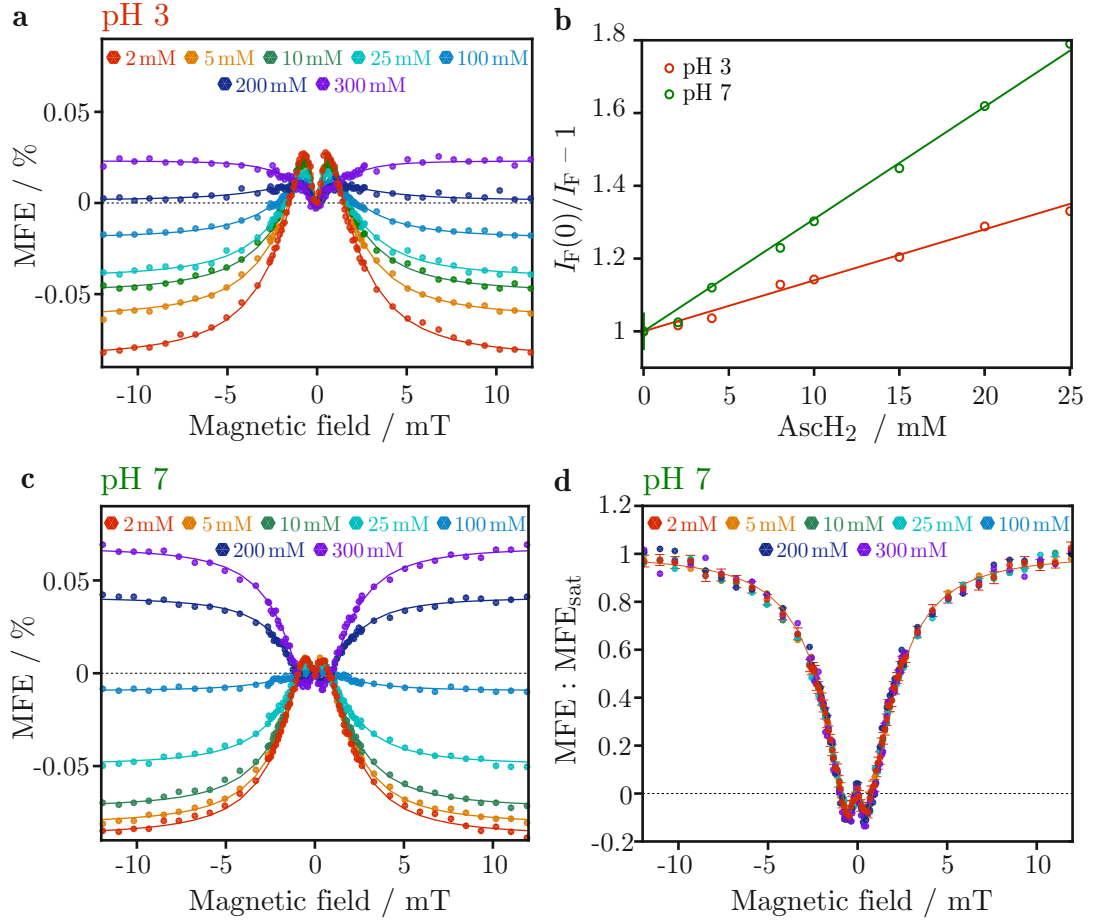


FIGURE 3.6: PF MARY plots for 10 μ M FMN with AscH₂ concentration varying from 2–300 mM for solutions of a) pH 3 and c) pH 7. d) PF MARY plots from c) normalised with respect to their saturated magnetic field effects (MFE_{sat}). 99% confidence intervals for 2 mM dataset are given (red) and generally all of the normalised profiles fall within this range. Dots indicate the experimental points and solid lines denote the fit by the composite lineshape model as discussed in the main text. By introduction of NaCl and NaAscH, the ionic strength of formate- and phosphate-buffered samples were fixed at 0.1 M for [AscH₂] $\leq$ 25 mM. The pH of the higher concentration solutions were adjusted through the addition of NaOH and HCl; the ionic strength was unregulated for these measurements. All measurements were conducted using the experimental arrangement introduced in Chapter 2. b) Stern Volmer plots for solutions of FMN/AscH₂ at pH 3 (red) and pH 7 (green), with fixed ionic strength (0.1 M). Representative error bar is shown at AscH₂ = 0 mM.

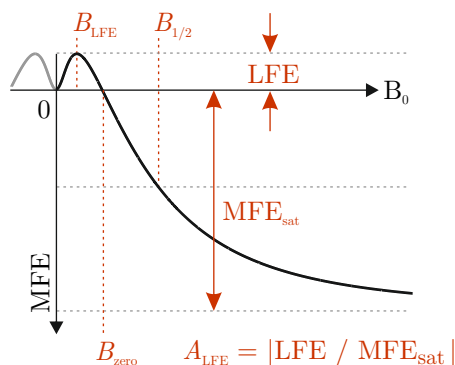


FIGURE 3.7: Typical magnetic field effect (MFE) plot showing the external magnetic field strength dependence. The figure depicts the characteristic shape parameters: $B_{1/2}$, A_{LFE} , B_{LFE} and B_{zero} (red) as discussed in the main text.

is in striking contrast to the analogous concentration study conducted at pH 3 where the shape of the profiles undergo significant alteration upon transitioning from a system of predominantly triplet-born to singlet-born RPs, culminating in the disappearance of the LFE. However, even at lower concentrations, the MARY lineshapes and the associated parameters vary systematically with ascorbic acid concentration, $[AscH_2]$. Figure 3.8 summarises these trends for concentrations of up to 25 mM (resulting in largely triplet-born RPs) at pH 3 and 7.

3.4.4 Low quencher concentration: mainly T-pathway MFEs

The data in Figure 3.8 reveal that for pH 3, all field intensity related parameters ($B_{1/2}$, B_{LFE} and B_{zero}) are higher than for pH 7. Moreover, the A_{LFE} at pH 3 is also higher by a factor of 3–4 as compared to pH 7. All of the characteristic parameters increase with quencher concentration at pH 3, whilst they are unchanged for neutral conditions. The values for the shape parameters have been extracted for the (hypothetical) limit of zero ascorbic acid concentration where the effects of degenerate electron exchange and other concentration-dependent processes disappear, see Table 3.1. For pH 3, the zero-concentration values were extrapolated for an offset exponential function fitted to the concentration-dependent data (solid red lines) in Figure 3.8. The fits for the data at pH 7 in Figure 3.8 are indicated by dotted green lines.

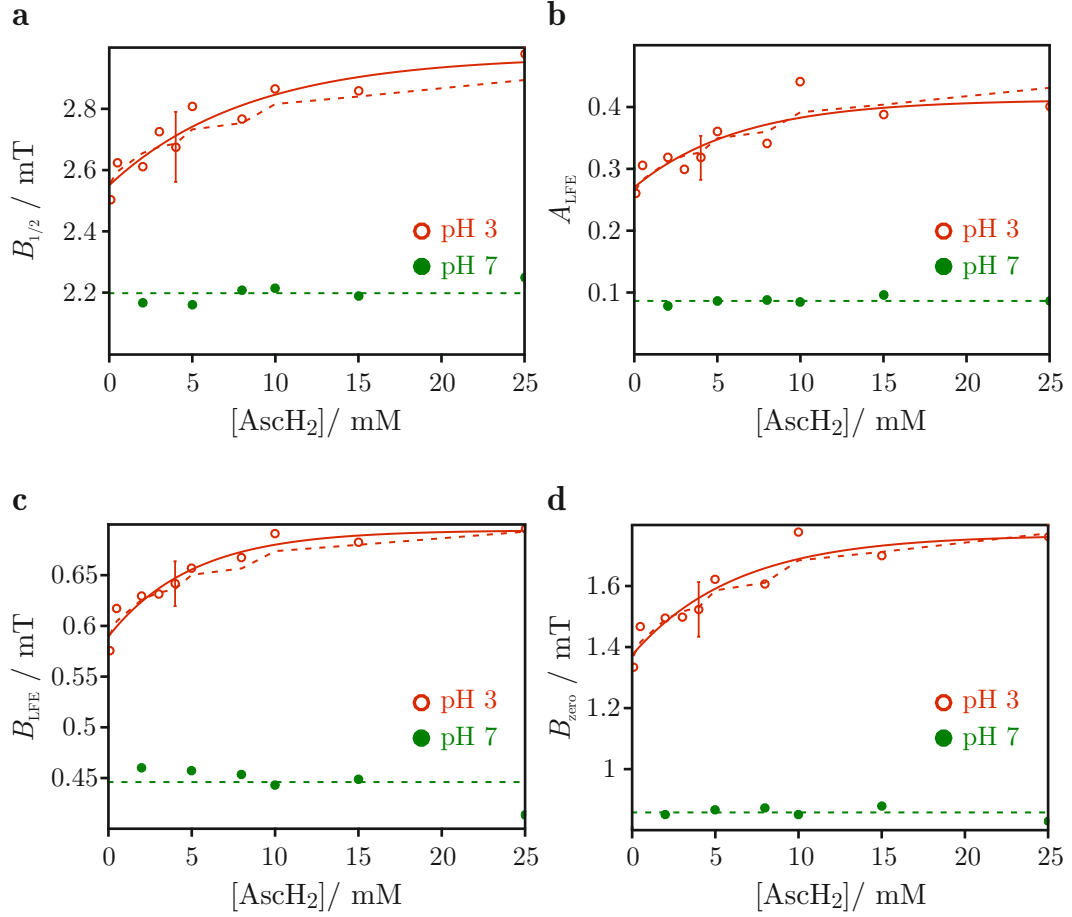


FIGURE 3.8: Concentration dependence for the characteristic shape parameters describing the MFE profiles arising from solutions of $10 \mu\text{M}$ FMN/2–25 mM AscH_2 at pH 3 (open red circles) and pH 7 (filled green circles). The studied shape parameters are: a) $B_{1/2}$, the magnetic field strength corresponding to half the saturated MFE (by the hyperfine mechanism); b) A_{LFE} , the ratio between the low field effect (LFE) and saturated MFE; c) B_{LFE} , the magnetic field strength corresponding the maximal LFE magnitude; d) B_{zero} , the magnetic field strength corresponding to the cross-over between the LFE and so-called ‘normal’ MFE regimes. A representative 99% confidence interval is shown for $[\text{AscH}_2] = 5 \text{ mM}$ at pH 3. By introduction of NaCl and NaAsCH, the ionic strength of formate- and phosphate-buffered samples were fixed at 0.1 M for $[\text{AscH}_2] \leq 25 \text{ mM}$. Dotted green lines indicate the average shape parameters extracted for the MFE profiles at pH 7, see Tables 3.1 and 3.2. The red dotted lines denote the shape parameters at pH 3 arising from the composite lineshapes produced by the two-state model discussed in the main text. An offset exponential function (solid red line) was used for an empirical fit to the concentration dependence and for extrapolation to the hypothetical zero-quencher limit, see Table 3.1.

TABLE 3.1: Summary of the MARY shape parameters for triplet-born RPs formed by the T-pathway. These values were determined by extrapolation of the data in Figure 3.8 (pH 3, solid red lines and pH 7, dotted green lines) to the hypothetical zero-quencher limit where RPs are exclusively triplet born.

T-pathway MFE	$B_{1/2}/\text{mT}$	A_{LFE}	B_{LFE}/mT	$B_{\text{zero}}/\text{mT}$
pH 3	2.55 ± 0.04	0.27 ± 0.02	0.59 ± 0.01	1.37 ± 0.04
pH 7	2.20 ± 0.01	0.09 ± 0.01	0.45 ± 0.01	0.86 ± 0.01

3.4.5 High quencher concentration: mainly S-pathway MFEs

From Figures 3.6a and 3.8, it is apparent that the MARY lineshapes derived from FMN/AscH₂ (pH 3) are dependent on quencher concentration. Upon increasing the ascorbic acid concentration, the MARY curve initially broadens and eventually gives rise to a comparably featureless profile which is devoid of the phase inversion found for the LFE.

One possible explanation for the concentration-dependent lineshape alteration is degenerate electron exchange (dee) akin to the observations of Justinek et al. [110]. However, this interpretation can be dismissed on the basis of the associated rate constants which are too low ($k_{\text{dee}} \approx 10^6 \text{ M}^{-1}\text{s}^{-1}$ [111]) as to impact the spin evolution on the timescale of tens to hundreds of nanoseconds for sub-molar quencher concentrations.

A decomposition of the MARY profiles by a procedure that is analogous to a target fit reveals that the lineshapes actually correspond to superpositions of two MARY curves with different (yet similar) width and LFE characteristics, see Figure 3.9. As before, each curve is modelled by a double Lorentzian function and its relative weight in the composite curve is governed by the probability that the RP is singlet ($P_{0\text{S}}$) or triplet born. Two effective species, *i.e.* MARY lineshapes, are required to model the main features of the data: a triplet-born species (MARY_{T-pathway}), as found above in the context of the zero-concentration limit, and a singlet-born pair (MARY_{S-pathway}). The predicted shape parameters for these species are as follows: triplet, $B_{1/2} = 2.56 \text{ mT}$ and $B_{\text{LFE}} = 0.59 \text{ mT}$; singlet, $B_{1/2} = 2.31 \text{ mT}$ and $B_{\text{LFE}} = 0.47 \text{ mT}$. More complicated models involving further geminate pair species show signs of overfitting by F-tests. The characteristic parameters for the field sensitivity of FMN/AscH₂ singlet-born RPs formed at pH 3 and 7 are summarised in Table 3.2.

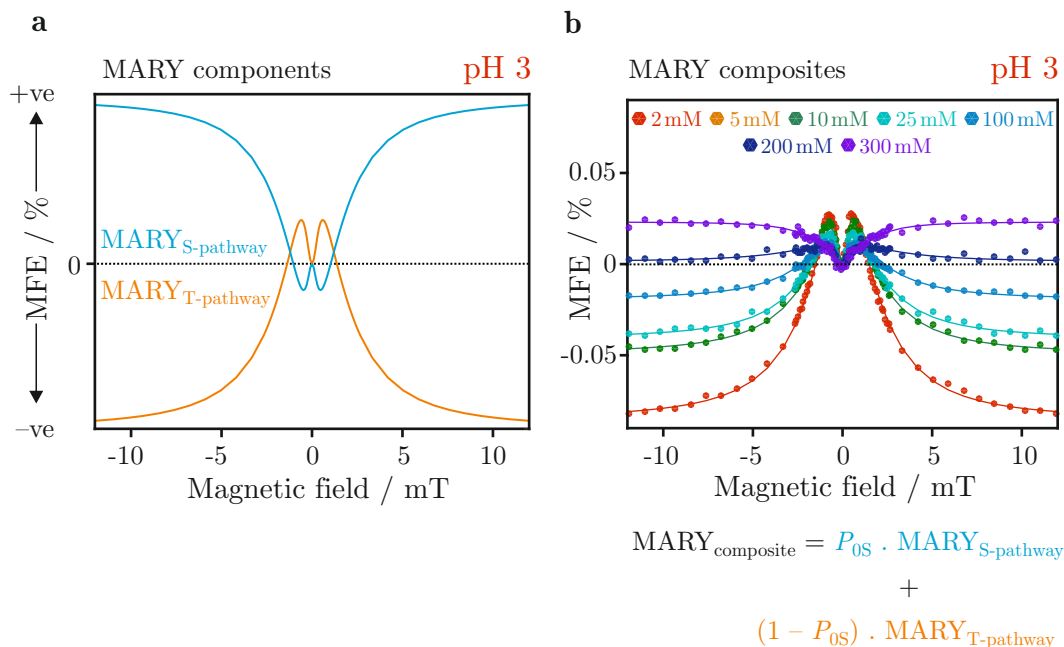


FIGURE 3.9: a) Singlet-born and triplet-born RP MFE profiles determined by target analysis (see text) for the b) FMN/AscH₂ PF MARY plots at pH 3. Solid lines in b) indicate the fits (MARY_{composite}) by superposition of MARY_{S-pathway} and MARY_{T-pathway} in a). Data in b) and fits were previously shown in Figure 3.6a.

It is interesting to note that the lineshape broadening for MARY curves derived from FMN/AscH₂ at pH 3 in the range of 2–25 mM quencher concentration can be reproduced by the two-species model. Dotted lines in Figure 3.8 represent the $B_{1/2}$, A_{LFE} , B_{LFE} and B_{zero} parameters for the compound plots arising from the fits. The actual fits are overlaid as solid lines in Figure 3.6a. In the limit of low quencher concentration (*i.e.* for FMN/AscH₂ with predominantly triplet-born RPs), the effective field dependence of the system can broaden beyond the $B_{1/2}$ values of the composite species. For high quencher concentration (*i.e.* in samples with majority singlet-born pairs), the field dependence of the overall system can become narrower than for the component MARY curves (see MARY_{S-pathway} and MARY_{T-pathway} in Figure 3.9a). The high- and low-quencher concentration behaviour can be switched with reversal of the relative $B_{1/2}$ values for the singlet- and triplet-born radical pairs.

Intriguingly, a combination of distinct but similar singlet- and triplet-born radical pairs can give rise to a peculiar and thus-far unobserved response to weak magnetic fields. The reaction yield can be tailored to be most sensitive to low magnetic field intensities without the usual pattern of the LFE.

TABLE 3.2: Summary of the MARY shape parameters for singlet-born RPs formed by the S-pathway. The values derived for pH 3 have been obtained from the target analysis outlined in the text. For pH 7, the field parameters for the S- and T-pathways are identical.

S-pathway MFE	$B_{1/2}/\text{mT}$	A_{LFE}	B_{LFE}/mT	$B_{\text{zero}}/\text{mT}$
pH 3	2.31	0.21	0.47	1.03
pH 7	2.20 ± 0.01	0.09 ± 0.01	0.45 ± 0.01	0.86 ± 0.01

3.4.6 Hyperfine interactions within the radical pair

An understanding of the hyperfine interactions of the transient radicals is essential for an interpretation of the MFEs. Whilst the hyperfine parameters of the ascorbyl radical are well-known, those for $\text{FMNH}^\bullet$ have not been experimentally established. Based on a shared isoalloxazine ring, the hyperfine parameters of FMN are expected to resemble lumiflavin, the electron paramagnetic resonance (EPR) spectra of which have been published [112, 113]. However, the published spectra are for methylated isoalloxazine cations (width = 5.72 mT) and correspond to an effective hyperfine coupling (Equation 2.12) of 1.63 mT, resulting in a predicted $B_{1/2}$ value of 2.7 mT by the Weller formula (Equation 2.11). This prompted the direct measurement of the $\text{FMNH}^\bullet$ EPR spectrum. The radical was generated by comproportionation of the fully reduced and ground state forms of FMN at neutral pH. Figure 3.10 shows the EPR spectra in H_2O and D_2O (where the protons at positions 3 and 5 are assumed to be substituted by deuterium), together with their global fits using EasySpin. The hyperfine parameters (a_{iso}) are close to these calculated by DFT (see Appendix 3) and are given in Table 3.3. Notably, the width of the EPR spectrum (4.81 mT), effective hyperfine strength and (thus) predicted $B_{1/2}$ values are considerably smaller for FMN than for the lumiflavin derivatives. The spectrum in H_2O exhibits alternating linewidth phenomena, which are probably a consequence of proton exchange reactions on the EPR timescale. These effects have not been taken into account in the simulation. Regardless of this phenomenon, it is clear that the hyperfine interactions in the FMN radical are weaker than for lumiflavin.

In the next section, the knowledge of the hyperfine interactions is used to rationalise the magnetosensitivity of the FMN/AscH₂ system, beginning with further discussion of the Weller predictions.

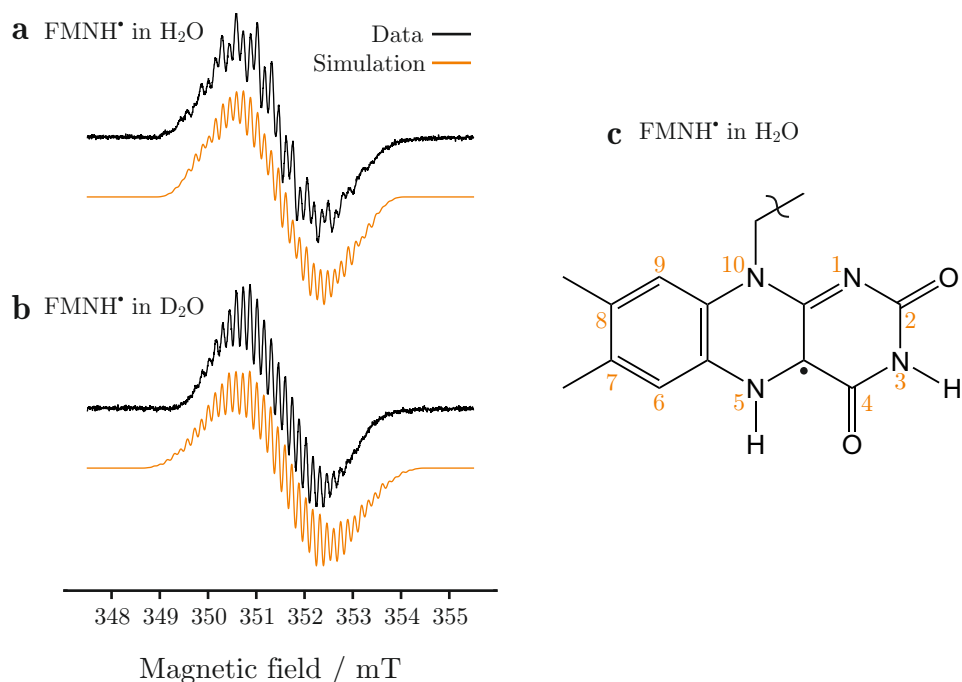


FIGURE 3.10: Experiments (black) and simulated (orange) EPR spectra of FMNH• in an aqueous phosphate buffered solution at a) pH 7 and b) PBS-buffered D₂O solution at pH 7.4 (a). Simulations of the EPR spectra were conducted using EasySpin. The radical was prepared by comproportionation of FMNH₂ and FMN as described in the experimental methods section. Spectra were accumulated over 10 hours. Hyperfine parameters were adjusted such that both spectra (normalised to the same amplitude) are simultaneously reproduced in the least squares sense. c) Chemical structure for neutral flavin radical, FMNH•.

3.4.7 Geminate pair assignment by spin chemistry

3.4.7.1 Discussion: Weller formula

The Weller equation introduced in Chapter 2 (Equation 2.11) provides the simplest prediction for the width of the MARY curves. For ease of reference in the following discussion, the formula is stated again.

$$B_{1/2(\text{Weller})} = 2 \frac{B_1^2 + B_2^2}{B_1 + B_2}$$

where B_x is the effective hyperfine coupling constant for radical $x = 1, 2$:

$$B_x = \left(\sum_i a_{xi}^2 I_{xi} (I_{xi} + 1) \right)^{1/2}$$

TABLE 3.3: Table of hyperfine parameters for the neutral flavin radical derived from the EPR spectra in Figure 3.10.

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
N ₅	1×1	0.60
N ₁₀	1×1	0.43
H ₅	$1 \times 1/2$	0.71
H ₆	$1 \times 1/2$	0.25
H(N ₁₀ –CH ₂)	$2 \times 1/2$	0.15
H(C8 α ₁₀ –CH ₃)	$3 \times 1/2$	0.30
H ₉	$1 \times 1/2$	0.10
H(C7 α ₁₀ –CH ₃)	$3 \times 1/2$	0.10
H ₃	$1 \times 1/2$	0.15

with I_{ix} being the spin quantum number of nuclear spin i with hyperfine coupling constant a_{ix} in radical x . The Weller formula provides a first order approximation for $B_{1/2}$ in RPs of intermediate lifetime (where the hyperfine couplings are comparable to the inverse of the RP lifetime) and an upper bound for RPs which are relatively long-lived with slow relaxation [25].

At both pH 3 and 7, the FMNH[•]/Asc^{•−} RP is the most thermodynamically stable protonation state. However, the relevant species for magnetosensitivity in the short-lived geminate RP (lifetime on the order of hundreds of ns) can differ from this equilibrium state such that the MARY profile might be associated with a different configuration. The TA studies (above) revealed that for the T-pathway at pH 7, the anionic semiquinone intermediate is protonated on the timescale of 1 μs , suggesting that FMN^{•−} is present in the geminate pair. Using the DFT-derived hyperfine values given in Appendix 3 for the flavin radical and EPR-derived ascorbyl radical parameters from [105], $B_{1/2}$ values were estimated for the neutral and anionic semiquinone combined with the anionic ascorbyl radical: $B_{1/2}(\text{FMN}^{\bullet-}/\text{Asc}^{\bullet-}) = 2.20 \text{ mT}$ and $B_{1/2}(\text{FMNH}^{\bullet}/\text{Asc}^{\bullet-}) = 2.32 \text{ mT}$. Excellent agreement is found for $B_{1/2}(\text{FMN}^{\bullet-}/\text{Asc}^{\bullet-})$ and the experimental value obtained at pH 7 (see Table 3.1, 2.20 mT). Whilst the singlet-born species at pH 3 from Table 3.2 (2.31 mT) is well-matched with $B_{1/2}(\text{FMNH}^{\bullet}/\text{Asc}^{\bullet-})$ by Weller, poor agreement is found by both Weller predictions for the experimentally determined 2.56 mT value for the T-pathway at pH 3.

Due to the form of the Weller equation, a minimum in $B_{1/2}$ is predicted for $B_2 = (\sqrt{2} - 1)B_1$ — see Figure 3.11. The corresponding $B_{1/2,\text{min}}$ occurs at $4(\sqrt{2} - 1)B_1$. This

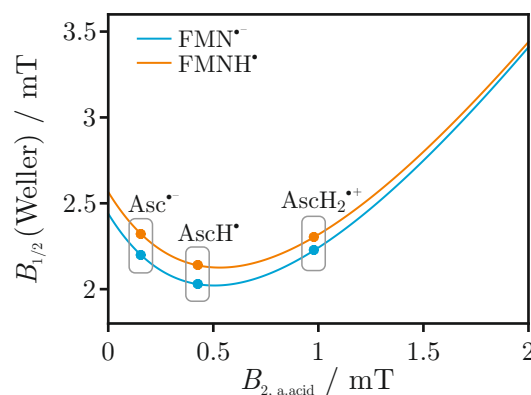


FIGURE 3.11: Plot of the Weller formula prediction for the $B_{1/2}$ of the MARY curves derived from the neutral (orange) and anionic (blue) flavin radical as a function of the effective hyperfine coupling of the RP partner, $B_{2,a,acid}$. The hyperfine parameters for the ascorbyl radicals are such that the predicted $B_{1/2}$ values are situated about the minimum of the Weller estimate.

suggests that for the flavin/ascorbyl radical pairs, an increase in hyperfine interactions for the ascorbyl component (*i.e.* by protonation) will only lead to very minor changes in $B_{1/2}$. In fact, changing $\text{Asc}^{\bullet-}$ to $\text{AscH}^{\bullet}$ to $\text{AscH}_2^{\bullet+}$ is expected to change $B_{1/2}$ by -0.1 mT (situated to the left of the minimum of the Weller prediction in Figure 3.11) and $+0.1$ mT (to the right of the minimum), respectively. Based on these considerations, no clear assignment of the ascorbyl radical protonation state is possible.

3.4.7.2 Discussion: Spin chemistry

In order to elucidate the processes determining the shape and size of the LFE, extensive simulations of the spin dynamics were performed by Dr. Daniel Kattnig. The theoretical insights obtained from these calculations, coupled with the experimental results above, enable certain aspects of the systems' photochemistry to be unveiled which are largely elusive to typical spectroscopic techniques.

Theory

MFES arise from changes in the intensity of an external magnetic field which cause changes in the singlet recombination yield of the geminate radical (ion) pair — see Figure 3.2a. In the experiments described in this chapter, the MFES are detected via the effect on the fluorescent flavin singlet excited state population of a continuously

excited sample. The recombination yield depends on the initial spin configuration, the relative motion of the radicals, the rates of (spin-selective) recombination, and the spin dynamics, which result from hyperfine, exchange and Zeeman interactions. These phenomena can be modelled in terms of the electron-nuclear spin density operator, $\hat{\rho}(r, t)$, whose equation of motion, the stochastic Liouville equation reads:

$$\frac{\partial}{\partial t} \hat{\rho}(r, t) = \left(i\hat{L}(r) + \hat{\mathcal{L}}(r) + \hat{K}(r) - \tau_{\text{sc}}^{-1} \right) \hat{\rho}(r, t). \quad (3.6)$$

Here, the first part:

$$\hat{L}(r) \hat{\rho}(r, t) = \left(\hat{L}_0 + \hat{L}_{\text{ex}}(r) \right) \hat{\rho}(r, t) = -[\hat{H}(r), \hat{\rho}(r, t)] \quad (3.7)$$

accounts for the coherent evolution under the Hamiltonian:

$$\hat{H}(r) = \hat{H}_{0,A} + \hat{H}_{0,B} + J(r) \left(2\hat{S}_A \cdot \hat{S}_B + \frac{1}{2} \right) \quad (3.8)$$

with $\hat{H}_{0,A}$ and $\hat{H}_{0,B}$ describing the Zeeman and hyperfine interactions for the radicals (see Chapter 1); and $J(r)$ denoting the exchange interaction, which depends on the inter-radical distance, r .

The second term in Equation 3.6, $\hat{\mathcal{L}}(r)$, models the relative stochastic motion of the radicals and, for continuous diffusion, is given by:

$$\hat{\mathcal{L}}(r) \hat{\rho}(r, t) = \frac{D}{r^2} \frac{\partial}{\partial r} r^2 \exp\left(-\nu(r)\right) \frac{\partial}{\partial r} \exp\left(+\nu(r)\right) \hat{\rho}(r, t). \quad (3.9)$$

D is the mutual diffusion coefficient and $u(r) = \nu(r)k_{\text{B}}T$ is the interaction potential. For Coulombic interactions between radicals:

$$u(r) = -k_{\text{B}}T \frac{r_c}{r}, \quad (3.10)$$

where r_c denotes the Onsager radius.

The third term in Equation 3.6 accounts for radical recombination according to the Haberkorn approach:

$$\hat{K}(r)\hat{\rho}(r,t) = \frac{w_S(r)}{2}[\hat{P}_S, \hat{\rho}(r,t)]_+. \quad (3.11)$$

$w_S(r)$ is the distance-dependent recombination rate for the singlet radical pair species to the ground state. The triplet recombination pathway is ignored as the lowest excited triplet state has a higher energy than the radical ion pair (see Chapter 1). $\hat{P}_S$ is the singlet projection operator.

The τ_{sc} -term in Equation 3.6 accounts for spin-unselective termination of the radicals and, effectively, the loss of coherence by spin relaxation by removing pairs with lifetimes exceeding τ_{sc} .

Generally, the solution of Equation 3.6 is non-trivial, which for all but the simplest spin systems requires numerous approximations. Here, contact approximations were employed for the fast-decaying exchange interaction and singlet recombination rate functions. These functions are only non-zero in a narrow shell surrounding the contact distance (σ), and thus are well-represented by:

$$J(r) \approx \frac{j}{4\pi r \sigma} \delta(r - \sigma) \text{ and } w_S(r) \approx \frac{k_S}{4\pi r \sigma} \delta(r - \sigma) \quad (3.12)$$

with k_S and j defined such that their integrated value is the same as for their distance-dependent counterparts. The benefit of this approximation is that the functional form of Equation 3.12 enables Equation 3.6 to be solved using a Green's function approach for large spin systems [114, 115].

Summary of model calculations

Some of the most striking observations from the simulations are as follows: firstly, all parameters ($B_{1/2}$, A_{LFE} , ...) are strongly influenced by the charges of the radicals due to their profound effect on the lifetime of the RP. For attractive interactions, the RP lifetime is decreased and all position parameters increase. Second, for repulsive Coulomb interactions, the exchange interaction does not significantly impact the shape

of the MARY curve. The RP dynamics for repulsive pairs appears to be dominated by recombination at the first encounter. Third, the effect of the exchange modulation on the LFE is observed to be generally weaker than that on $B_{1/2}$. Consequently, the LFE is a sensitive indicator of the charges of the RP rather than the exchange interaction. These features can be found in Figure 3.12 for the characteristic MFE parameters derived from calculations of the $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$ geminate pair's magnetosensitivity.

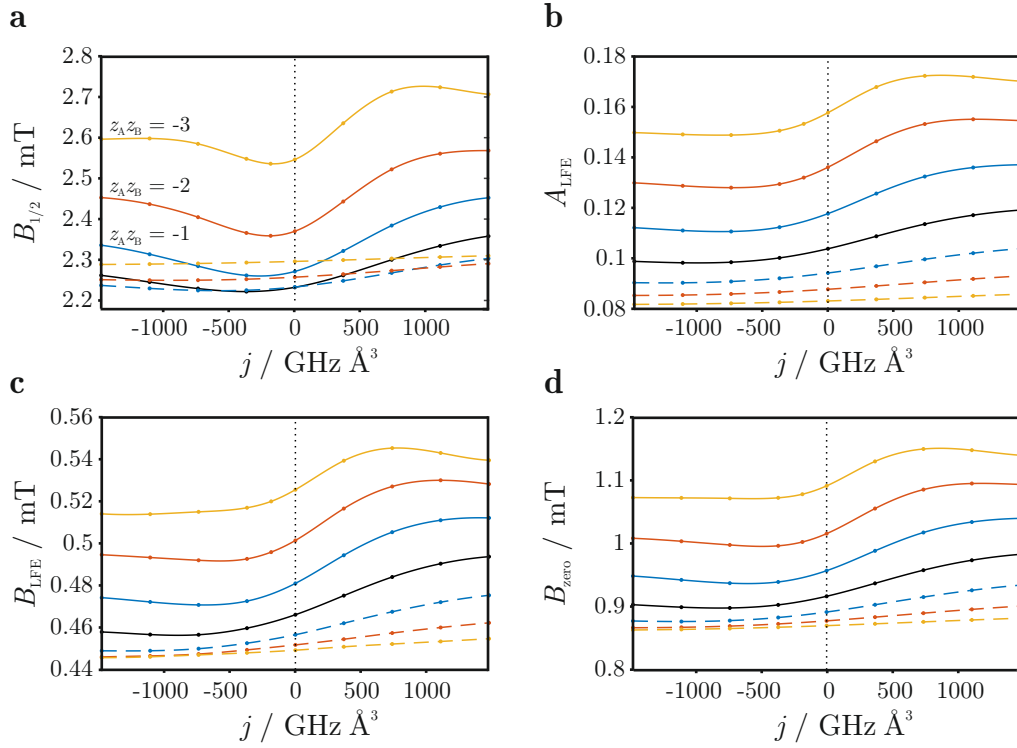


FIGURE 3.12: Dependence of the PF MFE shape parameters for the diffusing $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$ -pair on the strength of the contact exchange interaction and the charges of the radicals. Solid lines correspond to evaluation of the spin dynamics of the radical pair subject to attractive (yellow: $z_1 z_2 = -3$, orange: $z_1 z_2 = -2$, blue: $z_1 z_2 = -1$) or no Coulomb interactions (black); dashed lines profile for repulsive interactions (colours as for the attractive interactions but with the positive sign of $z_1 z_2$). The hyperfine parameters used for the calculations are given in Appendix 3. Additional parameters: $k_S = 1.6 \times 10^4 \text{\AA}^3 \text{ns}^{-1}$, $\tau_{\text{sc}} = 500 \text{ns}$, $r_c = 7 \text{\AA}$, $D = 129 \text{\AA}^2 \text{ns}^{-1}$, $\sigma = 7 \text{\AA}$. The shape parameters are independent of the initial spin configuration.

FMN/AscH₂ (pH 7): At pH 7, ascorbic acid actually exists as ascorbate, AscH^- , which upon photoreduction of FMN is converted to $\text{AscH}^\bullet$. $\text{AscH}^\bullet$ is a highly acidic species ($\text{p}K_a = -0.45$) and is expected to rapidly deprotonate to yield $\text{Asc}^{\bullet-}$. Furthermore, the TA data cannot preclude the formation of $(\text{FMNH}^\bullet\text{Asc}^{\bullet-})$ in a single hydrogen transfer event, that might occur in parallel to the aforementioned direct electron transfer process.

Thus, $(\text{FMN}^{\bullet-}\text{AscH}^\bullet)$, $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$ and $(\text{FMNH}^\bullet\text{Asc}^{\bullet-})$ are the most likely species governing the spin evolution in the short-lived geminate radical pair, giving rise to the observed MARY curves. The charge of $\text{FMN}^{\bullet-}$ corresponds for the most part to $z_1 = -3$ (besides 14% of $z_1 = -2$ due to protonation of the phosphate group) while that of $\text{AscH}^\bullet$ and $\text{Asc}^{\bullet-}$ are $z_2 = 0$ and -1 , respectively. For $\text{FMNH}^\bullet$, $z_1 = -2$ would apply. Markedly different MARY profiles are expected due to different charge interactions and hyperfine parameters.

The theoretical predictions for $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$ were the closest match to the MARY parameters for the experimental data (within $\pm 5\%$), see Figure 3.12.

Note that the effective hyperfine coupling strength of the protonated $\text{AscH}^\bullet$ is distinctly larger than that of $\text{Asc}^{\bullet-}$ but still smaller than that of $\text{FMN}^{\bullet-}$. In agreement with the Weller formula, the more comprehensive calculations have lower $B_{1/2}$ predictions for the $(\text{FMN}^{\bullet-}\text{AscH}^\bullet)$ -pair versus $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$, see Figure 5 in Appendix 3. Furthermore, the simulations also indicate a drastic reduction of the LFE amplitude (by more than a factor of two) and an overall shift of the MFE profile to lower field intensities. These calculations poorly model the experimental observations, suggesting that either the $(\text{FMN}^{\bullet-}\text{AscH}^\bullet)$ is rapidly deprotonated on a timescale faster than the spin evolution in the radical pair (*i.e.* nanoseconds) or does not form at all.

Simulations for the $(\text{FMNH}^\bullet\text{Asc}^{\bullet-})$ radical pair show $B_{1/2}$ to be well-predicted (assuming $z_1 z_2 = 2$), whilst distinctly larger deviations are found for the LFE parameters (Figure 6 in Appendix 3).

In conclusion, the experimental MARY profiles recorded at pH 7 are most likely attributed to $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$ by spin chemistry considerations. The best agreement was found for $k_S = 7.8 \times 10^4 \text{ Å}^3 \text{ ns}^{-1}$ and $k_{sc} = 1/\tau_{sc} = 0.97 \text{ μs}^{-1}$.

FMN/AscH₂ (pH 3): At pH 3, the LFE amplitude amounts to a remarkable 27% for the pure triplet-born radical pair (see Table 3.1). Compared to the data at pH 7, the field effects are also shifted to larger fields. The trends revealed by the model calculations suggest that these observations are probably indicative of an attractive interaction within the RP that increases the re-encounter probability. Under the influence of an

attractive interaction, the spin evolution is more susceptible to the effects of the exchange interaction which induces additional broadening and potentially boosts the LFE amplitude.

The TA measurements indicate that for low quencher concentrations at pH 3, the RP comprises the protonated FMN radical, $\text{FMNH}^\bullet$, which is generated from the protonated flavin triplet state ($\text{p}K_a = 4.4$). Consequently, the flavin radical is expected to be the mono-anion ($z_1 = -1$) throughout its entire lifetime, the charge resulting from the hydrogen phosphate in the ribityl side chain. In order for an attractive Coulomb potential to ensue under these conditions, the transient RP would have to contain the fully protonated $\text{AscH}_2^{\bullet+}$ ($z_2 = +1$), at least for the duration of the magnetosensitive lifetime (larger than several nanoseconds but less than several microseconds). In this picture, the $\text{AscH}_2^{\bullet+}$ would result from the undissociated AscH_2 (pH 3) by direct electron transfer. The mono-deprotonated $\text{AscH}^\bullet$ and the thermodynamically favoured $\text{Asc}^{\bullet-}$, on the other hand, would give rise to zero ($z_2 = 0$) and repulsive ($z_2 = -1$) interaction potentials, which are in qualitative (and quantitative) disagreement with the large and broad LFE found for the T-pathway.

Even under the assumption that the geminate pair is comprised of $(\text{FMN}^{\bullet-} \text{AscH}_2^{\bullet+})$, the simulations cannot quantitatively reproduce the large LFE (see Figure 7 in Appendix 3). Instead, the calculations stipulate that the binding potential would have to surpass that of a Coulomb interaction with $z_1 z_2 = -3$, suggesting that the process involves additional, presumably specific, interactions, *e.g.* hydrogen bonding or π - π stacking.

Thus, realising that the model of a radical pair diffusing in a Coulomb potential is not able to reproduce the experimental data, further calculations were conducted for a RP at a fixed inter-radical distance as would result from a strong binding interaction of the reactants, see Figure 3.13. Here the species are modelled to undergo exponential recombination kinetics (via the singlet state) as occurs in intramolecular, rigid radical pair systems. The τ_{sc} parameter is replaced by τ_{esc} , the effective lifetime of the complex with ‘esc’ denoting escape. As the triplet-born radical pair complex is more susceptible to decay/escape, it inherently has a broader MFE profile versus a singlet-born pair with identical parameters describing their spin dynamics. Very similar results are found for the different protonation states of the ascorbyl radical, making a definitive assignment difficult.

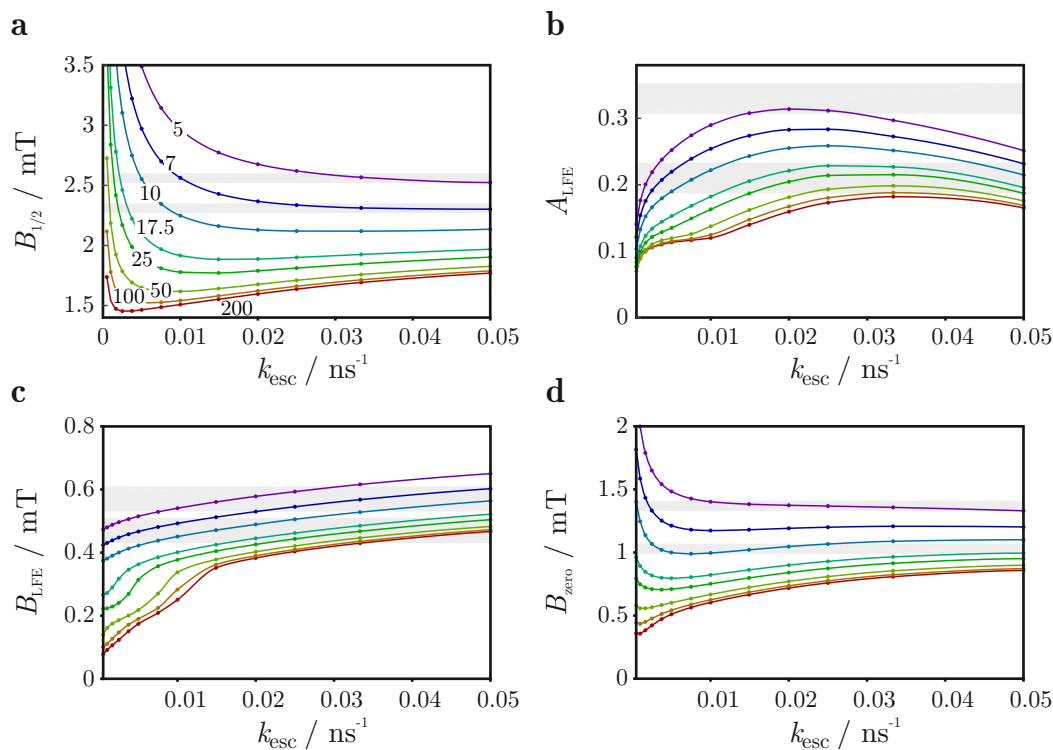


FIGURE 3.13: Shape parameters of the magnetic field effects arising from the bound ($\text{FMNH}^{\bullet}-\text{Asc}^{\bullet-}$) radical pair subject to spin selective recombination in the singlet state (rate constant k_S) and a homogenous decay process (rate constant k_{esc}). The colour of the lines follow k_S ; for a) the values of $\tau_S = 1/k_S$ in nanoseconds are indicated. The grey horizontal bars mark the experimentally determined parameters for the T- and S-pathways at pH 3 from Tables 3.1 and 3.2. The best-fitting parameter sets for the two radical pairs are reported in the text.

The best fits of the experimental data for the pH 3 studies are obtained using the following parameters for the ($\text{FMNH}^{\bullet}-\text{Asc}^{\bullet-}$) pair: T-pathway, $k_S = 1/5.1 \text{ ns}^{-1}$ and $k_{\text{esc}} = 1/40 \text{ ns}^{-1}$; S-pathway, $k_S = 1/10 \text{ ns}^{-1}$ and $k_{\text{esc}} = 1/115 \text{ ns}^{-1}$.

It should be highlighted that flavin-ascorbic acid complexes have been proposed by others [108]. However, these complexes are often elusive due to unaltered fluorescence and absorption characteristics. The complex could form following an electron transfer encounter of the excited state species which results in a bound radical pair, thus explaining the unperturbed ground state spectra for the system.

3.5 Conclusion and outlook

Sensitivity to fields comparable with the Earth’s magnetic field is of critical importance to the radical pair hypothesis for magnetoreception in animals. In this chapter, recombination of radical pairs resulting from photoinduced electron transfer reactions for FMN and ascorbic acid is demonstrated to be sensitive to weak magnetic fields of the order of several millitesla. The field dependence of the system exhibits a remarkable low field effect which peaks at external field intensities as low as 0.45 mT (*i.e.* fields smaller than associated with a household fridge magnet, or 10 times the Earth’s magnetic field). Relative LFE amplitudes observed here exceed those of known donor-acceptor systems, including biologically relevant entities such as cryptochromes (the putative magnetoreceptor of several migratory birds and other animals). This is unexpected as the reaction is formally bimolecular and, thus, subject to comparably low recombination quantum yields which typically prescribe small LFEs. The large LFEs, in part, arise due to the asymmetric distribution of the hyperfine coupling constants (as large hyperfine interactions in the flavin radical are accompanied by a single, strongly coupled proton with 0.176 mT only in the ascorbyl radical). Such a configuration is perceived to promote large LFEs from theoretical investigations [12]. In addition, the LFE can be boosted by attractive potentials of mean force as a result of Coulomb or specific interactions. Here, this effect is evident from the pH dependence of the magnetic field effect. In acidic solutions attractive interactions are active and the LFE reaches relative amplitudes of 27%, *i.e.* three times larger than at pH 7 for which the radicals are freely diffusing or experience repulsive Coulomb interactions.

At pH 7, the relative shape of the system’s field dependence is constant with increasing ascorbic acid concentration whilst the phase changes as a consequence of the bimolecular quenching reaction out-competing the intersystem crossing of $^1\text{FMN}^*$. The invariance in the shape parameters for the MFEs indicates that the transient, field-sensitive radical pair is identical for the singlet and triplet pathways. From a combination of the MARY profiles, transient absorption data and theoretical calculations, $(\text{FMN}^{\bullet-} \text{Asc}^{\bullet-})$ is assigned to be the main radical pair.

At pH 3, particularly large low field effects are observed which cannot be rationalised on the basis of radical ions diffusing in a Coulomb potential. However, the data are in agreement with the picture of a bound radical pair with a lifetime of the order of

100 ns, suggesting that specific interactions are opposing the free diffusion of the pair. In contrast to pH 7, the shape of the MARY curves are strongly dependent on the ascorbic acid concentration. This phenomenon reflects the involvement of changing proportions for different radical pairs in the magnetosensitive reaction state, differing by their reactivity. Whilst further investigation into the underlying mechanism is clearly required (*e.g.* by ultrafast absorption spectroscopy), the process allows for high sensitivity to weak magnetic fields which result not only from large intrinsic LFEs but also from the superposition of slightly different field effect curves. For intermediate ascorbic acid concentration the largest relative LFE is observed, which amounts to 40% of the saturated MFE. At higher ascorbic acid concentration, the effect changes sign and atypical compound lineshapes arise due to a composite of singlet- and triplet-born radical pair species. A steeper low-field response with an effective $B_{1/2}$ of less than 1 mT can be achieved, thereby giving optimised sensitivity to weak magnetic fields.

Preliminary studies on the magnetosensitivity of the flavin adenine dinucleotide (FAD) / AsCH₂ system have also been conducted, see Figure 3.14. FAD has previously been demonstrated to form a triplet-born, intramolecular radical pair following photoinduced electron transfer between the flavin and adenine groups [41]. The addition of tryptophan to solutions containing FAD can boost the size of the observed MFEs by forming intramolecular/intermolecular RP systems, *i.e.* F[•]—Adenine[•]/F[•]—Adenine + Tryptophanyl[•] [102]. Furthermore, it appears that replacing tryptophan with ascorbic acid in such a system can also enhance the magnitude of the LFE, presumably due to the LFE-favourable nature of its radicals (Figure 3.14c). Promotion of the LFEs derived from FAD by ascorbic acid in this manner could be relevant to improved low-field response of cryptochromes in magnetoreception, thus meriting further investigation of the mechanistic details.

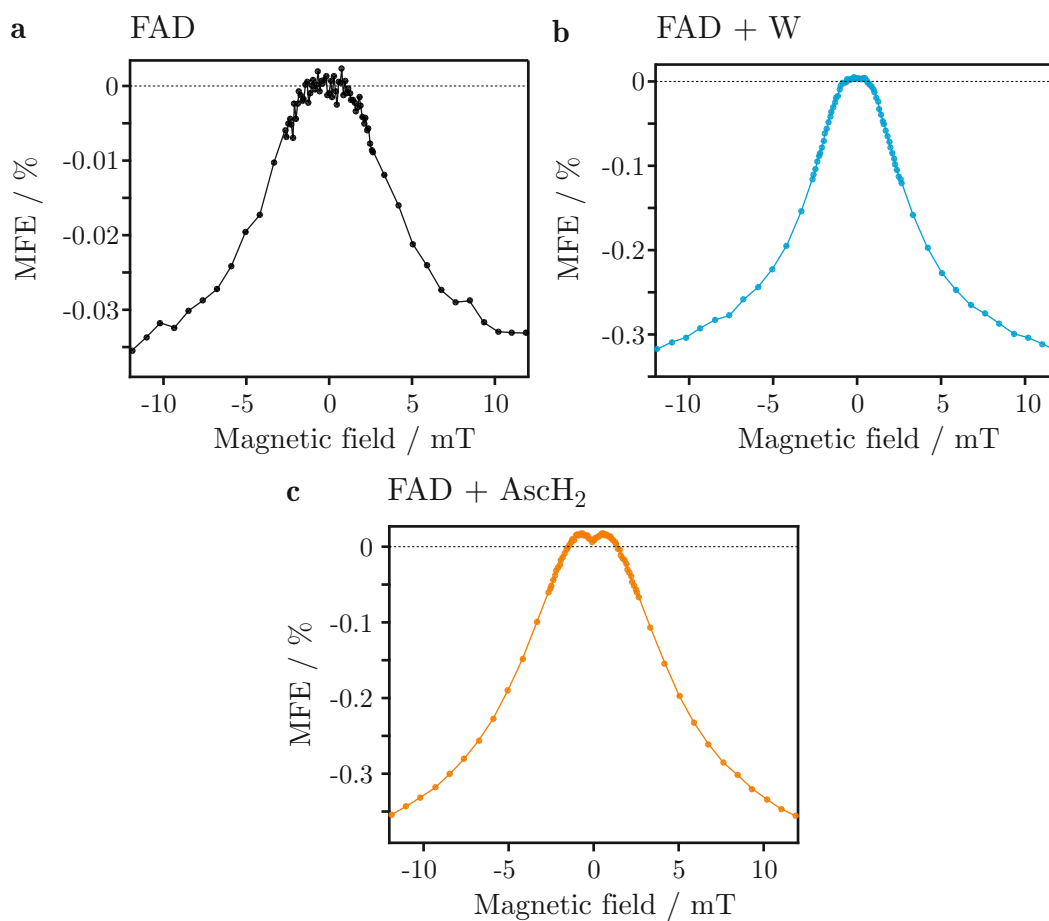


FIGURE 3.14: Transfer of a) intramolecular magnetosensitivity in FAD to intermolecular radical pairs involving b) tryptophan (W) and c) ascorbic acid (AscH₂). PF MARY plots for a) 10 μ M FAD (pH 7, aq), b) 10 μ M FAD/0.5 mM W (pH 7, aq) and c) 10 μ M FAD/0.5 mM AscH₂ (pH 4, aq). Measurements recorded using the experimental arrangement introduced in Chapter 2 (405 nm, 350 mW).

Chapter 4

Chemical amplification of MFEs

Critics of the cryptochrome radical pair hypothesis for magnetoreception often raise concerns regarding the detection of what are likely to be exceptionally small anisotropic MFEs derived from the Earth’s weak geomagnetic field (50 μ T) [12]. This aspect was even raised by Ritz et al. in the seminal paper which proposes the cryptochrome-based magnetosensing mechanism [13]:

“The theoretical considerations in this article address a possible primary magnetoreception mechanism in animals, leaving the question open whether and how the primary effects will be increased through subsequent amplification processes.” - Ritz et al., 2000, Biophys. J.

In their proposal, amplification of the field-dependent signals would be inherent to the visual transduction pathway itself. Others have envisaged that the magnetic field experienced by a radical pair-based sensor could be increased by adjacent magnetic nanoparticles containing magnetite [53–55]. MagR, an exotic rod-like protein complex and an alternative to the conventional cryptochrome hypothesis, is also postulated to exploit such an amplification [116]. The complex consists of an arrangement of 20 Fe-S clusters along the central axis which produces a permanent magnetic moment experienced by 10 cryptochrome proteins located in the outer layer.

In this chapter it is demonstrated that the primary magnetic field effect on flavin photoreactions can be subjected to chemical amplification by (previously dismissed [117]) slow termination reactions under conditions of continuous photoexcitation. The nature

of the amplification is revealed by studies of the intermolecular flavin/tryptophan and flavin/ascorbic acid radical pair photocycles. Work presented in this chapter has been conducted in collaboration with Dr. Daniel Kattnig and has been published in [118].

4.1 Experimental methods

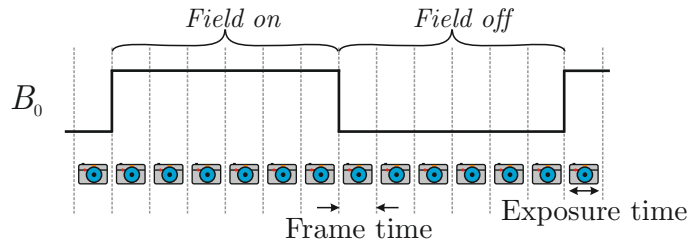
To study the chemical amplification of MFEs in flavin-based intermolecular radical pairs, two experimental arrangements were used. Figure 4.1 depicts the synchronisation of the applied magnetic field (B_0) and fluorescence measurements of systems using Apparatus 1 and 2 as discussed next.

4.1.1 Apparatus 1: Time-resolved MFEs

An inverted fluorescence microscope (Nikon, Ti-E) was used for time-resolved MFE measurements of the intermolecular RP photocycles studied in this chapter. Samples were fed into a flow cell (optical pathlength of 0.5 mm) constructed from a silicon rubber gasket (McMaster-Carr) and two coverslips (Menzel) [119]. Before each measurement, fresh sample was admitted to the cell and the flow was stopped. Static samples were illuminated by the attenuated and expanded beam of a 470 nm diode laser (Vortran Laser Technology, Stradus 473-80, 80 mW). Furthermore, the excitation light was focused at the back of an oil immersion objective (Nikon, CFI Apo TIRF 60 $\times$ Oil, N.A. 1.49) and enabled trans-illumination of the sample. By the use of neutral-density filters, the power entering the rear of the objective was varied between 0.24 and 3.7 mW. Fluorescence was collected through the same objective used for sample illumination, transmitted through a dichroic mirror (Semrock, Brightline FF497-Di03) and a band-pass filter (Semrock, Brightline 550/88), then recorded with an electron-multiplying CCD camera (Andor Technology, iXon+ DV680E). Magnetic fields of up to 27 mT were generated by a solenoid wound around a 3 mm diameter ferrite core and placed 1 mm above the sample in a non-metallic mount. Measurements using a custom-built gauss-meter revealed a field rise time of $\lesssim 70 \mu\text{s}$. A digital delay pulse generator (Quantum Composers, 9520) synchronised the data acquisition, magnetic field switching and an optical shutter (Thorlabs, SH05) to initiate photoexcitation at Time = 0 s (and not before).

For all studies using Apparatus 1 in this chapter (bar those in Figure 4.13), the fluorescence was recorded for an exposure time of 5 ms per frame and the interval between frames was 7 ms (frame rate ~ 143 Hz). 16,000 frames were measured for each experiment and the magnetic field was switched between zero and a preset value every 80, 160, 240 or 320 frames depending on the sample. The ‘field on/off’ interval is the time period that the magnetic field is on or off before switching to the other field state.

a Apparatus 1: Time-resolved MFEs



b Apparatus 2: Spectrally-resolved MFEs

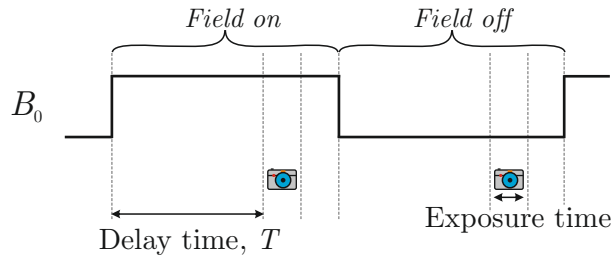


FIGURE 4.1: Depictions of the magnetic field-synchronised fluorescence measurements of samples using a) Apparatus 1 and b) Apparatus 2.

4.1.2 Apparatus 2: Spectrally-resolved MFEs

Magnetic field effects on fluorescence spectra were recorded using the same setup as in Chapter 2 (see Figure 2.2). However, the delay time (T) between setting the magnetic field and recording the sample fluorescence was varied beyond the previously fixed 50 ms. As before, an exposure time of 20 ms was used.

Apparatus 1 has a superior fluorescence collection efficiency to Apparatus 2 and thus lower exposure times can be employed in the former for comparable signal-to-noise to the latter. This translates into the possibility of a higher time resolution for monitoring the time evolution of the MFE by Apparatus 1. The studies are supplemented by spectrally-resolved MFE measurements using Apparatus 2.

4.1.3 Materials

Flavin mononucleotide (FMN), hen egg white lysozyme (HEWL), tryptophan (TrpH), indole, tris(2-carboxyethyl)phosphine (TCEP), citric acid, ascorbic acid (AscH_2), and glycerol were obtained from Sigma Aldrich and used without further purification. Samples were prepared with ultrapure water (Milli-Q, $18.2 \text{ M}\Omega \cdot \text{cm}$).

4.2 MFE enhancement and reduction

Initial studies using Apparatus 1 were conducted on the previously explored intermolecular model systems of the flavin/tryptophan radical pair in a cryptochrome (see Chapter 2).

4.2.1 MFE enhancement - Flavin/HEWL

Time-resolved fluorescence-detected magnetic field effects for the FMN/HEWL photoreaction are shown in Figures 4.2a and b. During the experiment, the sample was subjected to continuous illumination by a 470 nm laser and a stepping applied magnetic field which switched between 0 and 27 mT every 1.68 s (see cartoon depiction in Figure 4.2). The prompt fluorescence of the sample was recorded continuously and the overall decrease in Figure 4.2a is attributed to a combination of re-equilibration of the photocycle, photobleaching reactions and diffusion. Superimposed on the decaying trend is a small modulation in the fluorescence intensity. The modulations and the time-dependent magnetic field applied to the sample are synchronous. Furthermore, the relative size of the modulations do not change significantly with time as their amplitudes decrease with the decrease in the overall fluorescence intensity. The time-resolved MFE (as defined in Equation 2.2) was averaged over multiple field cycles to obtain the averaged response to a single field step, see Figure 4.2b. Following the ‘normal’ MFE behaviour of triplet-born SCRPs in aqueous solutions of FMN/HEWL, switching the field on/off causes a reduction/increase in the fluorescence. See Chapter 2 for further discussion on the origin of the primary field response for this system as explained using the general photochemical scheme for bimolecular triplet-born RPs in Figure 2.1.

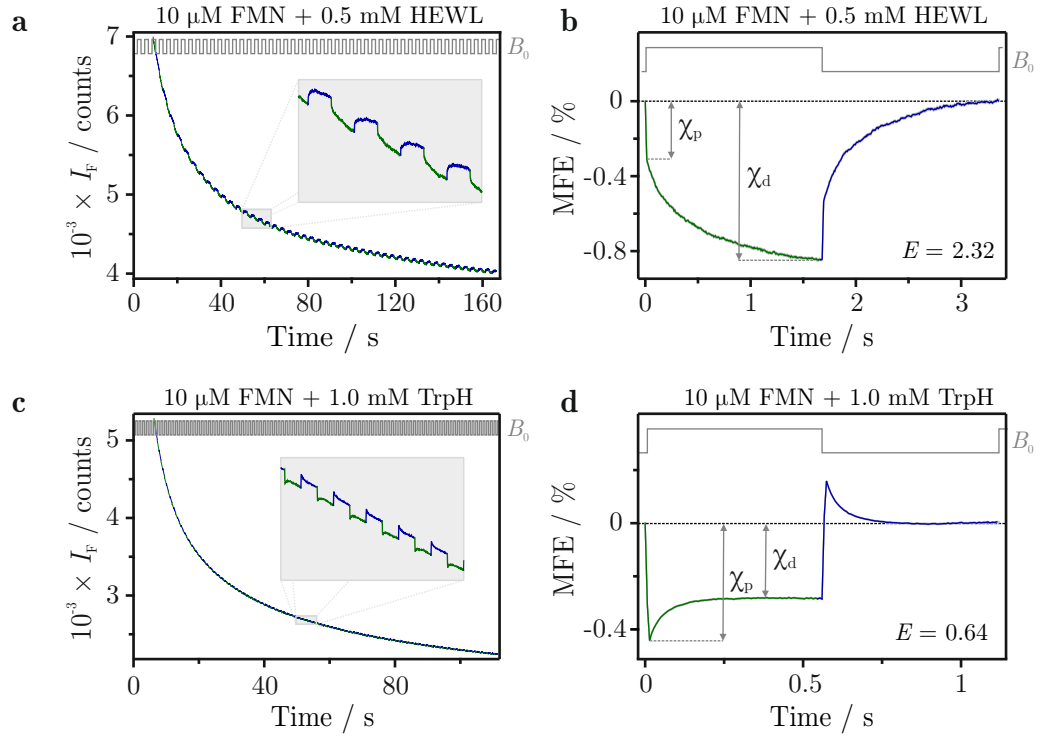


FIGURE 4.2: a) and b) 10 μ M FMN/0.5 mM HEWL in aqueous solution (pH 4.0). c) and d) 10 μ M FMN/1.0 mM TrpH in aqueous solution (pH 6.2). a) Fluorescence intensity profile for the sample subjected to continuous photoexcitation (470 nm, 0.24 mW) initiated at Time = 0 s and step-wise changes of the external magnetic field (B_0) between 0 and 27 mT (as represented pictorially) every 1.68 s. b) Average MFE responses to individual magnetic field steps depicting a prompt MFE (χ_p) and a slowly established delayed MFE (χ_d). c) and d) are analogous to a) and b), showing the time-resolved MFE measurements for FMN/TrpH, but with a higher photoexcitation power (3.7 mW) and field switching every 0.56 s. The data in the field-on and field-off periods in all graphs are shown in green and blue, respectively. In b) and d), the slow background decay of the fluorescence, visible in a) and c), has been subtracted. Measurements were conducted using Apparatus 1.

Curiously, the time-dependent MFE in Figure 4.2b has two components. Following the MFE as a function of time after switching on the magnetic field, a fast, prompt MFE (χ_p) is reached within < 1 ms. The MFE is then slowly enhanced with an exponential time constant of $\tau = 470 \pm 10$ ms to a delayed MFE (χ_d). A complementary pattern of fast and slow phases is observed when the field is switched off. The amplification factor, E , defined as χ_d/χ_p , is 2.32 ± 0.007 . χ_p can be assigned to the system reaching a new photostationary state at a rate which is limited by diffusion-controlled re-encounter of the free radicals $F^{\bullet-}$ and $D^{\bullet+}$. The timescale of this process is estimated as 20-100 μ s (based on a re-encounter rate constant of $10^{10} \text{ M}^{-1}\text{s}^{-1}$ and 1-5 μ M photostationary concentrations of $F^{\bullet-}$ and $D^{\bullet+}$). Measurements of the fluorescence intensity and MFE time profiles for the field-on and field-off periods are depicted in green and blue, respectively.

4.2.2 MFE reduction - Flavin/tryptophan

Analogous time-resolved MFE studies were conducted for FMN/TrpH (with tryptophan donor present in free amino acid form). As for FMN/HEWL, fast and slow phases in the response of the MFE to field switching are observed (Figure 4.2c and d). However, the initial MFE is now reduced with time and the evolution occurs on a faster timescale such that $E = 0.644 \pm 0.0003$ and $\tau = 55 \pm 1$ ms.

The standard error for the MFE response associated with the reactions of FMN/HEWL, from 448 averages, and FMN/TrpH, from 2375 averages, are denoted by (barely-visible) grey shaded regions around the averaged profiles. Averaged MFE responses to field-on and field-off steps are symmetrical and superimposable as shown in Figure 4.3.

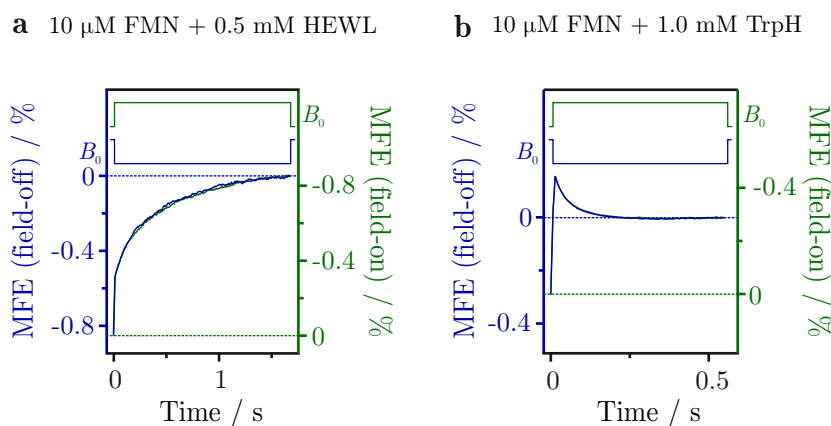


FIGURE 4.3: Overlaid average MFEs for 27 mT field-on (green) and field-off (blue) steps. a) 10 μ M FMN/0.5 mM HEWL (aq, pH 4.0); and b) 10 μ M FMN/1.0 mM TrpH (aq, pH 6.2). Data presented in the figure were obtained by Apparatus 1 and are re-representations of the data in Figures 4.2b and d.

4.2.3 Dependence on magnetic field intensity

Figure 4.4 shows the MARY plots for the MFEs derived from FMN/HEWL photoreactions as a function of magnetic field for different delay times, T , between changing the magnetic field and recording the MFE ($T = 10\text{ ms}$ – 5 s , Apparatus 2). In agreement with Figure 4.2b, the magnitude of the negative MFE becomes larger with increasing T . The magnetic field strength, $B_{1/2}$, corresponding to half of the maximum magnitude of the ‘normal’ MFE was obtained by Lorentzian fitting for each profile. A fixed, T -independent $B_{1/2}$ value of 6.5 mT is found. This is consistent with MFEs originating from hyperfine interactions in the flavin/tryptophan radical pair, augmented by spin dephasing (see Chapter 2). Furthermore, the invariance in $B_{1/2}$ suggests that the amplification does not originate in the field-dependent spin mixing (geminate pair) region of the photocycle. This is an important insight into the unexpected amplification phenomenon.

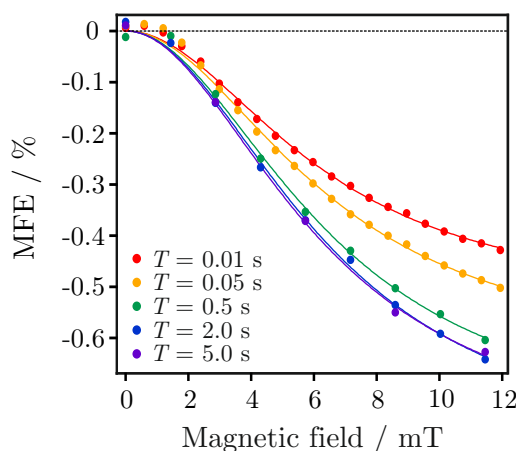


FIGURE 4.4: Dependence of the MFE on the magnetic field strength for $10\text{ }\mu\text{M}$ FMN/ 0.5 mM HEWL (aq) for different delay times (T) between switching the field on and measuring the fluorescence. The solid lines are Lorentzian fits to the data used to determine the $B_{1/2}$ values. Measurements were conducted using Apparatus 2 (405 nm at 270 mW).

4.3 Slow radical termination reactions

As discussed, the typical photocycle in Figure 2.1 used to rationalise MFEs derived from triplet-born bimolecular RPs cannot explain the slow phase response observed in Figures 4.2b, d and 4.4. The enhanced effects found in continuously photoexcited systems actually originate in slow termination reactions that return the free radicals to their respective ground states. These processes occur in addition to recombination of the radicals and on a much longer timescale than previously considered in magnetically sensitive RP reactions. The typical flavin/donor intermolecular RP photocycle was augmented to include these additional reactions (see coloured arrows, Figure 4.5a), whereby the rate constants corresponding to the removal of the flavin and donor radicals are given by k_F and k_D , respectively. For a simplicity in the following discussions, the termination reactions are assumed to follow first-order kinetics. As might be expected, when $k_F = k_D$, the extra k_F - and k_D -reaction pathways do not significantly affect the overall kinetics of the system. The field-response is identical to that in the absence of the additional routes, the transition to the new stationary state is fast ($\sim 100 \mu\text{s}$) and step-wise as typically expected, see Figure 4.5bi). However, when $k_F \neq k_D$, the prompt effect is followed by a slow phase. If $k_F < k_D$, the additional termination processes yield an enhancement of the MFE ($E > 1$, Figure 4.5bii)); whereas if $k_F > k_D$, the primary MFE is reduced with time ($E < 1$, Figure 4.5biii)).

Qualitatively, this MFE reduction for $k_F > k_D$ can be understood as the dominant, additional $F^{\bullet-}$ decay pathways serve to, overall, reduce the difference in fates for the singlet and triplet geminate RP. The MFE enhancement is attributed to removal of the flavin radical's recombination partner which leads to accumulated flavin radical concentration and accumulated, amplified MFEs.

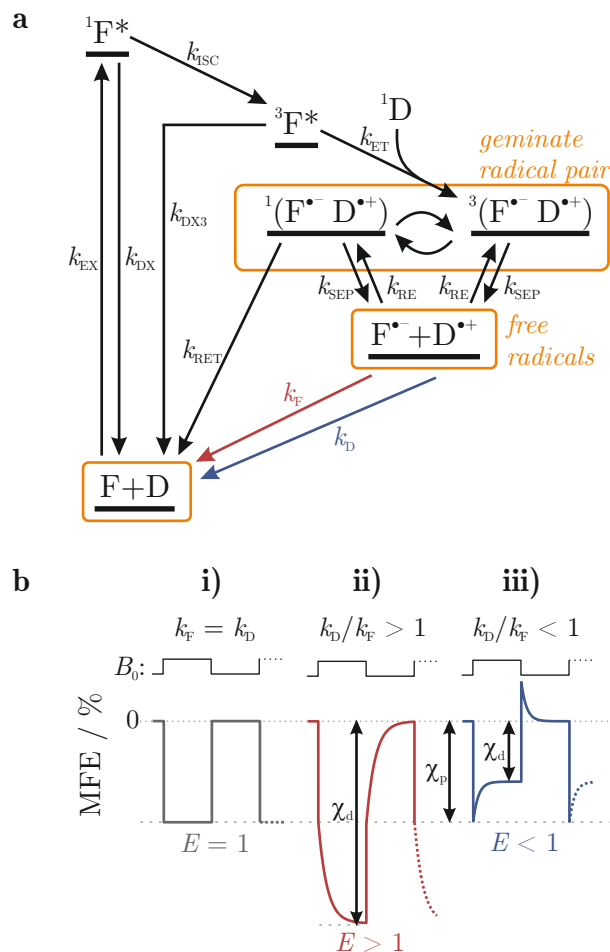


FIGURE 4.5: a) Augmented photocycle from Figure 2.1 for the intermolecular radical pair formed between a flavin (F) and an electron donor (D). The slow reaction steps labelled with rate constants k_F and k_D are responsible for the amplification of the MFE. The rate constants associated with the other processes are indicated and defined in Table 4.1. b) Characteristic responses of the flavin excited state, $^1F^*$, simulated (see next section) for sudden on/off switching of an external magnetic field (B_0): i) $k_F = k_D$ ($E = 1$, grey); ii) $k_F < k_D$ ($E > 1$, red); iii) $k_F > k_D$ ($E < 1$, blue). The amplification factor is defined as $E = \chi_d/\chi_p$ where χ_p and χ_d are the prompt and delayed MFEs, respectively.

4.3.1 Numerical calculation of MFEs

Using an approach analogous to that presented by Murakami et al., the time-dependence of the photocycle in Figure 4.5a subject to step-wise modulations in the intensity of an external magnetic field has been modelled via appropriate rate equations [41].

Field-dependent singlet-triplet interconversion of the singlet (S) and triplet (T_0, T_+, T_-) spin states of the geminate radical pair (curly arrows in Figure 4.5a) is the origin of the photocycle's magnetosensitivity. The field-independent rate equations for species other than the geminate pair are as follows:

$$\frac{d[F]}{dt} = -k_{EX}[F] + k_{DX}[^1F^*] + k_{DX3}[^3F^*] + k_{RET}[S] + k_F[F^{\bullet-}] \quad (4.1a)$$

$$\frac{d[^1F^*]}{dt} = -(k_{ISC} + k_{DX})[^1F^*] + k_{EX}[F] \quad (4.1b)$$

$$\frac{d[^3F^*]}{dt} = -(k_{ET}[D] + k_{DX3})[^3F^*] + k_{ISC}[^1F^*] \quad (4.1c)$$

$$\frac{d[F^{\bullet-}]}{dt} = -(k_{RE}[D^{\bullet+}] + k_F)[F^{\bullet-}] + k_{SEP}([S] + [T_0] + [T_+] + [T_-]) \quad (4.1d)$$

$$\frac{d[D]}{dt} = -k_{ET}[^3F^*][D] + k_{RET}[S] + k_D[D^{\bullet+}] \quad (4.1e)$$

$$\frac{d[D^{\bullet+}]}{dt} = -(k_{RE}[F^{\bullet-}] + k_D)[D^{\bullet+}] + k_{SEP}([S] + [T_0] + [T_+] + [T_-]). \quad (4.1f)$$

Full definitions for the rate constants above are given in Table 4.1. In the absence of a magnetic field, spin mixing by the hyperfine mechanism interconverts the singlet state (S) with all three triplet substates (T_0, T_+, T_-) with the rate constant, k_{HFM} :

$$\begin{aligned} \frac{d[S]}{dt} = & -(3k_{HFM} + k_{RET} + k_{SEP})[S] + k_{HFM}([T_0] + [T_+] + [T_-]) + \\ & \frac{1}{4}k_{RE}[F^{\bullet-}][D^{\bullet+}] \end{aligned} \quad (4.2a)$$

$$\begin{aligned} \frac{d[T_0]}{dt} = & -(3k_{HFM} + k_{SEP})[T_0] + k_{HFM}([S] + [T_+] + [T_-]) + \\ & \frac{1}{4}k_{RE}[F^{\bullet-}][D^{\bullet+}] + \frac{1}{3}k_{ET}[^3F^*][D] \end{aligned} \quad (4.2b)$$

$$\begin{aligned} \frac{d[T_+]}{dt} = & -(2k_{\text{HFM}} + k_{\text{SEP}})[T_+] + k_{\text{HFM}}([S] + [T_0]) + \frac{1}{4}k_{\text{RE}}[F^{\bullet-}][D^{\bullet+}] + \\ & \frac{1}{3}k_{\text{ET}}[{}^3F^*][D] \end{aligned} \quad (4.2c)$$

$$\begin{aligned} \frac{d[T_-]}{dt} = & -(2k_{\text{HFM}} + k_{\text{SEP}})[T_-] + k_{\text{HFM}}([S] + [T_0]) + \frac{1}{4}k_{\text{RE}}[F^{\bullet-}][D^{\bullet+}] + \\ & \frac{1}{3}k_{\text{ET}}[{}^3F^*][D]. \end{aligned} \quad (4.2d)$$

At high magnetic field, the T_+ and T_- states become energetically isolated from S and T_0 by the Zeeman interaction. This disables S - $T_{\pm}$ interconversion such that the rate equations for the geminate pair become:

$$\begin{aligned} \frac{d[S]}{dt} = & -(k_{\text{HFM}} + 2k_{\text{RM}} + k_{\text{RET}} + k_{\text{SEP}})[S] + k_{\text{HFM}}[T_0] + k_{\text{RM}}([T_+] + [T_-]) + \\ & \frac{1}{4}k_{\text{RE}}[F^{\bullet-}][D^{\bullet+}] \end{aligned} \quad (4.3a)$$

$$\begin{aligned} \frac{d[T_0]}{dt} = & -(k_{\text{HFM}} + 2k_{\text{RM}} + k_{\text{SEP}})[T_0] + k_{\text{HFM}}[S] + k_{\text{RM}}([T_+] + [T_-]) + \\ & \frac{1}{4}k_{\text{RE}}[F^{\bullet-}][D^{\bullet+}] + \frac{1}{3}k_{\text{ET}}[{}^3F^*][D] \end{aligned} \quad (4.3b)$$

$$\begin{aligned} \frac{d[T_+]}{dt} = & -(2k_{\text{RM}} + k_{\text{SEP}})[T_+] + k_{\text{RM}}([S] + [T_0]) + \frac{1}{4}k_{\text{RE}}[F^{\bullet-}][D^{\bullet+}] + \\ & \frac{1}{3}k_{\text{ET}}[{}^3F^*][D] \end{aligned} \quad (4.3c)$$

$$\begin{aligned} \frac{d[T_-]}{dt} = & -(2k_{\text{RM}} + k_{\text{SEP}})[T_-] + k_{\text{RM}}([S] + [T_0]) + \frac{1}{4}k_{\text{RE}}[F^{\bullet-}][D^{\bullet+}] + \\ & \frac{1}{3}k_{\text{ET}}[{}^3F^*][D]. \end{aligned} \quad (4.3d)$$

Here k_{RM} is the rate constant for incoherent spin mixing by the relaxation mechanism. S - T mixing by the relaxation mechanism is also active at weak/zero fields but as $k_{\text{RM}} \ll k_{\text{HFM}}$, its contributions have been omitted from Equations 4.2a-d.

The prompt fluorescence intensity detected in the experiments is proportional to the flavin singlet excited state concentration, thus PF MFEs were simulated by profiling

[$^1\text{F}^*$]. MATLAB's stiff, backward-differentiation ordinary differential equation solver, ode15s, was employed for the numerical calculations.

Calculations were performed using the rate constants in Table 4.1 with varied k_F and k_D (10^{-1} – 10^3 s^{-1}). In determining the enhancement factor, E , the final, delayed MFE (χ_d) response is obtained by the steady state solution to Equations 4.1, 4.2 and 4.3. The prompt MFE (χ_p) is defined as the field-effect arising immediately after magnetic field switching and was determined by an extrapolation of the exponential fit for the slow phase of MFE to Time = 0 s.

Calculated χ_p , χ_d and $E = \chi_d/\chi_p$ values for a solution of 10 μM FMN/0.5 mM HEWL are plotted in Figures 4.6a, b and c, respectively. The plots are given as a function of k_D and k_F . From the broad study, E is found to depend principally on the ratio of k_D/k_F . Surprisingly large steady-state enhancements (~ 5) can result from these slow termination processes. The actual values of k_F and k_D sets the timescale (characterised by an exponential time constant, τ) for reaching χ_d (Figure 4.6d). No simple dependence of τ on k_F and k_D could be derived but there appears to be a stronger dependence on the latter¹. τ was not evaluated where $k_F = k_D$ due to the absence of a slow phase following the prompt MFE response under these conditions, hence the white diagonal in Figures 4.6d and 4.7d.

¹Attempting to fit τ to combinations of ' $1/k_F$ ', ' $1/k_D$ ' and ' $1/(k_F + k_D)$ ' yields: $\log_e(\tau) \approx 0.82\log_e(1/k_D) + 0.23\log_e(1/k_F)$. However, as seen for other calculations with a different pump power in Figure 4.7d, this expression is not generally applicable.

TABLE 4.1: Values of the rate constants used in calculations of amplified MFEs for the intermolecular flavin photocycle in Figure 4.5a. The results are presented in Figures 4.6 ($k_{\text{EX}} = 1.36 \times 10^4 \text{ s}^{-1}$) and 4.6 ($k_{\text{EX}} = 2.72 \times 10^3 \text{ s}^{-1}$).

Rate constant	Value	Comment
k_{EX}	$1.36 \times 10^4 \text{ s}^{-1}$	= Apparatus 1, 470 nm, 3.7 mW.
	$2.72 \times 10^3 \text{ s}^{-1}$	= Apparatus 1, 470 nm, 20% $\times$ 3.7 mW. Photoexcitation is approximated to follow first order kinetics in the weak absorbance limit of the Beer-Lambert Law.
k_{DX}	$1 \times 10^8 \text{ s}^{-1}$	Decay of the flavin excited singlet state ($^1\text{F}^*$) by processes other than ISC [34].
k_{ISC}	$1 \times 10^8 \text{ s}^{-1}$	Intersystem crossing from $^1\text{F}^*$ to $^3\text{F}^*$ [35].
k_{ET}	$1.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	Reaction of the flavin excited triplet state ($^3\text{F}^*$) with an electron donor, D. The numerical value was derived from Stern-Volmer type experiments on FMN + TrpH using transient absorption spectroscopy [120].
k_{DX3}	$4 \times 10^5 \text{ s}^{-1}$	Decay of ($^3\text{F}^*$) by processes other than quenching by D. The numerical value was taken from transient absorption experiments on FMN, see Appendix 3.
k_{SEP}	$2 \times 10^8 \text{ s}^{-1}$	Separation of the geminate radical pair components to form free radicals. The value is based on a typical geminate radical pair lifetime of 100 ns in aqueous solution.
k_{HFM}	$8 \times 10^7 \text{ s}^{-1}$	Coherent singlet-triplet interconversion of the spin states of the geminate radical pair by the hyperfine mechanism as determined by Murakami et al. [41].
k_{RM}	$2 \times 10^6 \text{ s}^{-1}$	Incoherent singlet-triplet interconversion of the spin states of the geminate radical pair by the relaxation mechanism as determined by Murakami et al. [41].
k_{RE}	$1.87 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$	Re-encounter of free radicals to form the geminate radical pair. The value is obtained from the Smoluchowski equation (with an effective encounter distance, $R^* = 20 \text{ \AA}$) and the Stokes-Einstein relation ($T = 295 \text{ K}$; species radius, $R = 3.5 \text{ \AA}$; $\eta = 1 \times 10^{-2} \text{ kg m}^{-1} \text{ s}^{-1}$).
k_{RET}	$1 \times 10^8 \text{ s}^{-1}$	Average spin-selective rate of reverse electron transfer of the in-cage radical pair to form the ground state reactants.

$$k_{\text{EX}} = 1.36 \times 10^4 \text{ s}^{-1}$$

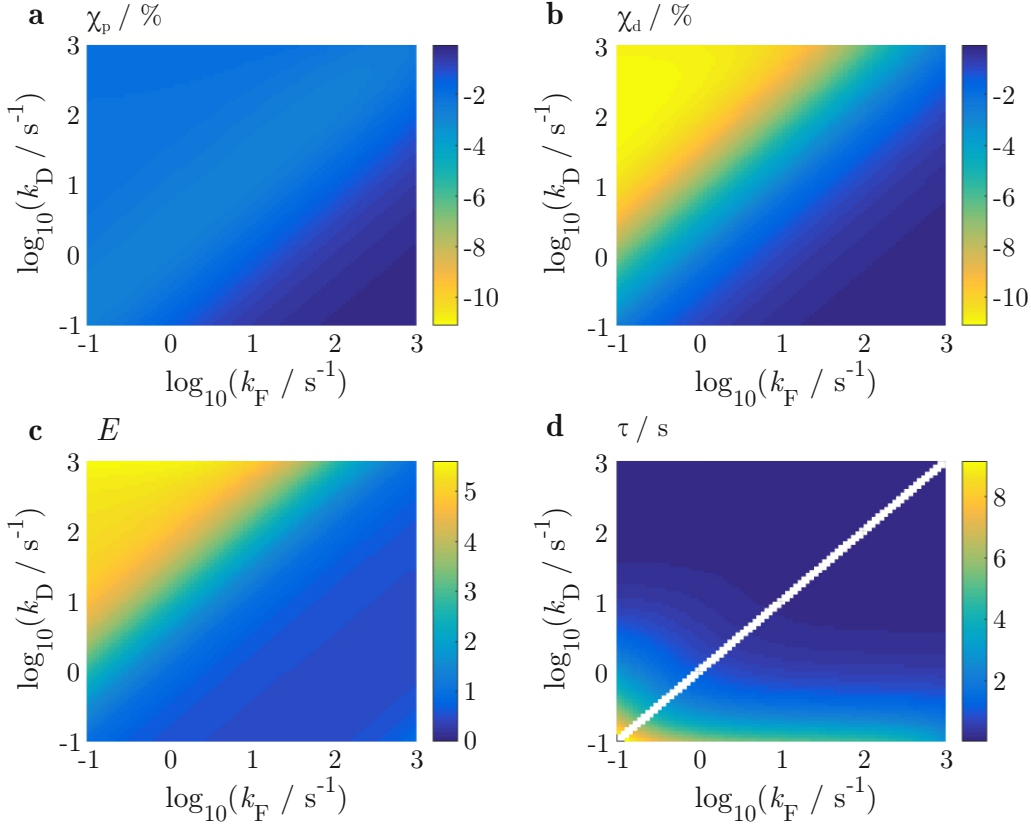


FIGURE 4.6: Density plots of the a) prompt MFE (χ_p), b) delayed MFE (χ_d), c) amplification factor (E) and d) time constant (τ) of the MFEs simulated for the intermolecular F/D radical pair, given as a function of the radical termination constants k_F and k_D . The simulations were performed using the rate constant values in Table 4.1 with $k_{\text{EX}} = 1.36 \times 10^4 \text{ s}^{-1}$.

Interestingly, lower light intensities were found to yield larger amplification factors. The calculated behaviour for a sample subjected to 20% of the photon flux (*i.e.* $k_{\text{EX}} = 2.72 \times 10^3 \text{ s}^{-1}$) is shown in Figure 4.7. Amplification factors of up to 24 are observed in the studied range of k_F and k_D values. There is a strong qualitative difference in the time constant dependence on the varied rate constants for Figure 4.7. A ‘valley’ is present in the time constant density plot for $\log_{10}(k_F/\text{s}^{-1}) = -1$ to 0 and $\log_{10}(k_D/\text{s}^{-1}) = -1$ to 0. This ‘valley’ broadens along the $\log_{10}(k_D/\text{s}^{-1})$, $\log_{10}(k_F/\text{s}^{-1})$ axes and the diagonal with decreasing laser pump power (*i.e.* decreasing k_{EX}).

$$k_{\text{EX}} = 2.72 \times 10^3 \text{ s}^{-1}$$

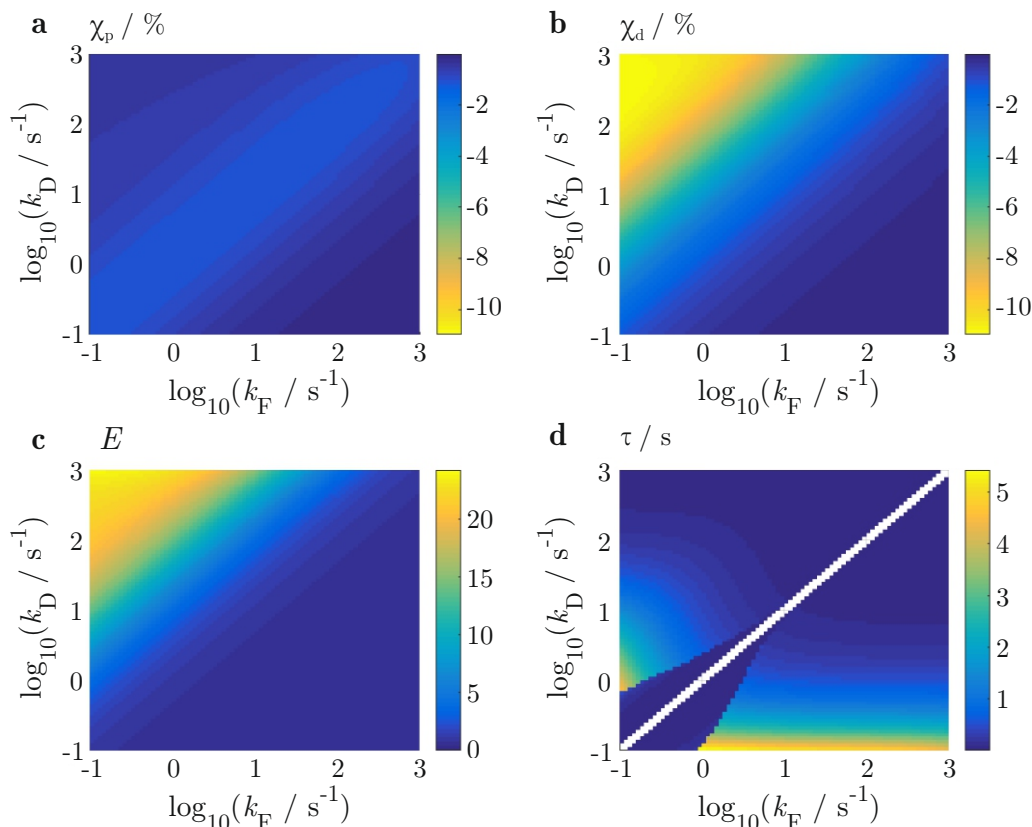


FIGURE 4.7: Density plots of the a) prompt MFE (χ_p), b) delayed MFE (χ_d), c) amplification factor (E) and d) time constant (τ) of the MFEs simulated for the intermolecular F/D radical pair, given as a function of the radical termination constants k_F and k_D . The simulations were performed using the rate constant values in Table 4.1 with sample subjected to 20% the photon flux subjected to the ‘sample’ in Figure 4.6 (*i.e.* $k_{\text{EX}} = 2.72 \times 10^3 \text{ s}^{-1}$).

4.4 Barebones model of MFE amplification

Although the numerical calculations for the kinetic scheme in Figure 4.5a are comprehensive, the physical origins for the enhanced effects are perhaps lost. Thus, a simpler scheme containing only four species which lead to the same qualitative behaviour is considered. In this section, the barebones model of MFE amplification is introduced. This model is used to derive a simple expression which relates the enhancement factor, E , to k_D and k_F .

The reaction scheme underlying the barebones intermolecular amplifier is shown in Figure 4.8a. It is comprised of the diamagnetic, ground-state fluorophore (F), magnetosensitive geminate pair (G) and free radical states $F^{\bullet-}$ and $D^{\bullet+}$. MFEs arise by the

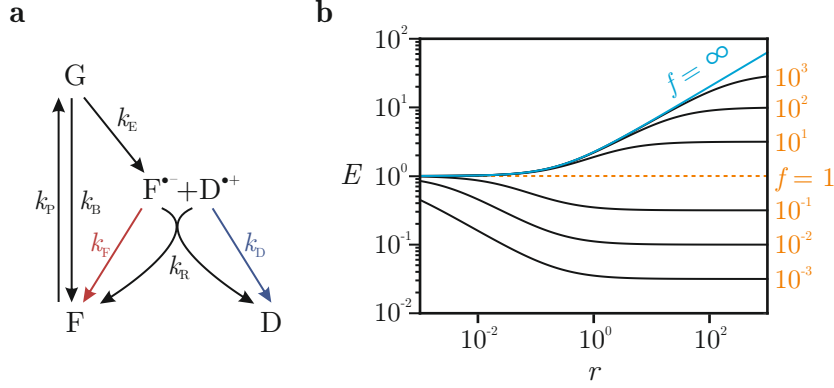


FIGURE 4.8: a) Simplified reaction scheme for magnetic field effects on intermolecular flavin/donor reactions. The curved arrows denote a bimolecular process. b) Dependence of the amplification factor, E , on the parameter r (as defined in the text). The values of $f = k_D/k_F$ are indicated in orange.

field-dependent reaction of G to the ground state species, which has a rate constant of $k_B(B_0)$. The rate equations governing the kinetics of the barebones model are:

$$\frac{d[F]}{dt} = -k_P[F] + k_B(B_0)[G] + k_F[F^{\bullet-}] + k_R[F^{\bullet-}][D^{\bullet+}] \quad (4.4a)$$

$$\frac{d[G]}{dt} = -(k_B(B_0) + k_E)[G] + k_P[F] \quad (4.4b)$$

$$\frac{d[F^{\bullet-}]}{dt} = k_E[G] - k_F[F^{\bullet-}] - k_R[F^{\bullet-}][D^{\bullet+}] \quad (4.4c)$$

$$\frac{d[D^{\bullet+}]}{dt} = k_E[G] - k_D[D^{\bullet+}] - k_R[F^{\bullet-}][D^{\bullet+}]. \quad (4.4d)$$

As aforementioned, the attainment of the prompt MFE (χ_p) is rate-limited by radical-radical recombination and is not affected by the slow termination reactions. Thus, the prompt MFE can be evaluated from the photostationary state of the system where $k_F = k_D = 0$. Assuming negligible steady-state population of the short-lived geminate pair (G), *i.e.* $[F]_{\text{total}} = [F] + [F^{\bullet-}]$, the photostationary concentration for the F ground state ($[F]_{\text{PS}}$) is:

$$[F]_{\text{PS}}(k_F = k_D = 0) = \frac{2[F]_{\text{total}}k_Rk_B + k_E(k_P + 2[F]_{\text{total}}k_R) - \sqrt{k_Ek_P(k_Ek_P + 4[F]_{\text{total}}k_R(k_B + k_E))}}{2(k_B + k_E)k_R}. \quad (4.5)$$

More generally, when $k_F \neq 0$ and $k_D \neq 0$, the steady-state concentrations include the amplifying/reducing effects of the termination reactions and the following is derived:

$$[F]_{PS}(k_F \neq 0, k_D \neq 0) = \frac{k_D k_E k_P + k_F(k_B + k_E)(k_D + 2[F]_{total} k_R)}{2k_F(k_B + k_E)k_R} - \frac{\sqrt{k_D(k_D(k_F(k_B + k_E) + k_E k_P)^2 + 4[F]_{total} k_R k_F k_E(k_B + k_E)k_P)}}{2k_F(k_B + k_E)k_R}. \quad (4.6)$$

Since the field-dependent photochemistry in the barebones model arises due to k_B 's magnetic field dependence, the MFE on the prompt fluorescence of the system can be defined as the fractional change in the photostationary concentration of F produced by a fractional change in k_B . Thus, the prompt and delayed MFEs ($\chi_i, i = p, d$) are derived as:

$$\chi_i = \frac{(d[F]_{PS})/[F]_{PS}}{(dk_B)/k_B}, \quad (4.7)$$

where $[F]_{PS}$ is given by Equations 4.5 and 4.6 for the prompt MFE, χ_p , and the delayed MFE, χ_d , respectively. Furthermore, the amplification factor is then evaluated as:

$$\begin{aligned} E &= \frac{\chi_d}{\chi_p} \\ &= \frac{\sqrt{k_D k_E k_P (k_E k_P + 4[F]_{total} k_R (k_B + k_E))}}{\sqrt{k_D (k_F (k_B + k_E) + k_E k_P)^2 + 4[F]_{total} k_R k_F k_E k_P (k_B + k_E)}} \\ &= \frac{\sqrt{1 + \frac{4[F]_{total} k_R (k_B + k_E)}{k_P k_E}}}{\sqrt{(1 + \frac{k_F (k_B + k_E)}{k_P k_E})^2 + \frac{4[F]_{total} k_R k_F (k_B + k_E)}{k_D k_P k_E}}}. \end{aligned} \quad (4.8)$$

Since k_D and k_F are assumed to be small compared to the other rate constants, Equation 4.8 can be simplified as $(1 + k_F(k_B + k_E)/(k_P k_E))^2 \rightarrow 1$. The following is obtained:

$$E = \sqrt{\frac{1 + 4r}{1 + 4r/f}}, \quad (4.9)$$

where $f = k_D/k_F$, $r = (k_R[F]_{total})/(\phi_E k_P)$ and $\phi_E = k_E/(k_E + k_B)$ denotes the quantum yield for the formation of free radicals. When $4r \gg 1$ and $4r \gg f$, Equation 4.9

implies that $E \approx \sqrt{f} = \sqrt{k_D/k_F}$. Figure 4.8b depicts the dependence of E on r for different ratios of k_D and k_F . When $r \gtrsim 100$ *i.e.* high flavin concentration and weak photoexcitation conditions, E is generally found to be proportional to $\sqrt{k_D/k_F}$.

The barebones model emphasises the non-restrictive requirements for the chemical amplification of magnetic field effects. Enhanced effects arise for cyclic systems containing a magnetosensitive radical pair which undergoes non-spin-selective termination of the radicals with unequal rates.

4.5 Rate constants

Using full numerical calculations as outlined in Section 4.3.1, appropriate fitting of the MFE traces enabled the determination of the rate constants for the overall effect of the termination reactions. For the FMN/HEWL case ($k_{EX} = 8.82 \times 10^2 \text{ s}^{-1}$, 0.24 mW photoexcitation), $k_F = 1.1 \text{ s}^{-1}$ and $k_D = 4.7 \text{ s}^{-1}$; and for FMN/TrpH ($k_{EX} = 1.36 \times 10^4 \text{ s}^{-1}$), $k_F = 66 \text{ s}^{-1}$ and $k_D = 12 \text{ s}^{-1}$. For these two systems, amplification factors from the barebones model are predicted to be $\sqrt{k_D/k_F} = 2.1$ and 0.43, respectively. These values agree reasonably well with the experimental amplification factors, $E = 2.3$ and 0.64. The better match for the FMN/HEWL system versus FMN/TrpH could be attributed to the lower pump power used to photoexcite the former. This would result in a larger r -value and thus find the system closer to the regime of $E \rightarrow \sqrt{k_D/k_F}$ in the barebones model. It is worth noting that the reduction in pump power must be more significant than the expected reduction in k_R for the slowly diffusing ‘large’ protein versus ‘small’ amino acid scenario.

k_D is found to be lower for the flavin/lysozyme reaction versus flavin/tryptophan (free amino acid). This presumably arises due to the lower accessibility of the TrpH residues in the protein for electron transfer than the free form. Different k_F for the two reactions can be ascribed to two factors: 1) sample pH and 2) the lower photoexcitation power for studying MFEs originating from the photoreactions of flavin/HEWL (pH 4.0) versus flavin/tryptophan (pH 6.2). Further discussion for the influence of these factors on the chemical amplification of MFEs are presented in the next section.

4.6 Control of MFE amplification: efficiency of termination reactions

Since the enhanced MFE response depends on the relative rates of the termination reactions of the radicals, it is deduced that the extent of the enhancement can be controlled by external factors affecting their efficiency. In the following sections, this is achieved by: 1) the introduction of reducing agents; 2) altering the O_2 content of the system; 3) varying the pump power and the flavin concentration; 4) adjusting the sample pH; and 5) changing the solvent viscosity.

4.6.1 TCEP: reducing agent

As expected, the addition of 50 μM of the reductant tris(2-carboxyethyl)phosphine, TCEP, to the FMN/TrpH reaction increases the E -factor for the MFE by increasing the rate of reduction of the TrpH radical ($k_D \uparrow$). E increases from 0.644 ± 0.0003 (Figure 4.9) to 0.89 ± 0.02 (Figure 4.9).

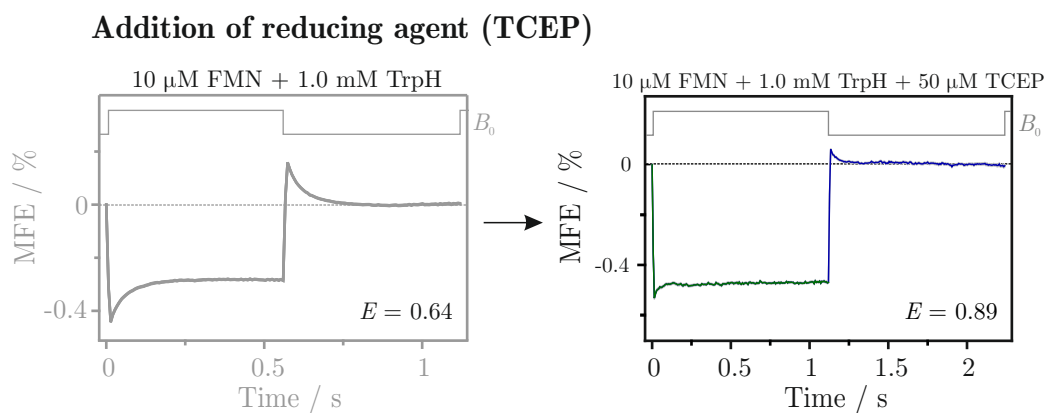


FIGURE 4.9: Effect of adding a reducing agent (TCEP). Average time-resolved MFEs to individual magnetic field steps for 10 μM FMN/1.0 mM TrpH (aq) in the absence (left, grey, same as Figure 4.2d) and presence of 50 μM tris(2-carboxyethyl)phosphine, TCEP (right). Apparatus 1, 470 nm at 3.7 mW, $B_0 = 0$ to 27 mT with the field switching every 0.56 s (left) and 1.12 s (right).

4.6.2 O_2 : oxidising agent

For the flavin radicals, one of the most important termination reactions contributing to k_F is expected to be the direct reaction with molecular oxygen (O_2). To confirm this,

experiments were performed on deoxygenated aqueous solutions of 10 μM FMN/1.0 mM TrpH prepared in a glove box under a nitrogen atmosphere (Figure 4.10).

Consistent with the interpretation from above, an increased amplification factor (E) is found in the absence of oxygen because of the reduced rate of removal of flavin radicals (k_F). However, the averaged MFE trace in Figure 4.10b appears to contain an initial period of MFE reduction followed by amplification. One possible explanation for the mixed shape is that the probed volume contains radical pairs in both locally reducing (*i.e.* $k_D > k_F$) and oxidising (*i.e.* $k_D < k_F$) environments. Peculiar shapes such as this are also obtained in numerical calculations when introducing bimolecular recombination processes not shown in Figure 4.5a.

Further complications arise because deoxygenation affects several regions in the photocycle (unlike the addition of TCEP which is expected to react predominantly to reduce the donor radical). With the removal of oxygen, the flavin triplet photostationary concentration increases and leads to appreciable triplet-triplet annihilation ($^3\text{F}^* + ^3\text{F}^* \rightarrow \text{F} + ^1\text{F}^*$) and subsequently delayed fluorescence ($^1\text{F}^* \rightarrow \text{F} + h\nu'$); this process drives the slow recovery of the overall fluorescence intensity following the minimum seen at ~ 25 s in Figure 4.10a. Furthermore, in the absence of oxygen, the flavin radicals are longer-lived and result in higher depletion of the ground state reactions. This results in weaker $^1\text{F}^*$ fluorescence intensities and the samples are ‘darker.’ Oxygen is desirable as it effectively “closes” the photocycle and drives favourable cycle times.

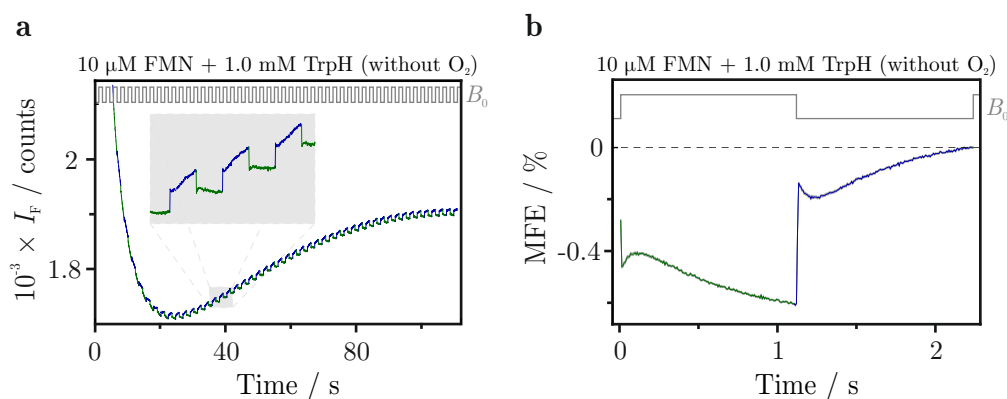
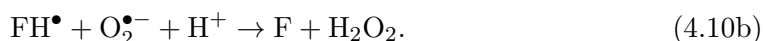


FIGURE 4.10: a) Fluorescence intensity profile (I_F) for oxygen-free 10 μM FMN/1.0 mM TrpH (aq) obtained by Apparatus 1 (470 nm at 3.7 mW, $B_0 = 0$ to 27 mT with the field switching every 1.12 s). b) Average time-resolved MFE for an individual magnetic field step.

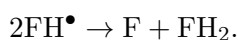
4.6.3 Pump power and flavin concentration

Increasing either the concentration of FMN or the pump power of photoexcitation results in a reduction in E for the FMN/tryptophan reaction, implying a decrease in k_D/k_F (see Figure 4.11). Both observations can be understood by considering the flavin radical oxidation step in further detail. As discussed above, one route is oxidation by molecular oxygen. This reaction is auto-catalytic and proceeds as follows [121, 122]:



The reaction of $\text{FH}^\bullet$ with O_2 in Equation 4.10a is slow and has a rate constant of $k_{\text{O}_2} \approx 1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. Equation 4.10b, the oxidation of $\text{FH}^\bullet$ by the superoxide ($\text{O}_2^{\bullet-}$) formed in Equation 4.10a occurs much more rapidly as $k_{\text{O}_2^{\bullet-}} \approx 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, hence resulting in the auto-catalytic behaviour for the overall process.

Furthermore, another important route for the removal of flavin radicals is disproportionation as first highlighted in Equation 2.9 [121, 122]:



The disproportionation and reverse comproportionation reactions have rate constants of $k_{\text{dis}} = 5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{com}} = 1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, respectively. Comproportionation ($\text{F} + \text{FH}_2 \rightarrow 2\text{FH}^\bullet$) followed by radical recombination and oxidation of the fully reduced flavin by activated oxygen species such as hydrogen peroxide (Equation 4.11) are two pathways which recover the oxidised flavin, “closing” the photocycle.



Both oxidation by O_2 and disproportionation reactions result in a supralinear dependence of the oxidation rate constant k_F on the flavin radical concentration due to the

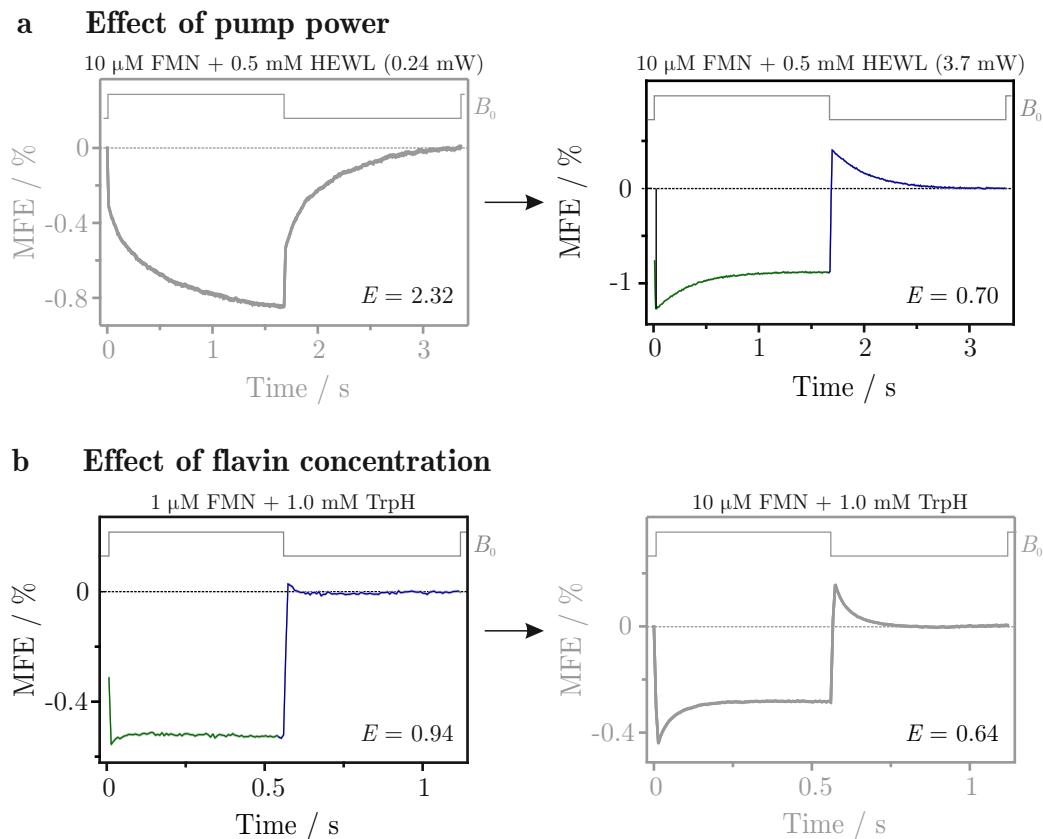


FIGURE 4.11: a) Effect of pump power. Average time-resolved MFEs to individual magnetic field steps for 10 μM FMN/0.5 mM HEWL (aq) with a photoexcitation (470 nm) power of 0.24 mW (left, same as Figure 4.2b) and 3.7 mW (right). b) Effect of flavin concentration. Average time-resolved MFEs to individual magnetic field steps for FMN/1.0 mM TrpH (aq) with a flavin concentration of 1 μM (left, same as Figure 4.2d) and 10 μM (right). The pump power was fixed at 3.7 mW. Data in a and b were obtained using Apparatus 1 ($B_0 = 0$ to 27 mT with the field switching every 1.68 s (a) and 0.56 s (b)).

auto-catalytic nature of the first (Equations 4.10a and b) and the second-order kinetics of the second (Equation 2.9). Increasing the pump power of photoexcitation and increasing the total flavin concentration (in presence of excess donor species) causes an increased flavin radical concentration and increased k_F . In Figure 4.11a the time-resolved MFE response of the FMN/HEWL system switches from $E > 1$ to $E < 1$ with increasing pump power (0.24 mW $\rightarrow$ 3.7 mW, left to right). The E -factor for MFEs derived from FMN/1.0 mM TrpH (aq) decreases upon increasing the total flavin concentration (1 μM $\rightarrow$ 10 μM , left to right).

4.6.4 pH

Furthermore, the rate of flavin oxidation by molecular oxygen is strongly dependent on the protonation state ($pK_a = 8.5$ for $\text{FMNH}^\bullet$) and therefore pH. The equivalent reaction to Equation 4.10a for the anionic flavin radical $\text{F}^{\bullet-}$ has a rate constant, $k_{\text{O}_2} \approx 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, that is four orders of magnitude faster than for $\text{FH}^\bullet$. This allows for E to be controlled by changing the pH of the solution. In the FMN/TrpH system (Figure 4.12), lowering the pH from 6.2 (unbuffered) to 2.7 (citric acid buffer, ionic strength = 11 mM) switches the time-resolved MFE from $E < 1$ to $E > 1$ as k_{F} is decreased.

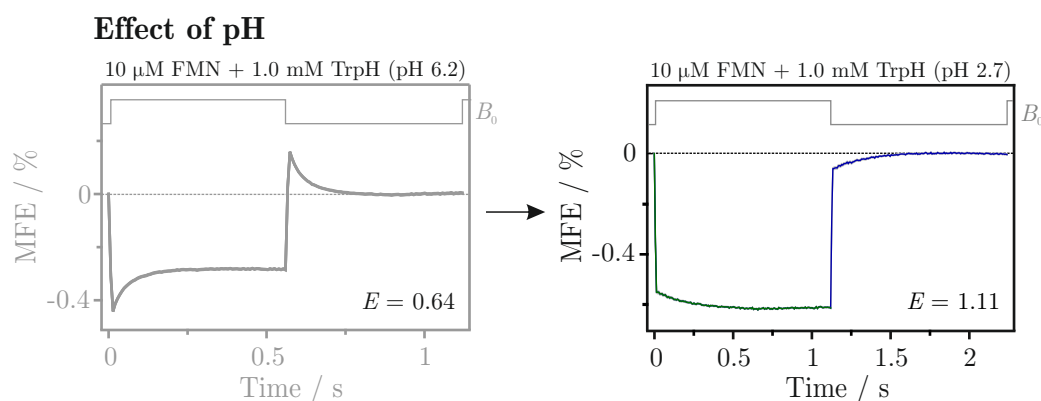


FIGURE 4.12: Varying pH. Average time-resolved MFEs to individual magnetic field steps for 10 μM FMN/1.0 mM TrpH (aq) at pH 6.2 (left, same as Figure 4.2d) and 2.7 (right, citric acid buffer, ionic strength = 11 mM). Apparatus 1, 470 nm at 3.7 mW, $B_0 = 0$ to 27 mT with the field switching every 1.12 s.

4.6.5 Solvent viscosity

The presence of 20% glycerol (v/v) boosted E for the FMN/HEWL reaction from 2.32 ± 0.007 to 3.55 ± 0.02 , with τ increasing from 470 ms to 2.2 s (Figure 4.13). An increase in the solvent viscosity appears to reduce k_{D} and k_{F} in such a way that $k_{\text{D}}/k_{\text{F}}$ is increased. To profile the slow time evolution of the MFE, the field was switched every 7.04 s.

4.7 Chemical amplification of LFEs

Chemical amplification of MFEs in continuously irradiated systems is not exclusive to the ‘normal’ MFEs presented thus far. Consequently, enhanced effects were explored

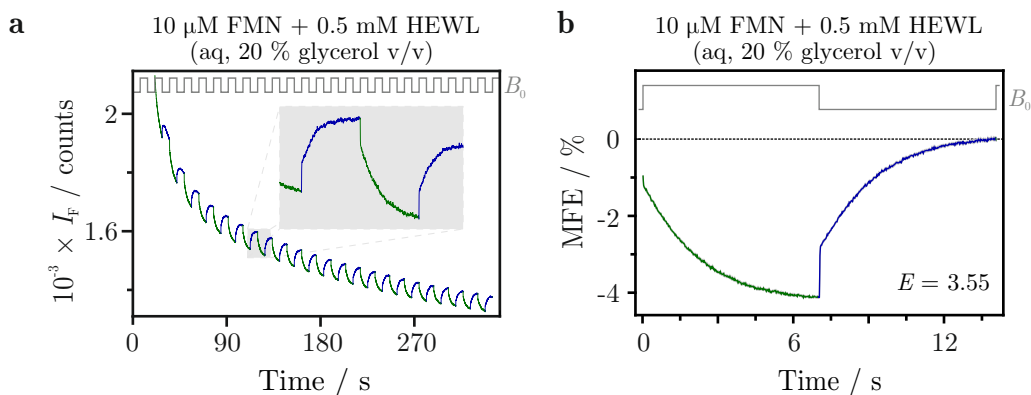


FIGURE 4.13: a) Fluorescence intensity profile (I_F) for 10 μM FMN/0.5 mM HEWL (aq, 20% v/v) obtained by Apparatus 1 (470 nm at 0.24 mW, $B_0 = 0$ to 27 mT). b) Average time-resolved MFE to an individual magnetic field step with the field switching every 7.04 s.

in the context of the Low Field Effect (see Figure 4.14). The prompt LFE studied in Chapter 3 for the flavin/ascorbic acid radical pair is particularly large and warranted the study of potential ‘delayed’ enhancement. For magnetic fields in the range of -12 mT to 12 mT, the field dependence of the prompt MFE recorded using Apparatus 2 for a delay time $T = 50$ ms and exposure time of 20 ms is shown in Figure 4.14a.

Time-resolved MFE studies of this system reveal amplification behaviour at both 0.7 mT (in the low-field region) and 27 mT (for the ‘normal’ MFE). The timescale for the amplification is slightly shorter for the high-field region, $\tau = 170$ ms compared to 190 ms in the low-field region, see Figures 4.14b and c. Furthermore, close examination of these profiles reveal that E is increased at low fields as the prompt MFE is much reduced whilst the delayed MFEs are of a comparable magnitude. The E -factor increases from 1.98 ± 0.02 at 27 mT to 5.6 ± 0.3 at 0.7 mT, here exemplifying that the amplification of MFEs is much greater for low fields versus high fields. The origin of this behaviour can be understood, once more, by the supralinear dependence of the disproportionation and auto-catalytic oxidation channels for the removal of flavin radicals. An increased (or reduced) free flavin radical concentration is expected by the action of the prompt MFE at high fields of 27 mT (or low fields of 0.7 mT), hereby giving rise to a non-linear increase (or decrease) in the rate of $\text{F}^{\bullet-}$ decay and a consequent decrease (or increase) in E -factor. The differing time constants for the delayed MFEs at low and high fields are also in agreement with this model. The shorter time constant for the delayed MFE arising from a low field (0.7 mT) versus high field (27 mT) also agrees with a higher k_F

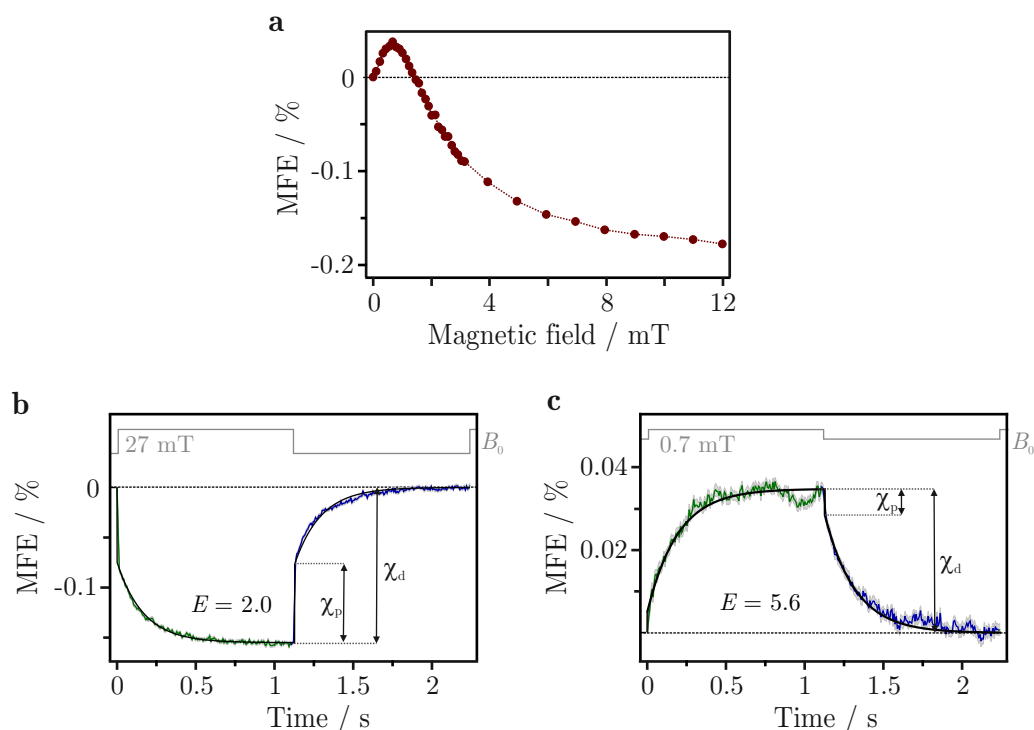


FIGURE 4.14: a) Dependence of the MFE on the magnetic field strength for 10 μM FMN/0.5 mM AsCH₂ with a 50 ms delay time between switching the field on and measuring the fluorescence. The positive MFE at fields below 1.4 mT is the Low Field Effect. Average time-resolved MFEs to individual magnetic field steps for b) $B_0 = 0$ to 27 mT and c) $B_0 = 0$ to 0.7 mT. The field was switched every 1.12 s. Apparatus 1, 470 nm at 0.24 mW.

value for the former (see k_F - and k_D -dependence of τ in Section 4.3.1).

4.8 Switched light intensity

Enhanced magnetic field effects (both in the ‘normal’ and low-field region) are proposed to originate in the unequal rates for radical termination reactions which impact the establishment of photostationary states. If true, the mechanism should also be able to affect other processes which shift the photocycle’s pseudo-equilibrium. One of the most straightforward ways to achieve such changes is to alter the pump power of photoexcitation (for a fixed magnetic field strength).

Clearly, the prompt effects of changing the applied magnetic field strength and varying the pump power for photoexcitation are fundamentally different. As highlighted in Figure 4.15 and previously discussed, in the regime of the ‘normal’ MFE, increasing the applied field reduces the extent of spin mixing within the geminate pair and leads to

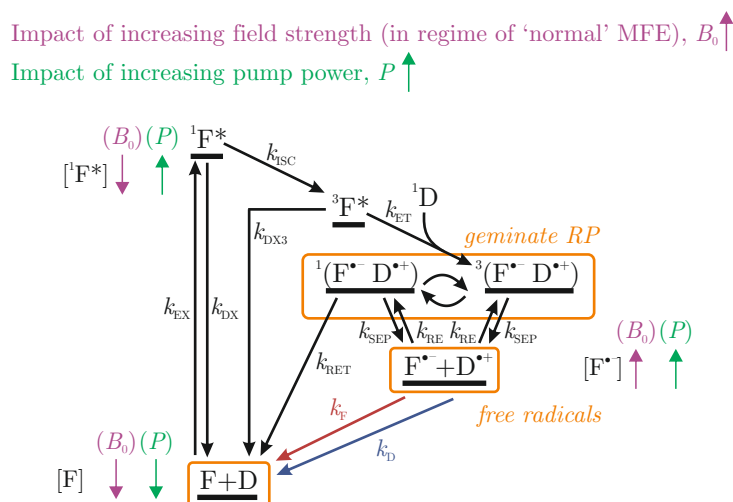


FIGURE 4.15: Photochemical reactions scheme for the intermolecular radical pairs formed between a flavin (F) and an electron donor (D). The (augmented) scheme was first shown in Figure 4.5a. In this depiction, the effects of the increasing the magnetic field strength in the field regime of the ‘normal’ MFE (B_0 , purple) and increasing the photoexcitation pump power (P , green) on the photostationary concentrations are highlighted.

an increase of the $F^{\bullet-}$ free radical concentration and depletion of the flavin ground (F) and singlet excited ($^1F^*$) states. However, increasing the laser pump power leads to a prompt effect of increasing $^1F^*$ and $F^{\bullet-}$, whilst only F is depleted.

Furthermore, consider the scenario in which $k_F < k_D$, the dominant (slow) termination reaction acts to remove the flavin radical recombination partner such that the flavin radical concentration is accumulated during the ‘delayed’ timeframe following the ‘prompt’ change. Thus F and $^1F^*$ become ever more depleted. On altering the magnetic field strength, the prompt and delayed changes in photostationary concentration occur in the same sense (*i.e.* in depletion) so that amplified magnetic field effects are observed. In contrast, on changing the applied laser power, the prompt and delayed effects are opposite in nature. Thus, for systems in which $k_F < k_D$ (*e.g.* FMN/HEWL, Figure 4.2b), enhancing magnetic field effects ($E > 1$) and reducing-type power variation effects ($E_{\text{power}} < 1$) are predicted. The opposite is true for the scenario where $k_F > k_D$ (*e.g.* FMN/W, Figure 4.2d): diminishing magnetic field effects ($E < 1$) and enhancing power variation effects ($E_{\text{power}} > 1$) are expected.

Using Apparatus 2, experiments were conducted on a sample of 10 μM FMN/0.5 mM HEWL (aq), illuminated using a 405 nm diode laser and subjected to switching phases of high power (350 mW, Time = 0 to 1 s) and low power ($y\% \times 350$ mW, Time = 1 to

2 s). $y\%$ is the percentage of the high power intensity for which the low power phase is equivalent (*i.e.* $y\% = 50\%$, means 175 mW). An exposure time of 20 ms was used.

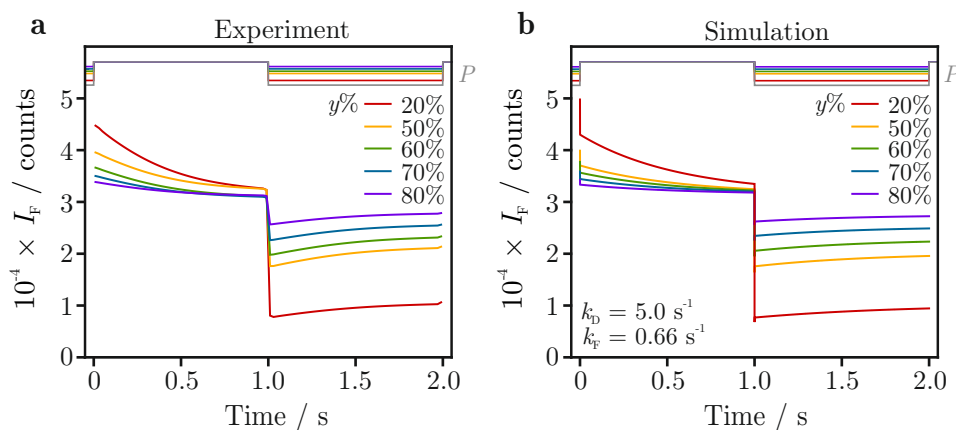


FIGURE 4.16: a) Fluorescence intensities of an aqueous solution of 10 μM FMN/0.5 mM HEWL recorded using a 405 nm diode laser (Apparatus 2). The laser power (350 mW) was periodically attenuated by 20–80 % after 1.0 s of irradiation and returned to 350 mW after a further 1.0 s. b) Simulation of the experiment using $k_D = 5.0 \text{ s}^{-1}$, $k_F = 0.66 \text{ s}^{-1}$ and other parameter values as in Table 4.1 using the method outlined in Section 4.3.1.

The FMN/HEWL system, previously shown to exhibit enhancing MFEs ($E > 1$), displays diminishing-type behaviour in response to laser power switching (Figure 4.16a). In contrast to the field switching-type experiments, it is noteworthy that increasing and decreasing the pump power results in asymmetric responses. However, this behaviour is in fact unsurprising with further consideration and is reproduced by the fits derived from numerical calculations shown in Figure 4.16b. Consider the radical pair system at the end of the high power phase: the system reaches ‘equilibrium’ concentrations of the ground state flavin and the free radicals. Upon switching to low power, the $[^1\text{F}^*]$ concentration undergoes a ‘prompt’ depletion (see Figure 4.17a). At the very instant of power attenuation, there still exists the high power photostationary concentration of the radicals. The $[^1\text{F}^*]$ concentration increases as it ‘equilibrates’ with the free radicals undergoing recombination via an encounter pair (millisecond timescale, seen in Figure 4.16b but not Figure 4.16a due to the 20 ms exposure time) and the slow termination reactions (hundreds of milliseconds timescale). The $[^1\text{F}^*]$ concentration is fed from the radical ‘equilibrium’ concentration.

On switching from low to high power of photoexcitation, the relative diminishment of the prompt effect with time is larger as it corresponds to a greater relative shift from the final photostationary state. The relative difference in the enhancement of system changes to

low-high and high-low power switching is reduced with decreasing ratio of k_D/k_F where $k_D > k_F$ and for increasing similarity between high and low power photoexcitation (*i.e.* $y\% \rightarrow 100\%$).

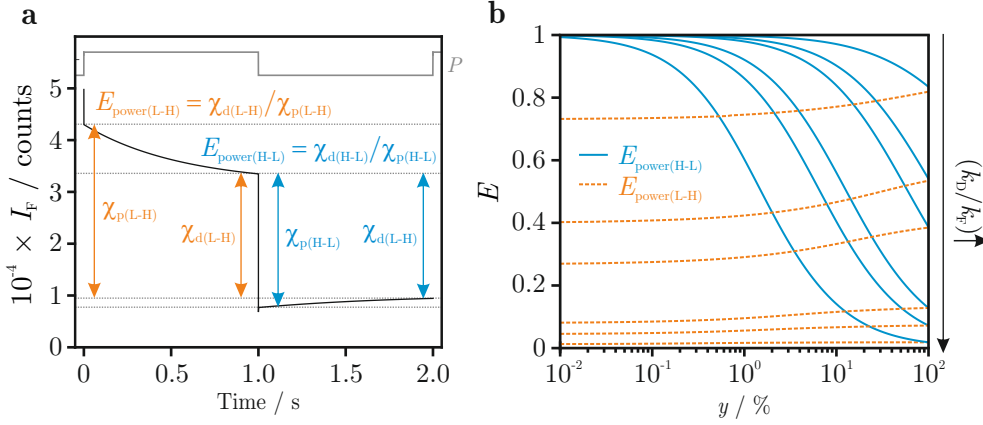


FIGURE 4.17: a) Typical fluorescence intensity profile for the intermolecular F/D system where $k_D > k_F$. Orange: definition of the enhancement factor ($E_{\text{power(L-H)}}$) for low to high power switching from the prompt ($\chi_{\text{p(L-H)}}$) and delayed ($\chi_{\text{d(L-H)}}$) intensity changes. Blue: definition of the enhancement factor ($E_{\text{power(H-L)}}$) for high to low power switching from the prompt ($\chi_{\text{p(H-L)}}$) and delayed ($\chi_{\text{d(H-L)}}$) intensity changes. b) General trend for the low:high pump power ratio dependence ($y\% = 100\% \times P_{\text{low}}/P_{\text{high}}$) of the enhancement factor for high-low and low-high power switching with varying ratio of k_D/k_F .

4.9 Conclusion and outlook

In this chapter, it has been demonstrated that slow processes associated with the removal of the free radicals can give rise to the chemical amplification of magnetic field effects in continuously photoexcited systems involving intermolecular radical pairs. The result is the possibility of MFE enhancement and reduction over long timescales as compared to the geminate RP lifetime. These effects were indiscernible in prior studies due to the use of time-resolved detection schemes employing pulsed light excitation sources or rapidly varying fields (*i.e.* high frequency field modulation with lock-in detection). Simulations and experiments have shown that the relative rates of termination of the free radical species control the nature of the enhanced magnetic field effects. If the fluorophore-derived radical is regenerated at a rate faster than the ionised quencher ($k_F > k_D$), diminishment of the MFE on the ground state concentration and the prompt fluorescence are observed ($E < 1$). In the opposite scenario, faster recovery of the ground state

quencher (D) leads to an enhancement of the MFE ($E > 1$). This enhancement can increase the size of the final MFE to a value which is several times larger than the prompt MFE. The enhancement factor, E , depends predominantly on the ratio of the free radical termination rates and thus even processes much slower than typically associated with spin chemistry can give rise to MFE amplification provided that k_D/k_F is large. MFE enhancement has been characterised for a number of flavin-based radical pairs. For FMN/HEWL, an E -value as large as 3.6 was achieved; whilst for FMN/ascorbic acid, 5.6 was found in the low-field region. Extensive studies for the FMN/tryptophan systems have shown that the enhanced MFE can be controlled by external factors influencing the termination reactions. The enhancement factor, E , for the MFE increases with decreasing pH, decreasing the excitation pump power, decreasing the fluorophore concentration, removing O_2 , and upon addition of an external reducing agent. Measurement of the LFE-optimal flavin/ascorbic acid system show that, under appropriate conditions, the enhancement of the MFE can be disproportionately large in the low field region than for saturating magnetic fields.

In light of typical radical pair lifetimes (0.1-1.0 μ s) and singlet-triplet interconversion frequencies (10-100 MHz), it has been suggested that time-dependent magnetic fields with a period of approximately 20 ms, *e.g.* the power line frequency, appear static on the timescale of the radical pair mechanism. Thus, epidemiological conclusions reached at static magnetic fields have been assumed true for systems subjected to extremely low frequency magnetic fields: an assumption that might not be valid as it disregards possibly substantial enhancement effects via recombination processes. In the future, the influence of 50 Hz oscillating magnetic field intensities should be studied with direct comparison to their static analogues. Chemical amplification is also expected to be important in elucidating radiofrequency effects on radical pair systems.

The mechanism underlying MFE amplification proposed in this chapter relies on non-spin-selective reactions only and is thus likely to be relevant for the majority of radical pair reactions (beyond flavin photochemistry). Furthermore, the phenomenon enables MFEs to be enhanced beyond the inherent limits of the radical pair mechanism.

In the final chapter, chemical amplification of MFEs are explored for cryptochromes and other intramolecular radical pair systems.

Chapter 5

Chemical amplification of MFEs in cryptochromes and related model systems

Chemical amplification of MFEs arising from the photochemistry of intermolecular flavin/donor systems was demonstrated in Chapter 4. Motivated by its potential implications as a possible signal amplification mechanism for magnetoreception, the exploration of enhanced magnetic field effects was extended to study cryptochromes derived from *Arabidopsis thaliana* (plant) and ‘maquette’ model systems. This chapter is based on some work published in [118], conducted with Dr. Daniel Kattnig, Dr. Lauren Jarocha and Katherine Buckton.

5.1 Cryptochrome photochemistry

A brief introduction to the magnetic field-dependent photochemistry of cryptochrome (and related photolyase) proteins was presented in Chapter 1. These flavoproteins share a common photolyase homology region consisting of a buried flavin adenine dinucleotide (FAD) moiety and a chain of three tryptophan residues at proximal, medial and distal positions (TrpH_A , TrpH_B and TrpH_C , respectively). The general photochemical scheme for the radical pair chemistry of cryptochromes is depicted in Figure 5.1.

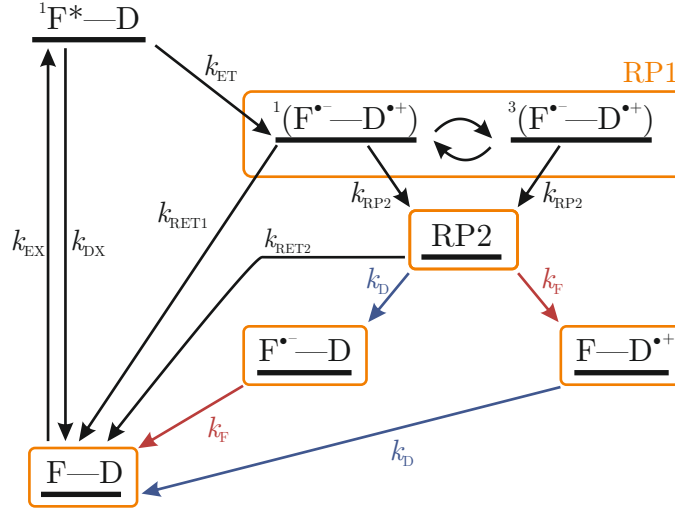


FIGURE 5.1: Photochemical reaction scheme for the formation of intramolecular radical pairs in cryptochromes, F—D, whereby F and D correspond to the flavin and distal residue of the tryptophan triad. After photoexcitation, the excited flavin singlet state abstracts an electron from the proximal tryptophan to form a radical pair, which subsequently undergoes ultrafast electron transfer along the tryptophan triad to yield RP1, $F^{\bullet-}-D^{\bullet+}$, in the singlet state. RP2 is a FAD-TrpH radical pair in which the flavin radical ($F^{\bullet-}$) is protonated or the tryptophan radical ($D^{\bullet+}$) is deprotonated or both; it is not magnetically sensitive. The slow reaction steps labelled with the rate constants k_D and k_F are responsible for the amplification of the MFE and correspond to the removal of the tryptophan and flavin radicals, respectively. Other rate constants in the photocycle are defined in Table 5.1.

$^1\text{FAD}^*$ forms by blue-light photoexcitation of FAD, and undergoes electron transfer with the proximal tryptophan to generate the $(\text{FAD}^{\bullet-} \text{TrpH}_A^{\bullet+} \text{TrpH}_B \text{TrpH}_C)$ radical pair. Ultrafast electron transfer along the tryptophan triad leads to the formation of $(\text{FAD}^{\bullet-} \text{TrpH}_A \text{TrpH}_B \text{TrpH}_C^{\bullet+})$, *i.e.* RP1, within tens of picoseconds. Note that the high efficiency for the first electron transfer step occurs before appreciable intersystem crossing of $^1\text{FAD}^*$ and results in a singlet-born RP. Transient absorption studies by Maeda et al. revealed the formation of secondary radical pairs (*i.e.* RP2) following deprotonation/protonation of the species in RP1 [49]. For a photolyase derived from *Escherichia coli* (*EcPL*), deprotonation of the tryptophan radical in RP1 was shown to produce $\text{RP2} = (\text{FAD}^{\bullet-} \text{TrpH}_A \text{TrpH}_B \text{TrpH}_C^{\bullet+})$. While *AtCry1* from *Arabidopsis thaliana* generated $\text{RP2} = (\text{FADH}^{\bullet} \text{TrpH}_A \text{TrpH}_B \text{TrpH}_C^{\bullet+})$ following tryptophan radical deprotonation and protonation of the anionic semiquinone by an adjacent aspartic acid residue. Studies conducted to date suggest that the presence of a suitable proton donor adjacent to $\text{FAD}^{\bullet-}$ is unique to *AtCry1*, as the photochemistry of other cryptochromes have been found to involve the same RP2 species as *EcPL* [123]. The time dependence for the subtraction of the difference absorption profiles, $\Delta A(t)$, in the presence and absence

of a 28 mT magnetic field show that the MFEs arise from field-dependent spin mixing in RP1. Presumably, coherences in the radical pair are lost on the ~ 10 μ s timescale for transitioning from RP1 $\rightarrow$ RP2. The lifetime of the RP2 state was found to be approximately 100 μ s.

As the radical pair is singlet-born, positive (and negative) MFEs are expected by prompt fluorescence for high (and low) magnetic field strengths. The cryptochrome radical pair photocycle in Figure 5.1 has been augmented to include slow termination reactions.

Unfortunately, the photochemistry and stability of cryptochromes make them unpromising candidates for characterising PF MFEs. By virtue of the singlet-born radical pair arising in these systems, the proteins are inherently dark with low fluorescence quantum yields ($\phi_F \approx 0.003$ for *AtCry3*, pH 7.5 at 298 K [124] versus $\phi_F = 0.26$ for FMN, pH 7 at 298 K [34]). Measured fluorescence intensities are also expected to be lowered by the formation of long-lived radical/‘dark’ states within the photocycle. Other expected issues include the cryptochrome samples’ susceptibility to photodegradation, aggregation and loss of the bound FAD cofactor. The optimised experimental procedures are presented in the Experimental methods of this chapter.

5.2 Flavomaquette

As well as studying cryptochromes themselves, flavomaquettes (synthetic protein-based model systems) have been designed to aid studies of intramolecular flavin/tryptophan-based photochemistry. The basic structure of the maquette studied here is shown in Figure 5.2a. Maquettes are minimalistic protein structures which are designed to mimic the fundamental behaviour of more complicated enzymes found in biology [125]. To this end, maquettes are incorporated with working arrays of cofactors to reproduce native-like function. The protein scaffold is comprised of a tetra- α -helix-bundle, stable over a wide range of conditions. Self-assembly of the helical chains is promoted by designing a peptide with sequences of polar (*e.g.* alanine, isoleucine, phenylalanine, leucine) and non-polar (*e.g.* aspartic acid, glutamic acid, asparagine, lysine, glutamine) residues at specific sites. Over the past two decades, numerous species have been incorporated to such protein scaffolds including: four hemes as a model for cytochrome *b* [126–128]; the covalent attachment of a porphyrin dimer akin to the fundamental component of

a photosynthetic reaction centre [129, 130]; and an iron-sulfur cluster (ferredoxin-heme maquette) [131]. From these design principles, Sharp et al. presented the first maquette of a biologically relevant process, being the light-activation of a flavin followed by intramolecular electron transfer with heme cofactors as observed in oxoreductases [132].

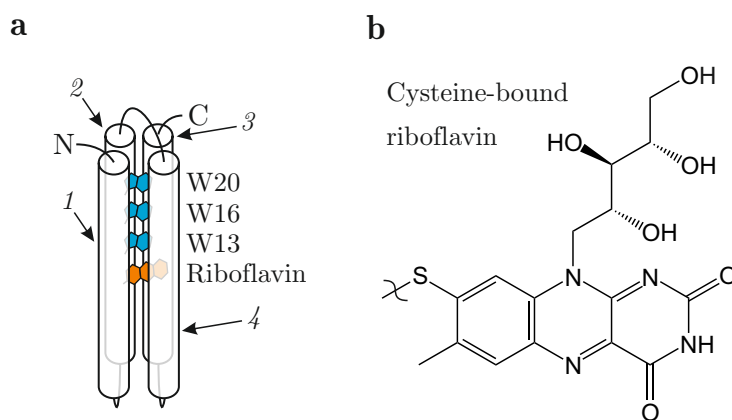


FIGURE 5.2: a) Structure of the 4- α -helical flavomaquette. Labels 1–4 signify different portions of the single polypeptide chain which are engineered to form a rod-like structure by self-assembly. Tryptophan residues can be incorporated at positions 13, 16 and 20 on peptide section-2. The riboflavin photoreceptor (orange) is covalently bound by a C–S linkage between C–8 on the isoalloxazine ring and a cysteine residue at position-9 on peptide section-2. b) Chemical structure of the cysteinyl-bound riboflavin.

Ultimately, flavomaquettes are envisaged which contain the characteristic flavin group and tryptophan triad. Collaborators in the Dutton Lab (University of Pennsylvania) have so far been able to produce single-tryptophan variants: maquette–W13 (MQ–W13), MQ–W16, and MQ–W20 whereby the tryptophan (W) has been incorporated one, two and three helical turns away from a covalently attached flavin. The numbering corresponds to the position of the tryptophan on α -helix 2 and the flavin-tryptophan separations for these species are 5.6 Å (MQ–W13), 11.2 Å (MQ–W16) and 16.8 Å (MQ–W20), being on a comparable lengthscale to the cofactors in cryptochromes. The flavin is covalently bound through C–8 to a cysteine group (position 9 on α -helix 2) on the maquette scaffold. By studying MQ–W13, MQ–W16 and MQ–W20, the effect of the electron donor-acceptor distance on the electron transfer efficiency and subsequent spin dynamics can be assessed. Furthermore, the ordered structures of maquettes are attractive due to their potential for alignment and the characterisation of anisotropic magnetic field effects. The photochemistry of these model systems are expected to closely resemble Figure 5.1.

5.3 Two-state model for primary MFEs

In Chapter 2, a simple two-state scheme for intermolecular radical pairs was presented to model the behaviour of prompt MFEs in regard to the pump power for photoexcitation, the fluorophore/photosensitiser concentration and the rate-limiting recombination of the radicals. For comparison, an analogous model has been developed for cryptochrome-derived MFEs that is also relevant to maquettes and other intramolecular radical pair systems.

To a good approximation, photostationary concentrations of the ground state, F—D, and RP2 vastly exceed those of the shorter-lived species in Figure 5.1. Thus,

$$[\text{F—D}]_{\text{total}} = [\text{F—D}] + [\text{RP2}] \quad (5.1)$$

where $[\text{F—D}]$, $[\text{RP2}]$ and $[\text{F—D}]_{\text{total}}$ are concentrations of the photostationary ground state, RP2, and total cryptochrome, respectively. The MFE expressions for the ground state and RP2 species are:

$$\text{MFE}(B_0, \text{F—D}) = \frac{[\text{F—D}(B_0)] - [\text{F—D}(0)]}{[\text{F—D}(0)]} \quad (5.2a)$$

$$\text{MFE}(B_0, \text{RP2}) = \frac{[\text{RP2}(B_0)] - [\text{RP2}(0)]}{[\text{RP2}(0)]}. \quad (5.2b)$$

In contrast to the intermolecular scenario, the photocycle's rate-limiting recombination step ($\text{RP2} \rightarrow \text{F—D}$) is unimolecular and follows first order kinetics:

$$\frac{d[\text{RP2}(B_0)]}{dt} = I_{\text{abs}}(B_0) \cdot \Phi_{\text{RP2}}(B_0) - k_{\text{RET2}}[\text{RP2}(B_0)] = 0. \quad (5.3)$$

Here $I_{\text{abs}}(B_0)$ is the rate of $\text{F—D} \rightarrow {}^1\text{F}^*\text{—D}$ excitation at B_0 ; $\Phi_{\text{RP2}}(B_0)$ is the magnetic field-dependent quantum yield for RP2 formation; and k_{RET2} is the rate constant for RP2 recombination to the ground state species. In the limit of low sample absorbance for the ground state, $A = \log_{10}(e)\alpha[\text{F—D}(B_0)]l \rightarrow 0$, $I_{\text{F—D}}(B_0) \rightarrow I_0\alpha[\text{F—D}(B_0)]l$ where α is the extinction coefficient of F—D at the pump wavelength; l is the pathlength; and I_0

is the rate of incident photons on the sample. Consequently, the RP2 photostationary concentration becomes:

$$[\text{RP2}(B_0)] = \frac{I_0 \alpha l \Phi_{\text{RP2}}(B_0) \cdot [\text{F—D}]_{\text{total}}}{I_0 \alpha l \Phi_{\text{RP2}}(B_0) + k_{\text{RET2}}} \quad (5.4)$$

Now, by substitution, the MFE Equations 5.2a and b can be re-expressed as:

$$\text{MFE}(B_0, \text{F—D}) = -\frac{\Phi_{\text{RP2}}(B_0) - \Phi_{\text{RP2}}(0)}{\Phi_{\text{RP2}}(B_0) + x} \quad (5.5a)$$

$$\text{MFE}(B_0, \text{RP2}) = x \frac{\Phi_{\text{RP2}}(B_0) - \Phi_{\text{RP2}}(0)}{\Phi_{\text{RP2}}(0)(\Phi_{\text{RP2}}(B_0) + x)}. \quad (5.5b)$$

where $x = k_{\text{RET2}}/(I_0 \alpha l)$. Thus, the magnitude of the MFE on the concentration of F—D and RP2 increases for decreasing and increasing x , respectively. Evaluation of the MFE in the limits of $x \rightarrow 0$ and $x \rightarrow \infty$ yields:

$$\lim_{x \rightarrow 0} \text{MFE}(B_0, \text{F—D}) = \frac{\Phi_{\text{RP2}}(0) - \Phi_{\text{RP2}}(B_0)}{\Phi_{\text{RP2}}(B_0)} \quad (5.6a)$$

$$\lim_{x \rightarrow 0} \text{MFE}(B_0, \text{RP2}) = 0 \quad (5.6b)$$

$$\lim_{x \rightarrow \infty} \text{MFE}(B_0, \text{F—D}) = 0 \quad (5.7a)$$

$$\lim_{x \rightarrow \infty} \text{MFE}(B_0, \text{RP2}) = \frac{\Phi_{\text{RP2}}(B_0) - \Phi_{\text{RP2}}(0)}{\Phi_{\text{RP2}}(0)} \quad (5.7b)$$

From Equation 5.5a, the magnitude of the PF MFE is largest for i) high pump powers (*i.e.* large I_0); and ii) low rate of RP2 recombination (*i.e.* low k_{RET2}). Unfortunately, high intensity photoexcitation inevitably promotes photodegradation and thus intermediate intensities are likely to be the best compromise. In contrast to intermolecular systems, the PF MFEs are independent of the fluorophore/photosensitiser concentration.

5.4 Barebones model of MFE amplification: cryptochromes

In addition to the differences observed in the characteristics of the primary MFEs derived from intermolecular and intramolecular radical pair systems, deviations also exist in their enhanced effects. Here, the barebones model is presented for an intramolecular radical pair system relevant to magnetoreception. In Figure 5.3a a simplified reaction scheme is depicted which reduces the comprehensive photocycle in Figure 5.1 to its bare bones. The model is comprised of the ground state ($F-D$), the $F^{\bullet-}-D^{\bullet+}$ RP (which encompasses RP1 and RP2 by ignoring the protonation states of the radicals), and the oxidised ($F-D^{\bullet+}$) / reduced ($F^{\bullet-}-D$) radical pair states.

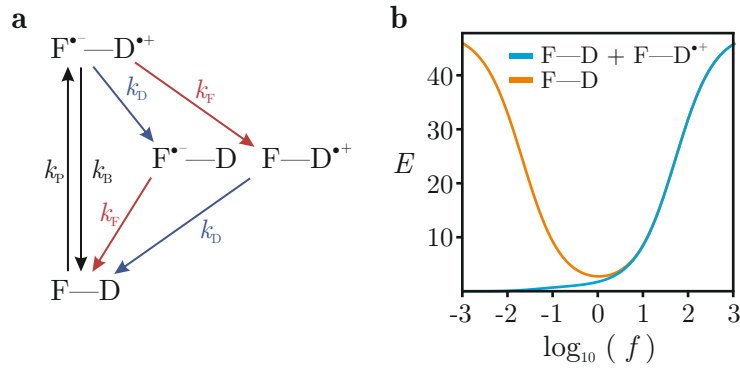


FIGURE 5.3: a) Simplified reaction scheme for magnetic field effects on intramolecular flavin/donor reactions relevant to cryptochrome. b) Dependence of the amplification factor, E , on $f = k_D/k_F$ for $\chi_P = 2\%$. The orange line denotes the MFE on the ground state population ($F-D$). The blue line gives the MFE on the total concentration of F irrespective of the oxidation state of the donor. When $f \geq 2.5$, the two calculations predict near-identical E -values.

The rate equations pertaining to the kinetics of the system are as follows:

$$\frac{d[F-D]}{dt} = -k_P[F-D] + k_B(B_0)[F^{\bullet-}-D^{\bullet+}] + k_F[F^{\bullet-}-D] + k_D[F-D^{\bullet+}] \quad (5.8a)$$

$$\frac{d[F^{\bullet-}-D^{\bullet+}]}{dt} = k_P[F-D] - (k_B(B_0) + k_F + k_D)[F^{\bullet-}-D^{\bullet+}] \quad (5.8b)$$

$$\frac{d[F-D^{\bullet+}]}{dt} = -k_D[F-D^{\bullet+}] + k_F[F^{\bullet-}-D^{\bullet+}] \quad (5.8c)$$

$$\frac{d[F^{\bullet-}-D]}{dt} = -k_F[F^{\bullet-}-D] + k_D[F^{\bullet-}-D^{\bullet+}] \quad (5.8d)$$

To a good approximation, the prompt MFE can be evaluated from the photostationary state of the system when $k_F = k_D = 0$:

$$[\text{F—D}]_{\text{PS}}(k_{\text{F}} = k_{\text{D}} = 0) = \frac{[\text{F—D}]_{\text{total}} k_{\text{B}}(B_0)}{k_{\text{B}}(B_0) + k_{\text{P}}} \quad (5.9)$$

where $[\text{F—D}]_{\text{total}} = [\text{F—D}] + [\text{F}^{\bullet-} \text{—D}^{\bullet+}] + [\text{F—D}^{\bullet+}] + [\text{F}^{\bullet-} \text{—D}]$, the total cryptochrome concentration. Including slow termination reactions, the photostationary ground state concentration becomes:

$$[\text{F—D}]_{\text{PS}}(k_{\text{F}} \neq 0, k_{\text{D}} \neq 0) = \frac{[\text{F—D}]_{\text{total}} (k_{\text{B}}(B_0) + k_{\text{A}} + k_{\text{D}})}{k_{\text{P}} + k_{\text{B}}(B_0) + k_{\text{F}} + k_{\text{D}} + (k_{\text{D}} k_{\text{P}})/k_{\text{F}} + (k_{\text{F}} k_{\text{P}})/k_{\text{D}}}. \quad (5.10)$$

As the fluorescence intensity is proportional to the ground state concentration, the PF MFE can be viewed as the fractional change in the ground state caused by field-dependent fractional changes in k_{B} . Thus, the prompt and delayed MFEs ($\chi_i, i = \text{p, d}$) are obtained by:

$$\chi_i = \frac{(d[\text{F—D}]_{\text{PS}})/[\text{F—D}]_{\text{PS}}}{(dk_{\text{B}})/k_{\text{B}}}, \quad (5.11)$$

where $[\text{F}]_{\text{PS}}$ is given by Equations 5.9 and 5.10 for the prompt MFE, χ_{p} , and the delayed MFE, χ_{d} , respectively. The enhancement factor for $\chi_{\text{p}} \rightarrow \chi_{\text{d}}$ is:

$$E = \frac{k_{\text{B}}(B_0)(k_{\text{B}}(B_0) + k_{\text{P}})(f^2 + f + 1)}{(k_{\text{F}}(1 + f) + k_{\text{B}}(B_0))(k_{\text{F}}(f^2 + f) + k_{\text{P}}f^2 + f(k_{\text{B}}(B_0) + k_{\text{P}}) + k_{\text{P}})} \quad (5.12)$$

where $f = k_{\text{D}}/k_{\text{F}}$. This can be simplified by a Taylor expansion with respect to k_{F} , assuming the termination rate to be relatively slow versus k_{B} and k_{P} :

$$E \approx \frac{f^2 + f + 1}{\kappa f^2 + f + \kappa} \quad (5.13)$$

where $\kappa = k_{\text{P}}/(k_{\text{B}}(B_0) + k_{\text{P}})$. As $0 \leq \kappa \leq 1$, MFE amplification (*i.e.* $E > 1$) is expected from Equation 5.13, regardless of whether $k_{\text{D}} > k_{\text{F}}$ or $k_{\text{D}} < k_{\text{F}}$, (see orange profile in Figure 5.3b). This contrasts with the behaviour found for the intermolecular RP systems explored in Chapter 4 (see Section 4.4).

However, some prompt fluorescence is also expected to result from the photoexcitation of $F-D^{\bullet+}$ in addition to $F-D$. The enhancement factor for the MFE on the total $^1F^*-D$ and $^1F^*-D^{\bullet+}$ fluorescence is:

$$E \approx \frac{f^2 + f + 1}{\kappa f^2 + f + \kappa} - \frac{1}{f(1 - \kappa) + \kappa}. \quad (5.14)$$

Now, as found for intermolecular systems, the overall E -factor depends on the relative ratio of the termination rates, $f = k_D/k_F$ (see blue profile in Figure 5.3b).

5.5 Experimental methods

Following Chapter 4, two methods were employed for the detection of MFEs in cryptochromes and maquettes by prompt fluorescence. See Figure 4.1 for schemes showing the synchronisation of the magnetic field and the fluorescence measurements. As highlighted below, the protocols were adapted and optimised for the protein measurements.

5.5.1 Apparatus 1

Cryptochrome: The time-dependence for the chemical amplification of MFEs in cryptochrome systems was recorded using the same inverted fluorescence microscope arrangement in Chapter 4. However, to minimise sample degradation, the microscope stage was cooled to 273 K. Furthermore, the laser was attenuated from the previous lower limit of 0.24 mW to 0.1 mW. Due to the sample's weak fluorescence, an exposure time of 40 ms was used, longer than the 7 ms typically employed in Chapter 4. The field was switched every 4.2 s.

5.5.2 Apparatus 2

Cryptochrome: To confirm that the recorded PF MFEs detected for cryptochromes were indeed derived from $^1F^*-D$, complementary measurements were made using the experimental arrangement first presented in Chapter 2. However, as measurable (10–100 μ M) concentrations of cryptochrome could only be obtained in relatively small quantities, static samples were studied in a cooled environment (283 K). As above, longer

exposure times were used in comparison to the previous studies on the intermolecular model systems: 20 ms (previous) $\rightarrow$ 0.5–1 s (here). Furthermore, the 450 nm diode laser (Power Technology) was attenuated to 1–1.74 mW for sample preservation and a 475 nm longpass filter was placed to reduce scattered light reaching the CCD camera. See Section 2.3.1 for further details.

In this chapter, Apparatus 2 has also been adapted for simultaneous temporal and spectral measurements of the prompt fluorescence MFEs. For these studies, the magnetic field and fluorescence measurements were synchronised as indicated for ‘time-resolved MFEs’ in Figure 4.1a. The samples were continuously irradiated in the presence of a stepping external magnetic field (between 0 and 12 mT). Prompt fluorescence was continuously recorded, yielding 600–1,800 frames per experiment (each with a 1 s frame time) synchronised with the switching magnetic field by the use of a function generator (Rigol DG1022). The duration of the field on and field off phases, *i.e.* the field on/off time, was 5 s.

Maquette: The benefit of simultaneous information on the time and spectral profile of the MFEs is that composite effects on the sample’s fluorescence from multiple species can be deconvoluted in terms of its constituent components. To this end, the static samples of the maquettes were subjected to continuous illumination by a diode laser (450 nm/55 mW or 405 nm/350 mW) with a stepping external magnetic field (0–12 mT) as for the cryptochrome measurements. The fluorescence derived from the samples was found to be much brighter than for cryptochromes and so 12,000–15,000 frames (40–50 ms) were recorded for each experiment.

5.5.3 Materials

Cryptochrome: Samples of *Arabidopsis thaliana* cryptochrome-1 (AtCry1) were acquired from Planet Biotechnology. The cryptochromes were expressed in *Nicotiana benthamiana* tobacco plants, then isolated following a sequence of purification steps. Prior to experiment, final preparation of the samples involved the use of centrifugal filter units (30 kD Ultra Centricon, Amicon) and a desalting column (Superdex 200 Increase 10/300 GL, Akta) to minimise the presence of free FAD as far as possible. For the measurements, AtCry1 ($\sim 100 \mu\text{M}$, verified spectrophotometrically) was contained in 10 mM Tris, 0.15 mM NaCl, 30 % glycerol v/v, pH 7.4.

Maquette: Maquette cryptochromes were prepared by collaborators in the Dutton Lab (University of Pennsylvania) following the single-chain tetrahelical design and protocols presented in [125]. Samples of 20 μM MQ-W13 and MQ-W20 were prepared in a pH 7.3 phosphate buffer containing 20 mM KH_2PO_4 / 100 mM KCl.

5.6 Results and discussion: Cryptochrome

Figure 5.4a shows the density plot for the fluorescence subtraction profile ΔI_F obtained by Apparatus 2 (450 nm at 1.74 mW); $\Delta I_F(\lambda, t) = I_F(\lambda, t) - I_F(\lambda, t_{\text{end}})$, where $I_F(\lambda, t)$ and $I_F(\lambda, t_{\text{end}})$ are the fluorescence profiles at Time = t in the field cycle and at the end of the field off period, respectively. The magnetic field was switched alternately on/off between 12 and 0 mT every 5 s, for which the fluorescence subtraction profile increased and decreased in synchrony. The positive sign of the effect induced by the applied magnetic field is in agreement with the observation of a singlet-born radical pair in previous studies. Averaging 500–600 nm for each time step yields the time-resolved MFE plot in Figure 5.4b. Here, the evolution of the MFE occurs over several seconds but is poorly resolved due to the 1.0 s exposure time.

Although the data in Figure 5.4a broadly support the exclusive contribution of $^1\text{FAD}^*$ to the magnetosensitive fluorescence, the assignment suffers somewhat from the S/N ratio. Thus, further measurements were recorded for a comparison of the fluorescence intensity profile at zero field versus subtraction following delay times of $T = 0.5, 1$ and 3.5 s, see Figure 5.5. Again, the subtraction signals increase with T and all closely resemble $I_F(0 \text{ mT})$ as well as prior measurements of cryptochrome fluorescence [101]. In fact, $\Delta I_F(12 \text{ mT})$ (red) for $T = 3.5 \text{ s}$ and $\Delta I_F(12 \text{ mT})$ (black, scaled) have been overlaid in Figure 5.5, lending further support to the assignment of $^1\text{FAD}^*$. The slow time dependence of the MFEs observed in Figures 5.4 and 5.5 imply slow termination reactions. These slow reactions are expected to cause substantial ground state depletion even under conditions of weak photoexcitation, thus being a further factor which results in reduced fluorescence signals.

Further assessment of the time-resolved MFE was conducted using Apparatus 2. Figure 5.6a shows the averaged response of *AtCry1* to a single field step (0–27 mT, field on/off time = 4.2 s). It displays an enhanced MFE with a time constant of $\tau = 3.4 \pm 0.9 \text{ s}$.

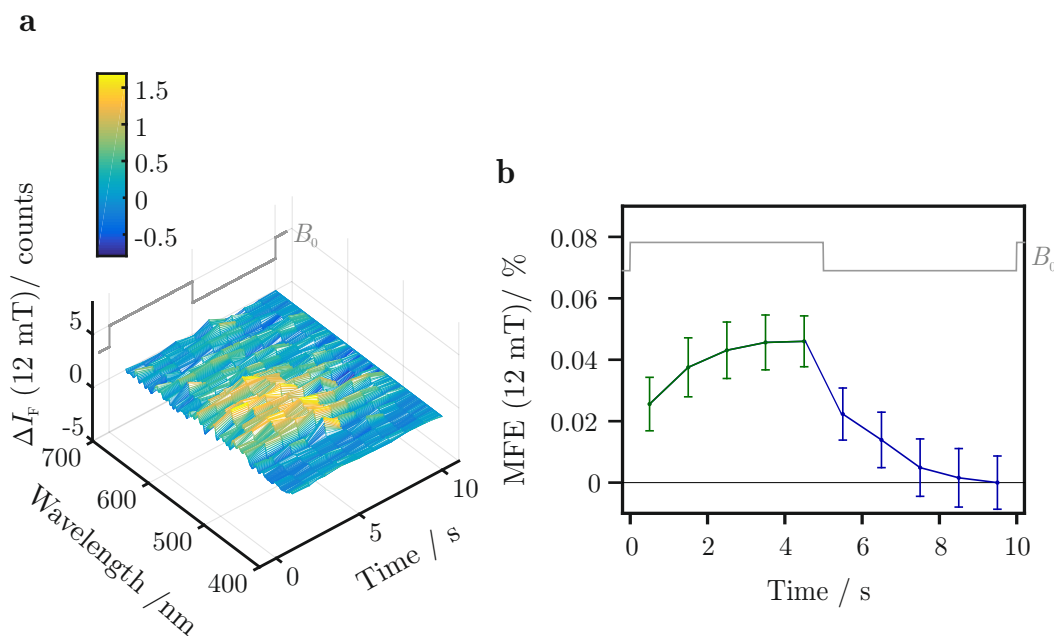


FIGURE 5.4: a) Density plot of the prompt fluorescence subtraction profile for *AtCry1* (aq, tris/NaCl buffer, 30 % glycerol v/v, pH 7.4) subjected to continuous photoexcitation (450 nm, 1.74 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT (as represented pictorially) every 5 s. Spectra corresponding to different time points have been smoothed using a 25 nm moving average filter. b) Average MFE (500-600 nm) response to one field on/off cycle showing enhanced-type behaviour ($E > 1$). The standard error of the averaged MFE is indicated. Measurements were conducted using Apparatus 2 with an exposure time of 1.0 s.

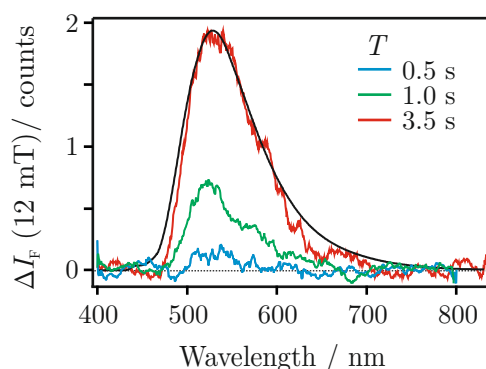


FIGURE 5.5: Changes in the fluorescence spectrum of *AtCry1* (aq, tris/NaCl buffer, 30 % glycerol v/v, pH 7.4) induced by a 12 mT magnetic field. Spectra corresponding to different delay times (T) after switching on a 12 mT magnetic field have been subtracted by the zero field spectrum and smoothed using a 25 nm moving average filter. The subtraction profiles (ΔI_F) increase in amplitude as T is increased from 0.5 to 3.5 s. The black trace is the fluorescence spectrum recorded in the absence of a magnetic field and scaled to match the maximum of the subtraction spectrum at $T = 3.5$ s (red trace). Measurements were conducted using Apparatus 2: 450 nm photoexcitation at 1 mW; exposure time = 0.5 s

The addition of 50 μM TCEP to accelerate the reduction of the $\text{TrpH}^{\bullet+}$ radical leads to a more pronounced enhancement which rises more rapidly ($\tau = 1.7 \pm 0.1 \text{ s}$). Thus it is deduced that the radical termination steps are such that $k_D > k_F$, as implied by Equation 5.14.

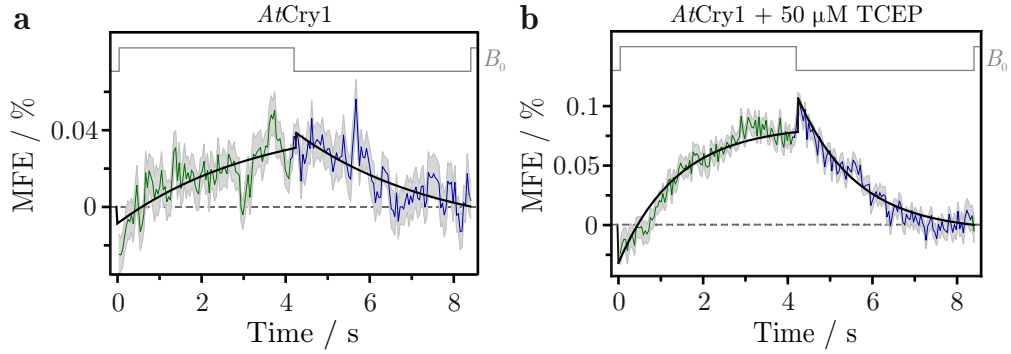


FIGURE 5.6: a) Average time-resolved MFE response to one cycle of field steps for *AtCry1* (aq, tris/NaCl buffer, 30 % glycerol v/v, pH 7.4). The enhanced MFE grows over a period of several seconds as a result of the slow radical termination reactions ($k_D > k_F$, as implied by Equation 5.14). b) as a, but with the addition of 50 μM TCEP. An accelerated reduction of the tryptophan radical by the TCEP boosts the MFE ($k_D \gg k_F$) and causes it to grow more rapidly. Measurements were conducted using Apparatus 1: 470 nm photoexcitation at 0.1 mW; exposure time = 40 ms.

These experiments also exhibit a small, overall-negative prompt MFE (clearer in Figure 5.6b) which is ascribed to free FAD in the sample. Photoexcited free FAD is postulated to react with surface-exposed tryptophan residues on the cryptochrome to generate triplet-born radicals. The negative prompt MFE obtained from the bimolecular radical pair obscures any prompt MFE from the cryptochrome. It is apparent that by means of the MFE amplification, the positive MFE of the protein system eventually dominates over this spurious component.

As well as being the first *in vitro* studies to show the chemical amplification of magnetic field effects in cryptochromes, these experiments are also the first studies to demonstrate fluorescence-based MFEs in such systems.

5.7 Numerical calculations of MFEs for cryptochromes

Numerical calculations for the field dependence of the cryptochrome photocycle presented in Figure 5.1 were conducted following the same method used in Chapter 4 for the intermolecular RP systems [41]. Table 5.1 gives the definitions and values of the rate constants associated with the rate equations which describe the kinetics of the system. The magnetosensitivity of the photoreaction was simulated through field-dependent rate equations for the RP1 spin substates (cf. Equations 4.2 and 4.3 in Chapter 4). All of the reaction steps in Figure 5.1 follow first order kinetics.

The prompt fluorescence intensity detected for cryptochrome samples is proportional to the flavin singlet excited state concentration irrespective of the redox state of the donor radical. Consequently, simulations for the time-dependent PF MFEs were obtained by monitoring the $[F-D]$ and $[F-D^{\bullet+}]$ concentrations. As before, numerical calculations were performed using MATLAB's ode15s solver for ordinary differential equations. Prompt (χ_p) and delayed (χ_d) MFEs, as well as the amplification factor (E) and the time constant for the evolution of $\chi_p \rightarrow \chi_d$ were determined as before in Chapter 4.

In Figure 5.7, the prompt MFE (a, χ_p), delayed MFE (b, χ_d), enhancement factor (c, E) and time constant (d, τ) obtained from a systematic evaluation for the time dependence of a system's field response with varying k_D/k_F is shown ($k_{EX} = 1 \times 10^3 \text{ s}^{-1}$). It appears that the enhanced magnetic field effects arising from intermolecular and intramolecular flavin-based RPs display the same qualitative behaviour (cf. Figure 4.6 in Chapter 4). A prompt MFE of $\sim 0.06\%$ is estimated for the system and found to be (largely) independent of k_D/k_F . The delayed MFE and amplification factor are observed to chiefly depend on the ratio of k_D/k_F , reaching $\chi_d = 4.1\%$ and $E = 68$ when $k_D \rightarrow 1 \times 10^3 \text{ s}^{-1}$ and $k_F \rightarrow 1 \times 10^{-1} \text{ s}^{-1}$. Familiar behaviour is also seen in the timescale simulated for the enhanced effects, with τ broadly decreasing with increasing values of k_D and k_F .

With a ten-fold reduction in the photoexcitation pump power ($k_{EX} = 1 \times 10^3 \text{ s}^{-1} \rightarrow 1 \times 10^2 \text{ s}^{-1}$), the prompt MFE decreases approximately ten-fold to $\sim 6 \times 10^{-3}\%$ (Figure 5.8a). This is in qualitative agreement with the two-state model presented above when $x \gg \Phi_{RP2}(B_0)$ in Equation 5.5. The enhancement factor follows a roughly ten-fold increase as the slow termination reactions result in accumulated, delayed MFEs which are

ultimately of a comparable magnitude to those found by the high power photoexcitation condition.

TABLE 5.1: Values of the rate constants used in calculations of amplified MFEs for the intramolecular cryptochrome photocycle in Figure 5.1. The results are presented in Figures 5.7 and 5.8.

Rate constant	Value	Comment
k_{EX}	$1 \times 10^3 \text{ s}^{-1}$	Pump power for calculations in Figs. 5.7,5.9,5.10.
	$1 \times 10^2 \text{ s}^{-1}$	Pump power for calculations in Fig. 5.8. Photoexcitation is approximated to follow first order kinetics in the weak absorbance limit of the Beer-Lambert Law, see Appendix 3.
k_{DX}	$1 \times 10^8 \text{ s}^{-1}$	Decay of the flavin excited singlet state ($^1\text{F}^*$) by processes other than electron transfer [34].
k_{ET}	$1 \times 10^{10} \text{ s}^{-1}$	Efficient electron transfer between donor and and flavin singlet excited state, $^1\text{F}^*$, to to form RP1 in the singlet state [47].
k_{RP2}	$2 \times 10^6 \text{ s}^{-1}$	Proton transfer processes for $\text{RP1} \rightarrow \text{RP2}$ [49].
k_{HFM}	$8 \times 10^7 \text{ s}^{-1}$	Coherent singlet-triplet interconversion of the spin states of the geminate radical pair by the hyperfine mechanism as determined by Murakami et al. [41].
k_{RM}	$2 \times 10^6 \text{ s}^{-1}$	Incoherent singlet-triplet interconversion of the spin states states of the geminate radical pair by the relaxation mechanism as determined by Murakami et al. [41].
k_{RET1}	$2 \times 10^6 \text{ s}^{-1}$	Reverse electron transfer of RP1 to form the ground state species [49].
k_{RET2}	$5 \times 10^4 \text{ s}^{-1}$	Reverse electron transfer of RP2 to form the ground state [49].

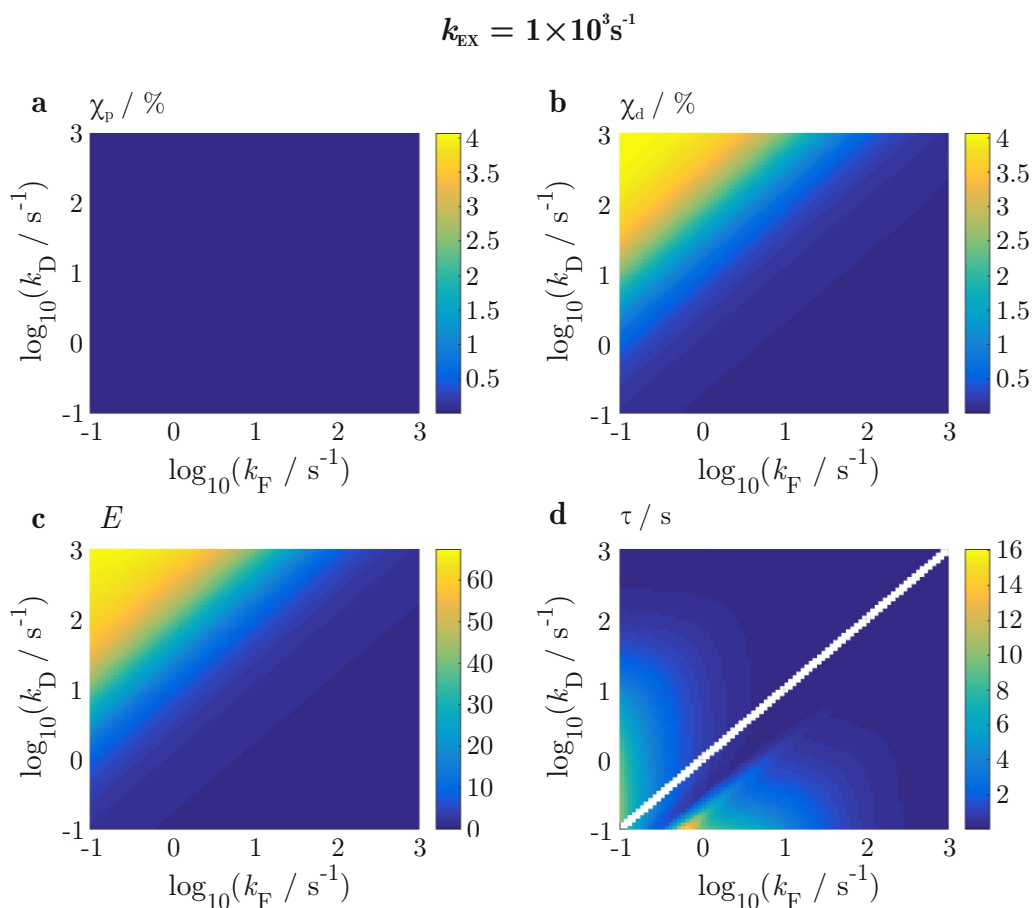


FIGURE 5.7: Density plots of the a) prompt component (χ_{p}), b) delayed component (χ_{d}), c) amplification factor (E) and d) time constant (τ) of the MFEs for the flavin ground state concentration (*i.e.* F—D, F—D $^{\bullet+}$). The plots are given as a function of the radical termination rate constants: k_{F} and k_{D} . Simulations were performed using the rate constant values in Table 5.1 with $k_{\text{EX}} = 1 \times 10^3 \text{ s}^{-1}$.

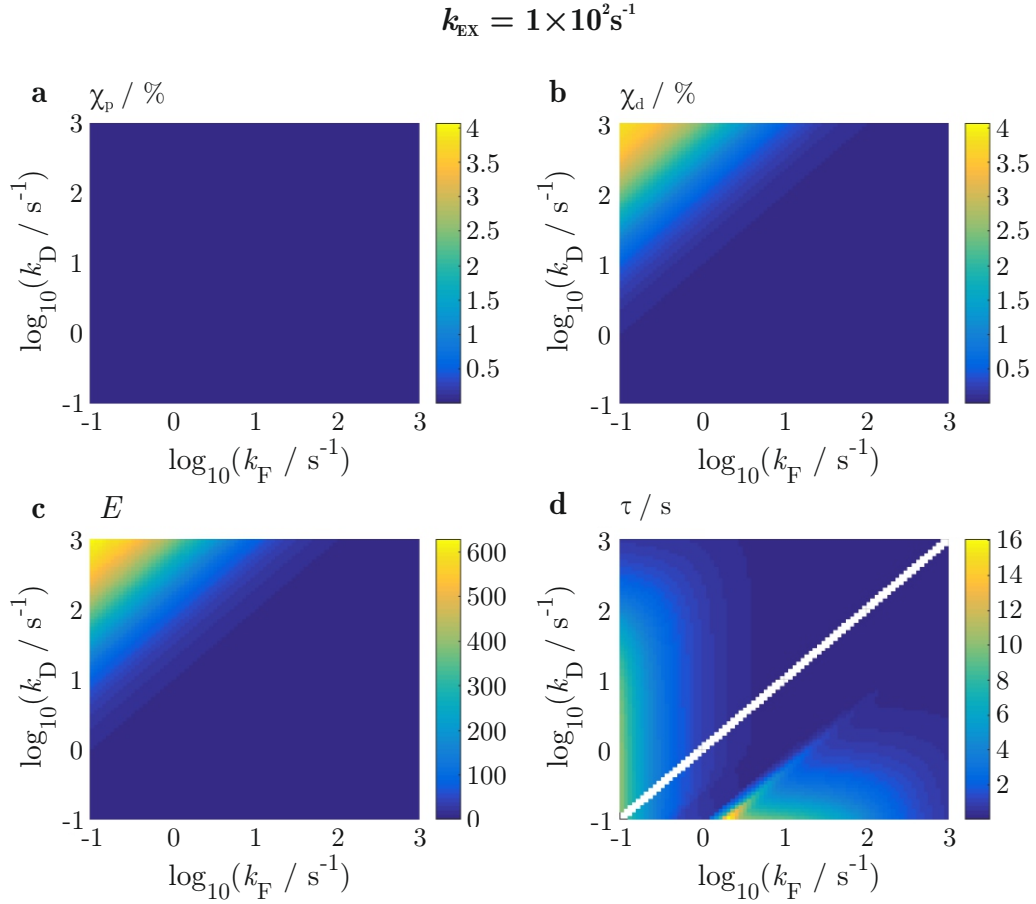


FIGURE 5.8: Density plots of the a) prompt component (χ_{p}), b) delayed component (χ_{d}), c) amplification factor (E) and d) time constant (τ) of the MFEs for the flavin ground state concentration (*i.e.* F—D, F—D $^{\bullet+}$). The plots are given as a function of the radical termination rate constants: k_{F} and k_{D} . Simulations were performed using the rate constant values in Table 5.1 with $k_{\text{EX}} = 1 \times 10^2 \text{ s}^{-1}$.

5.7.1 MFE calculations for the radicals: magnetic signalling in cryptochromes

In this chapter, enhanced magnetic field effects on the flavin ground state concentration have thus far been explored through experiments and calculations. However, it is the magnetosensitive flavin radical which is directly implicated in the cryptochrome hypothesis for magnetoreception. Figure 5.9 shows the k_D/k_F dependence for MFEs pertaining to the flavin radical concentration (*i.e.* $RP1 + RP2 + F^{\bullet-} \rightarrow D$). The enhancement factor, E , plot for MFEs associated with the concentration of $F^{\bullet-}$ -containing and F -containing species are identical (see Figures 5.7 and 5.9). Maximum steady state *relative* MFEs, χ_d , are found for $k_F \approx k_D$ (Figure 5.9b) and do not exceed the maximum value of the prompt MFE, χ_p , attained for $k_F \gg k_D$ (Figure 5.9a). Large enhancement factors are observed for $k_F \gg k_D$ where both the prompt and delayed MFEs are relatively small. Whilst this demonstrates that slow termination reactions can affect the field sensitivity of cryptochrome photoreactions, their role in the promotion of a magnetic sensor appears to be minor.

The effects of magnetic fields on chemical reactions in this thesis have largely been considered in terms of relative (percentage) MFEs, but this is probably not the relevant quantity for magnetic signalling. Note that even a large relative change in the steady-state concentration of the signalling state is unlikely to form the basis of a magnetic compass if that concentration is small. Thus, the critical quantity is more likely to be the absolute field-induced change in the concentration of the radicals present under steady-state illumination conditions. Weaver et al. developed this notion and presented a model in which the absolute number of neurotransmitter molecules resulting from the radical pair reaction is detected [133]. Following this concept, calculations were conducted for the prompt and steady-state changes in the concentration of the $F^{\bullet-}$ radical (from $RP1 + RP2 + F^{\bullet-} \rightarrow D$, as before). The results are shown in Figure 5.10: a,b for $k_{EX} = 1 \times 10^3 \text{ s}^{-1}$; and c,d for $k_{EX} = 1 \times 10^2 \text{ s}^{-1}$.

Critically, the largest delayed field-induced ($F^{\bullet-}$) concentration changes occur when $k_D \gg k_F$ (Figure 5.10b,d) and are significantly larger than the prompt effect for any values of k_D and k_F (Figure 5.10a,c). Furthermore, when the light intensity is reduced

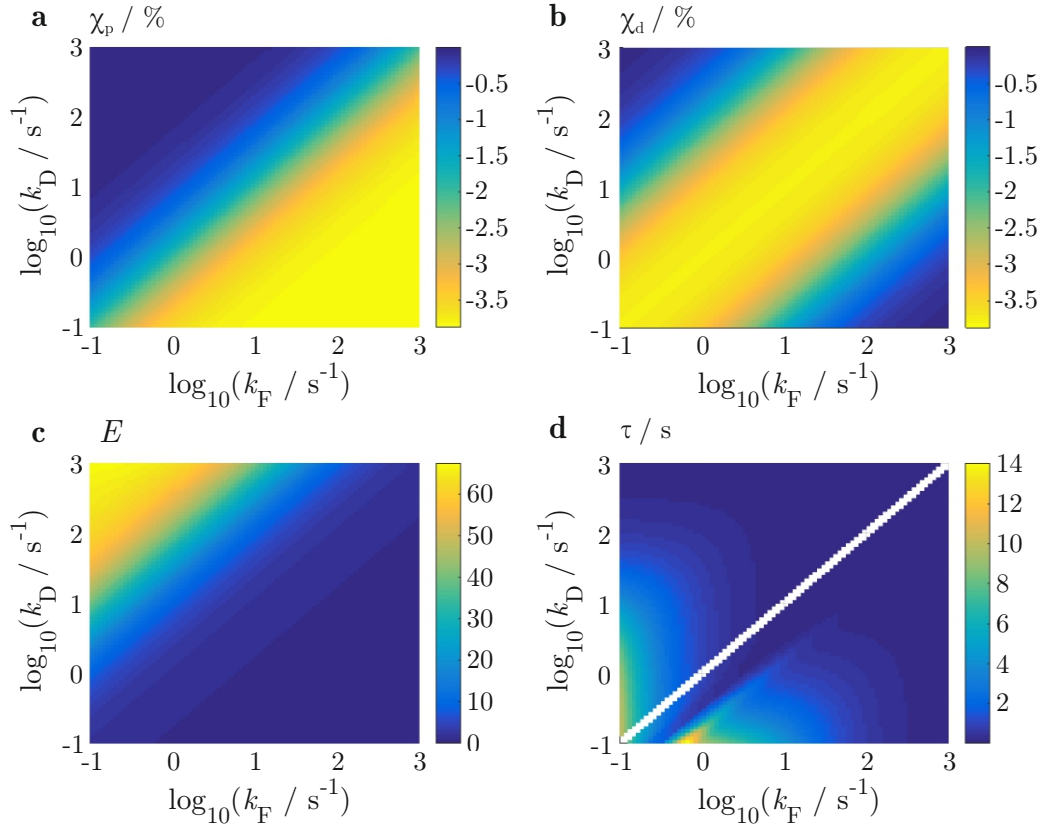


FIGURE 5.9: Density plots of the a) prompt component (χ_p), b) delayed component (χ_d), c) amplification factor (E) and d) time constant (τ) of the MFEs for the flavin radical concentration (*i.e.* $F^{\bullet-} \rightarrow D^{\bullet+}$, $F^{\bullet-} \rightarrow D$). The plots are given as a function of the radical termination rate constants: k_F and k_D . Simulations were performed using the rate constant values in Table 5.1 with $k_{EX} = 1 \times 10^3 \text{ s}^{-1}$.

(Figure 5.10a to c), a higher ratio of k_D/k_F is required to achieve a significant enhancement. Thus, the lower the light intensity, the more important is the amplification phenomenon.

The calculations presented in Figure 5.10 demonstrate the likely importance of enhanced magnetic field effects on the operational sensitivity of the putative avian chemical compass. Amplification is able to boost the absolute field-induced change in the signalling state by more than an order of magnitude. Consequently, the same signal-to-noise ratio can be derived from the enhanced MFEs with up to a two orders of magnitude reduction in the number of receptor molecules required for the primary MFEs [133]. Note again that for a chemical compass to be driven by the small changes induced by the Earth's magnetic field, an efficient amplification mechanism would appear to be critical to its success.

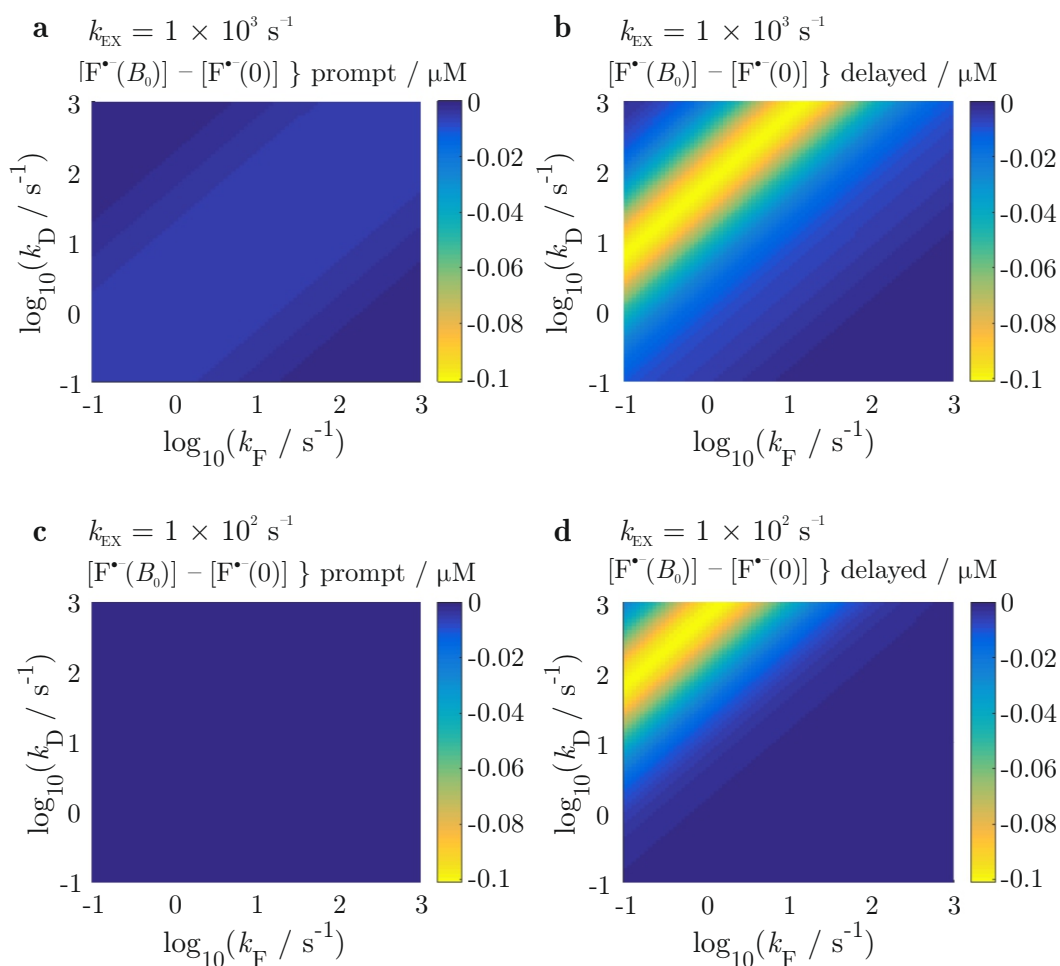


FIGURE 5.10: Density plots of the a) prompt b) delayed components of the absolute MFEs, $[F^{\bullet-}(B_0) - F^{\bullet-}(0)]$, for the flavin radical concentration (*i.e.* $F^{\bullet-} \rightarrow D^{\bullet+}$, $F^{\bullet-} \rightarrow D$). The plots are given as a function of the radical termination rate constants: k_F and k_D . Simulations were performed using the rate constant values in Table 5.1.

5.8 Results and discussion: Maquette

In this section the first results for the prompt fluorescence MFE studies on the novel flavomaquettes are presented. Whilst magnetosensitivity has been demonstrated for cryptochromes, the *in vivo* effects are relatively small and anisotropic responses have yet to be shown for any field strength, let alone those comparable to the Earth's magnetic field. To date, true chemical compass behaviour has only been characterised for the charge recombination reactivity of an artificial photosynthetic reaction centre, the carotenoid-porphyrin-fullerene (CPF) triad, for highly unphysiological conditions [104].

The feasibility of sample alignment for the flavomaquettes is highly attractive as it could facilitate studies of anisotropic MFEs in these systems. Furthermore, as the maquettes contain the same redox active residues as true cryptochrome proteins and can be aligned for study under ambient conditions (*i.e.* aqueous solutions and room temperature), chemical compass characterisation of this system could reveal important insights into the primary mechanism for avian magnetoreception.

5.8.1 MQ: absorption/emission

In Figure 5.11, the a) absorption and b) fluorescence profiles (solid orange lines) derived from cysteinyl-bound riboflavin for maquette-W13 are depicted. The presence of the sulfur atom on the isoalloxazine ring has a profound effect on the absorption spectrum (Figure 5.11a), merging the typical UV-Vis flavin transitions for $S_0 \rightarrow S_1$, $\pi \rightarrow \pi^*$ at 446 nm and $S_0 \rightarrow S_2$, $n \rightarrow \pi^*$ at 375 nm to one broad, red-shifted band centred at ~ 470 nm. However, the cysteinyl-bound flavin fluorescence profile is largely indiscernible from the 'free' form (compare Figures 1.4 and 5.11b).

Figure 5.11 also contains the a) absorption and b) fluorescence profiles of maquettes containing covalently bound lumichrome (dotted blue line). Analogous to free flavins, these species form by photodegradation following hydrogen abstraction from the ribityl group [134]. Lumichrome is also expected to have field-dependent photochemistry, thus potentially complicating MFE studies on maquettes. However, it is apparent from the absorption profiles in Figure 5.11a that sample illumination with wavelengths $\lambda > 440$ nm leads to the photoexcitation of riboflavin-containing maquettes (MQ-riboflavin) but not their lumichrome analogues (MQ-lumichrome). Selective

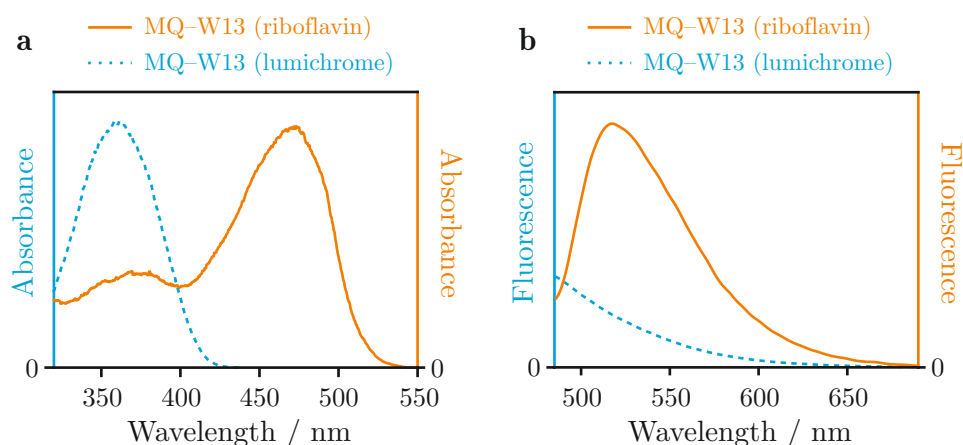


FIGURE 5.11: a) UV/vis and b) fluorescence profiles for MQW13 containing covalently bound riboflavin (solid orange line) and lumichrome (dotted blue line). The absorption profiles were obtained from fluorescence excitation spectra monitoring emission at 600 nm (riboflavin) and 455 nm (lumichrome). Fluorescence emission spectra were recorded following photoexcitation at 470 nm (riboflavin) and 400 nm (lumichrome, after subtraction of the riboflavin fluorescence component).

photoexcitation of the MQ–riboflavin subset is employed by the use of the 450 nm diode laser to study MFEs for the photoreactions of MQ–W13 and MQ–W20 in the following section. In order to study composite MFEs arising from simultaneous MQ–lumichrome and MQ–riboflavin photochemistry, a 405 nm diode laser is also used.

5.8.2 MQ–W13: 450 nm

The averaged time-resolved fluorescence-detected MFEs for the flavin-tryptophan photoreaction in MQ–W13 are shown in Figure 5.12 (Apparatus 2). During the experiment, the sample experienced continuous photoexcitation by a 450 nm laser and a stepping magnetic field which switched between 0 and 12 mT every 15 s. Prompt fluorescence from the sample was recorded continuously, with 750 frames measured per field on/off cycle. In Figure 5.12, the fluorescence subtraction profile is plotted, $\Delta I_F(\lambda, t)$. Fitting of the spectra to composites of the fluorescence profiles for MQ–Riboflavin and MQ–Lumichrome confirmed the presence of the former and the absence of the latter species. No discernible prompt response of the system’s fluorescence to the switching magnetic field can be seen, but rather a slow, accumulated rise and fall in signal which is in phase with the field modulation. The increase in fluorescence intensity during the 12 mT duration of the field cycle implies a singlet-born radical pair.

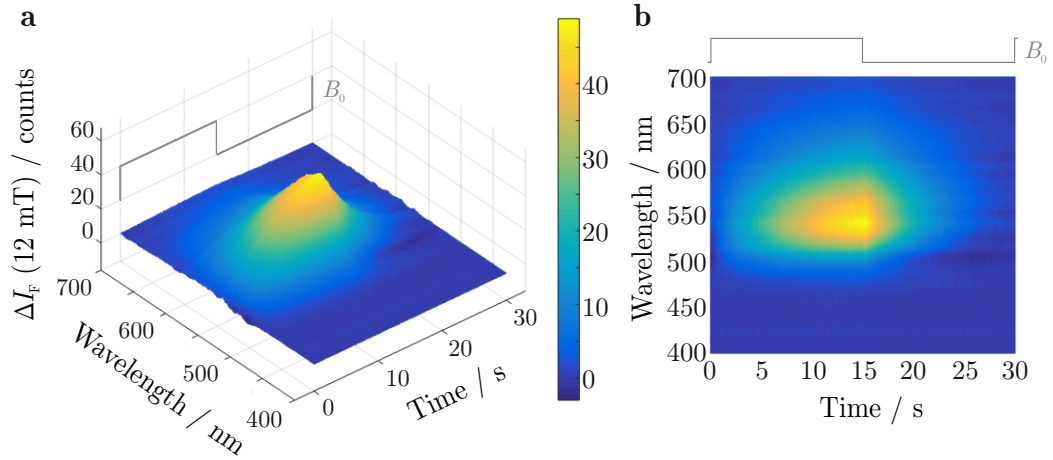


FIGURE 5.12: a) Density plot of the prompt fluorescence subtraction profile for 20 μM MQ-W13 (aq, phosphate/KCl buffer, pH 7.3) subjected to continuous photoexcitation (450 nm, 55 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT every 15 s. Spectra corresponding to different time points have been smoothed using a 20 nm moving average filter. Measurements were conducted using Apparatus 2. b) is an alternative representation of the same data as a).

Synchronous behaviour between the field and the recorded fluorescence is clearer still for the MFE profiles in Figure 5.13. The relative MFE is deconvoluted into MQ-riboflavin and (zero) MQ-lumichrome components. Remarkably, the MFE rises on the timescale of tens of seconds, which is orders of magnitude slower than for the other systems explored in this thesis, and is far from reaching its final value (χ_d) even after 15 s. Exponential fitting to the time-dependent MFE finds $\chi_d(t \rightarrow \infty) = 1.08 \pm 0.01 \%$ and $\tau = 11.5 \pm 0.2$ s.

The addition of 100 μM TCEP, a reducing agent, is expected to further enhance the MFE by increasing the ratio of k_D/k_F (see Equation 5.14). As above, following 450 nm photoexcitation, the MQ-W13+TCEP emission profile is exclusively derived from MQ-riboflavin and not MQ-lumichrome (Figure 5.14a). Furthermore, the singlet-born nature of the radical pair appears to be preserved. Figure 5.14b shows the time-resolved prompt fluorescence MFE. In agreement with the proposed amplification mechanism, $\chi_d(t \rightarrow \infty)$ is increased ($1.18 \pm 0.01 \%$). The timescale for the enhancement is also found to rise: $\tau = 17.2 \pm 0.2$ s.

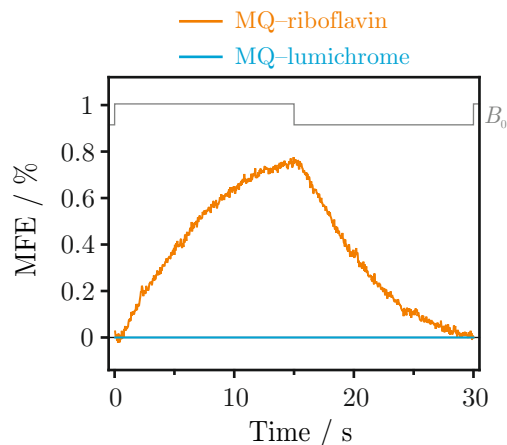


FIGURE 5.13: PF MFE responses for the MQ-riboflavin and MQ-lumichrome components of 20 μM MQ-W13 (aq, phosphate/KCl buffer, pH 7.3) subjected to continuous photoexcitation (450 nm, 55 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT every 15 s. MFEs have been deconvoluted using the reference spectra in Figure 5.11b. Measurements were conducted using Apparatus 2.

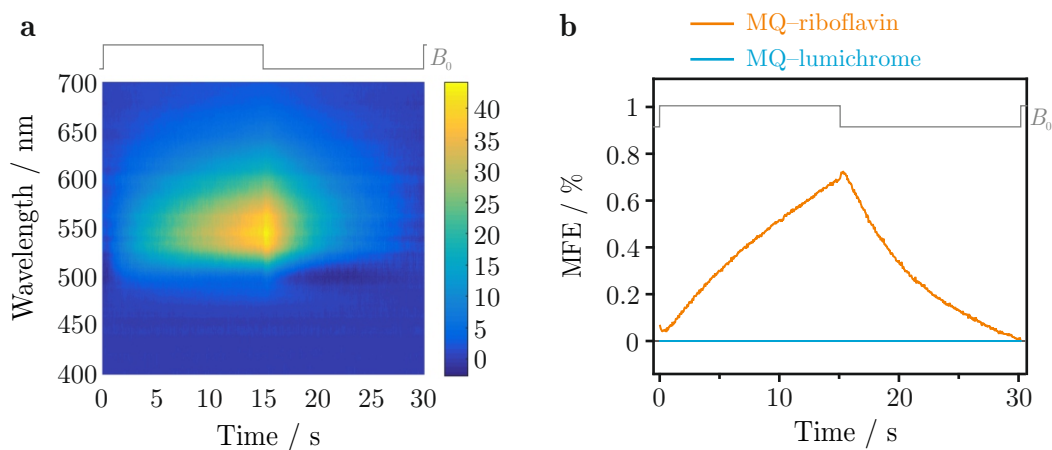


FIGURE 5.14: a) Density plot of the prompt fluorescence subtraction profile for 20 μM MQ-W13 + 100 μM TCEP (aq, phosphate/KCl buffer, pH 7.3) subjected to continuous photoexcitation (450 nm, 55 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT every 15 s. Spectra corresponding to different time points have been smoothed using a 20 nm moving average filter. b) PF MFE responses for the MQ-riboflavin and MQ-lumichrome components of the data in a). MFEs have been deconvoluted using the reference spectra in Figure 5.11b. Measurements were conducted using Apparatus 2.

5.8.3 MQ-W20: 450 nm

On going from MQ-W13 to MQ-W20, the flavin-tryptophan separation is increased by approximately 10 Å, corresponding to an estimated 10^7 -times decrease in the rate of photoinduced electron transfer by the Dutton-Moser ruler ($k_{\text{ET}} = 10^{11} \text{ s}^{-1} \rightarrow 10^5 \text{ s}^{-1}$) [135, 136]. Thus, MQ-W13 and MQ-W20 are expected to differ significantly in their spin dynamics.

The fluorescence subtraction profile for MQ-W20 contrasts with the monotonic field response observed in Figures 5.12 and 5.14a. During the field-on period, the sample fluorescence undergoes an increase and subsequent decrease in intensity. Complementary behaviour is found for the field-off phase as the fluorescence intensity first decreases and then increases with time. Whilst the overall behaviour differs markedly to MQ-W13, the magnetosensitive fluorescence is exclusively attributed to MQ-riboflavin.

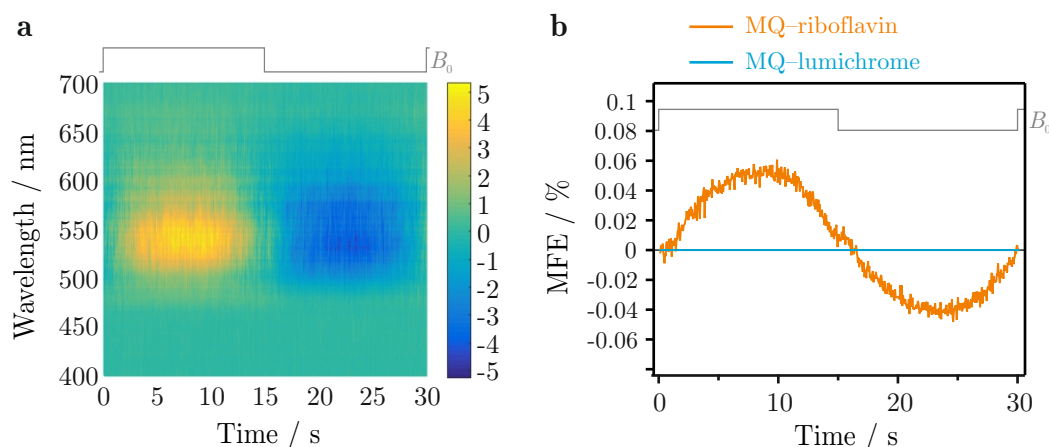


FIGURE 5.15: a) Density plot of the prompt fluorescence subtraction profile for 20 μM MQ-W20 (aq, phosphate/KCl buffer, pH 7.3) subjected to continuous photoexcitation (450 nm, 55 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT every 15 s. Spectra corresponding to different time points have been smoothed using a 20 nm moving average filter. b) PF MFE responses for the MQ-riboflavin and MQ-lumichrome components of the data in a). MFEs have been deconvoluted using the reference spectra in Figure 5.11b. Measurements were conducted using Apparatus 2.

One possible explanation is that MQ-W20 forms both singlet- (*i.e.* ‘the rise’) and triplet-born (*i.e.* ‘the fall’) radical pairs due to the existence of different conformations of the protein with varying electron transfer efficiency. As the composite field effect does not reach steady-state during the field cycle, it is not possible to determine the absolute

values of χ_d and τ for the two contributions. However, $\tau(\text{singlet}) < \tau(\text{triplet})$ can be deduced from the data.

The behaviour of MQ-W20 in Figure 5.15 can be explained by considering the effect of the rate of radical pair formation on MFE enhancement. Figure 5.16 shows the simulated effect of varying the rate of electron transfer, k_{ET} , on a) the time constant, τ , and b) the magnitude of the absolute, enhanced MFE derived from photoreactions of radical pair systems following Figure 5.1¹. The unique time dependence for ΔI_F observed in Figure 5.15 is realised where the formation of singlet-born radical pairs is faster than their triplet-born counterparts (*i.e.* $k_{\text{ET}}(\text{singlet}) > k_{\text{ET}}(\text{triplet})$), yet the size of their associated prompt fluorescence MFEs are comparable.

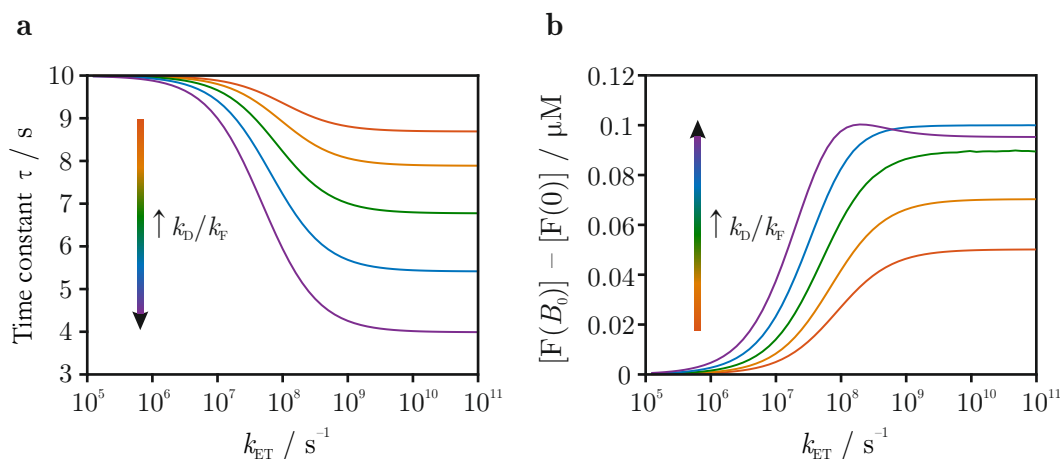


FIGURE 5.16: Dependence of the a) time constant (τ) and b) absolute MFE on the flavin ground state concentration, $[F(B_0) - F(0)]$, from numerical calculations of the photocycle in Figure 5.1 using the rate constant values in Table 5.1.

5.8.4 MQ-W13: 405 nm

The most exotic MFE enhancement profiles are obtained when both MQ-riboflavin and MQ-lumichrome photoreactions occur simultaneously. Figure 5.17 shows the averaged MQ-W13 fluorescence subtraction profile following continuous 405 nm (350 mW) irradiation in the presence of a stepping magnetic field (field on/off time = 10 s). On switching

¹Note that whilst the sign of the MFE for singlet- and triplet-born radical pairs have opposite phase, the magnitude of field-induced changes in concentration are identical assuming no other changes in the photocycle.

from 0 to 12 mT, both positive (yellow, 550–600 nm) and negative (blue, < 520 nm) subtraction bands are observed. Thus, at least two magnetosensitive fluorescent species must be present.

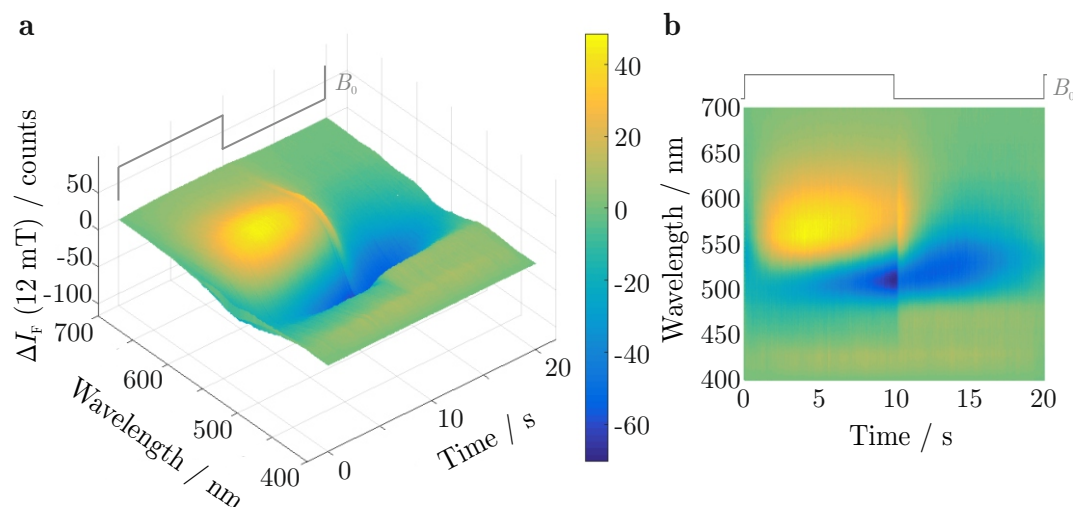


FIGURE 5.17: a) Density plot of the prompt fluorescence subtraction profile for 20 μ M MQ-W13 (aq, phosphate/KCl buffer, pH 7.3) subjected to continuous photoexcitation (405 nm, 350 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT every 15 s. Spectra corresponding to different time points have been smoothed using a 20 nm moving average filter. Measurements were conducted using Apparatus 2. b) is an alternative representation of the same data as a).

The subtraction and field-off fluorescence intensity spectra can be reproduced by linear combinations of the MQ-riboflavin and MQ-lumichrome profiles in Figure 5.11b. Figure 5.18 depicts how the overall MFE can be deconvoluted into its constituent parts. In contrast to the previous MQ-W13 studies by 450 nm excitation, the time dependence of the MFE associated with MQ-riboflavin is not monotonic (orange). A negative prompt MFE is discernible immediately after field switching. The slow component of the MFE is biphasic as for the MQ-W20 studies above. Unfortunately, the presence of a triplet-born radical pair species (deduced from the negative sign of the MFE) could be attributed to sample degradation. The efficiency of singlet-born RP formation diminishes with increasing flavin-tryptophan separation following the loss of the maquette's well-defined tetrahelical structure. MQ-lumichrome has no observable prompt MFE, and its monophasic enhanced field effects suggest that its photochemistry proceeds via triplet-born radicals. Exponential fitting to the time-resolved MFE reveals: $\chi_d = -0.0928 \pm 0.0004$ and $\tau = 2.05 \pm 0.03$ s.

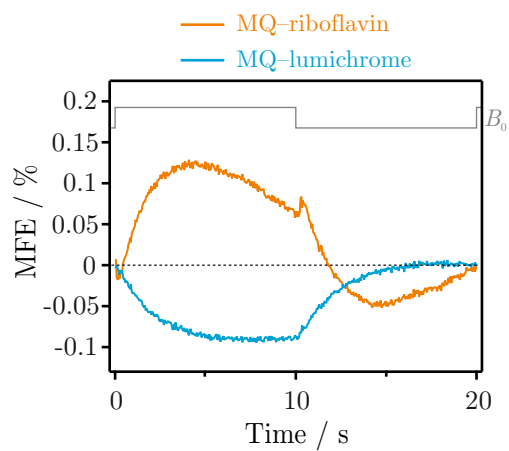


FIGURE 5.18: PF MFE responses for the MQ-riboflavin and MQ-lumichrome components of 20 μ M MQ-W13 (aq, phosphate/KCl buffer, pH 7.3) subjected to continuous photoexcitation (405 nm, 350 mW) and step-wise changes of the external magnetic field (B_0) between 0 and 12 mT every 15 s. MFEs have been deconvoluted using the reference spectra in Figure 5.11b. Measurements were conducted using Apparatus 2.

5.9 Conclusion and outlook

This chapter has explored the chemical amplification of MFEs derived from intramolecular radical pair systems relevant to cryptochromes, the putative receptor in the radical pair hypothesis for avian magnetoreception. Following a simple two-state treatment of the system's photocycle, the prompt magnetic field effects (χ_p) are found to be independent of sample concentration, but exhibit the same (qualitative) dependencies on photoexcitation and radical recombination as their intermolecular counterparts in Chapter 4. Further similarities are revealed by the barebones model and from numerical calculations as the delayed MFE (χ_d) depends critically on the relative rates of the termination reactions. The exceptional sensitivity of prompt fluorescence detection of MFEs is further demonstrated by simultaneous measurements of their spectral and temporal components, enabling further insights into the overall photochemistry of the studied systems.

Enhancement of MFEs has been demonstrated for novel flavomaquettes and *AtCry1*, becoming more pronounced following the addition of a reducing agent. This work provides direct experimental evidence for a possible mechanism of MFE amplification in cryptochromes. Furthermore, it should be noted that cellular environments are typically reducing in nature, thus supporting $k_D > k_F$ for enhanced flavin-based MFEs. Given the efficiency of the chemical amplification mechanism studied here, and elsewhere in this thesis, it would be surprising if evolutionary pressure had not resulted in its exploitation in magnetoreception.

Chapter 6

Conclusion

In this thesis, research into flavin photochemistry was motivated by its potential implications for magnetoreception in animals. Previously, light-induced flavin-tryptophan radical pair reactions in cryptochromes have been proposed to mediate this magnetic sense in navigation. Recent studies of the candidate magnetoreceptor and related model systems have focussed on characterising: magnetic field inclination-dependent reactivity of flavin-containing radical pairs (i.e. chemical compass behaviour); magnetic field effects (MFEs) on reactions at field strengths of a few millitesla and lower; and amplification processes deemed necessary to boost the inherently weak signals involved. The latter two aspects were explored in this thesis using a novel detection scheme for investigating MFEs on flavin photoreactions. The technique monitors the “prompt” fluorescence derived from the flavin singlet excited state ($^1F^*$) which becomes magnetosensitive under continuous sample illumination. Proof-of-principle studies were conducted on flavin-tryptophan radical pair model systems, showing that the fluorescence reflects the magnetic field dependence of the radical pair. Further proof for the validity of the approach was obtained by demonstrating the influence of internal (e.g. hyperfine interactions) and external (e.g. solvent viscosity, dielectric constant) factors on the spin dynamics of the geminate pair, which compared favourably with previous results using absorption-based methods. Due to the zero-background and scatter-insensitive nature of the measurements, magnetic field effects could be detected with high sensitivity on samples containing as little as 1 nM flavin.

Low field effects on the reactivity of the flavin-ascorbic acid intermolecular radical pair were seen to be especially pronounced and were ascribed to the near-negligible hyperfine interactions associated with the ascorbyl radical. Furthermore, the LFE was revealed to be a sensitive indicator for the charges of the radicals and could be altered by changing the sample pH. Direct control was exerted over the singlet- or triplet-born nature of the radical pair by variation of the electron transfer efficiency.

For continuously irradiated radical pair photocycles, slow (and typically neglected) processes associated with the termination of the radicals were found to give rise to MFE enhancement and diminishment over hundreds of milliseconds timescales and longer. Through calculation and experiments, it was demonstrated that the chemical amplification behaviour could be controlled by influencing the relative rates of termination of the free radical species (i.e. using reducing and oxidising agents; variation in pH; pump power and flavin concentration). Under appropriate conditions, the MFE enhancement was observed to be disproportionately larger at weak field strengths for the low field effect, a potentially useful trait in light of magnetoreception. In addition, MFE amplification has also been shown for cryptochrome samples, with enhanced MFE responses further boosted, as expected, by the addition of a reducing agent. As the requirements for MFE amplification are so simple, it is likely to be applicable to the majority of radical pair reactions, allowing MFEs to be enhanced beyond the previously perceived limits of the radical pair mechanism.

Appendix A: Estimating the rate of photoexcitation

The rate of photoexcitation, I_{Abs} , of species x can be estimated using the Beer-Lambert Law:

$$I_{Abs} = I_0(1 - e^{-\alpha lc}) \quad (1)$$

where I_0 is the rate at which incident light of wavelength λ strikes the sample, α is the normal extinction coefficient of species x for wavelength λ , l is the pathlength and c is the concentration of x .

Equation 1 can be evaluated as follows:

$$I_{Abs} = \frac{P}{h\nu N_A V} (1 - e^{-\log_e(10)\epsilon lc}) \quad (2)$$

where P is the pump power of photoexcitation, h is the Planck constant, ν is the frequency corresponding to light with a wavelength of λ , N_A is Avogadro's constant, V is the effective volume of laser illumination, ϵ is the decadic extinction coefficient.

Low sample absorbance (as is relevant to the systems studied in this thesis) leads to I_{Abs} being reasonably estimated as a first order process where:

$$I_{Abs} \approx \frac{P}{h\nu N_A V} \log_e(10)\epsilon lc = k_{abs}c. \quad (3)$$

For the MFE studies in Chapter 2: $P = 350 \text{ mW}$, $\lambda = 405 \text{ nm}$, $\nu = 7.14 \times 10^{14} \text{ s}^{-1}$, $V = 7.85 \times 10^{-7} \text{ dm}^3 \approx 10^{-6} \text{ dm}^3$, $\epsilon = 5000 \text{ M}^{-1} \text{ cm}^{-1}$ for flavins at 405 nm [46], $l = 1 \text{ mm}$; thus $k_{abs} = 1.74 \times 10^3 \text{ s}^{-1} \approx 10^3 \text{ s}^{-1}$.

Appendix B: Evaluating the limits of PF MFEs by the two-state model

In Chapter 2, the following expression for the PF MFE by the two-state model is stated:

$$\text{MFE}(B_0, \text{PF}) = \frac{\Phi_{\text{FR}}(0) \left(\sqrt{1 + z/\Phi_{\text{FR}}(0)} - 1 \right) - \Phi_{\text{FR}}(B_0) \left(\sqrt{1 + z/\Phi_{\text{FR}}(B_0)} - 1 \right)}{z/2 - \Phi_{\text{FR}}(0) \left(\sqrt{1 + z/\Phi_{\text{FR}}(0)} - 1 \right)}. \quad (4)$$

The equation can be evaluated in the limits of $z \rightarrow \infty$ or 0 using L'Hospital's Rule which states:

$$\lim_{z \rightarrow a} \frac{f(z)}{g(z)} = \lim_{z \rightarrow a} \frac{f'(z)}{g'(z)} \quad (5)$$

where $f(a)$ and $g(a)$ are both zero or ∞ , and $'$ denotes the derivative with respect to z .

For $z \rightarrow \infty$, $\lim_{z \rightarrow \infty} (f(z)/g(z)) = 0/0$. From Equation 4,

$$\frac{f'(z)}{g'(z)} = \frac{\frac{1}{2} \left(1 + \frac{z}{\Phi_{\text{FR}}(0)} \right)^{-1/2} - \frac{1}{2} \left(1 + \frac{z}{\Phi_{\text{FR}}(B_0)} \right)^{-1/2}}{\frac{1}{2} - \frac{1}{2} \left(1 + \frac{z}{\Phi_{\text{FR}}(0)} \right)^{-1/2}}, \quad (6)$$

thus $\lim_{z \rightarrow \infty} (f'(z)/g'(z)) = 0/\frac{1}{2} = 0$.

For $z \rightarrow 0$, $\lim_{z \rightarrow 0} (f(z)/g(z)) = 0/0$. From Equation 6, $\lim_{z \rightarrow 0} (f'(z)/g'(z)) = 0/0$.

Consequently, $\lim_{z \rightarrow 0} (f''(z)/g''(z))$ must be considered and is evaluated as follows:

$$\frac{f''(z)}{g''(z)} = \frac{-\frac{1}{4\Phi_{\text{FR}}(0)}\left(1 + \frac{z}{\Phi_{\text{FR}}(0)}\right)^{-3/2} + \frac{1}{4\Phi_{\text{FR}}(B_0)}\left(1 + \frac{z}{\Phi_{\text{FR}}(B_0)}\right)^{-3/2}}{\frac{1}{4\Phi_{\text{FR}}(0)}\left(1 + \frac{z}{\Phi_{\text{FR}}(0)}\right)^{-3/2}}, \quad (7)$$

and,

$$\lim_{z \rightarrow 0} \frac{f''(z)}{g''(z)} = \frac{-1/\Phi_{\text{FR}}(0) + 1/\Phi_{\text{FR}}(B_0)}{1/\Phi_{\text{FR}}(0)} = \frac{\Phi_{\text{FR}}(0) - \Phi_{\text{FR}}(B_0)}{\Phi_{\text{FR}}(B_0)}. \quad (8)$$

Appendix C: Evidence of lumichrome formation by flavin photoexcitation: mass spectrometry

Figure 1 shows the mass spectra before (a) and after (b) a 30 min PF MARY experiment on a static sample of 10 μ M FMN/0.5 mM HEWL, indicating an increase in lumichrome content due to continuous photoexcitation by a 405 nm diode laser at 350 mW. Spectrum a) suggests that lumichrome is present as a minor impurity in FMN (Sigma Aldrich). The spectra were recorded using a positive electrospray ionisation Waters Premier LCT mass spectrometer.

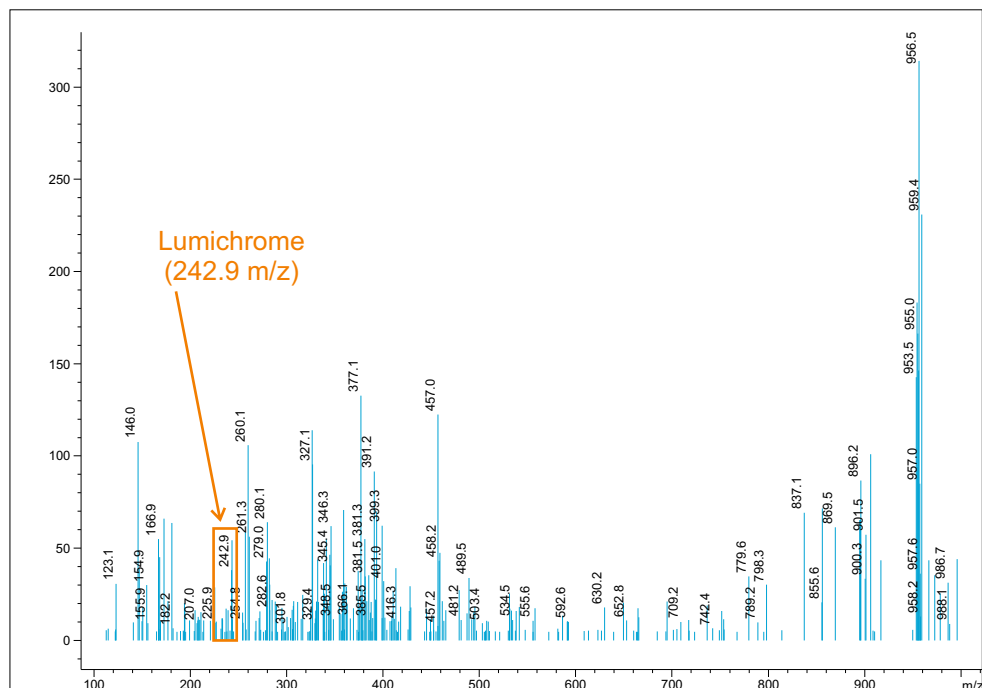
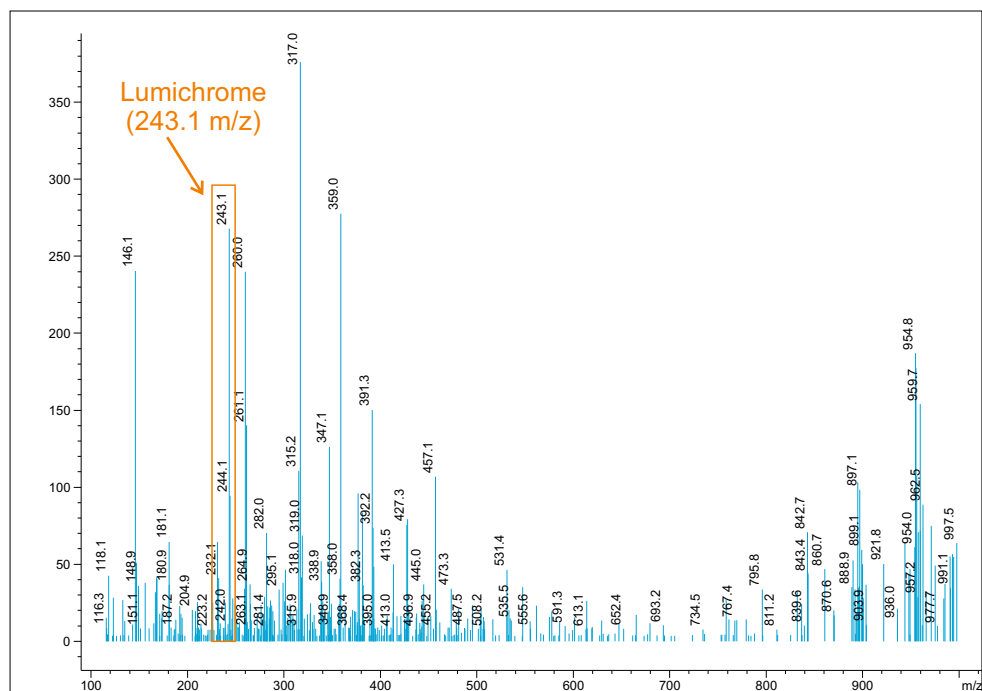
a FMN/HEWL prior to photoexcitation**b** FMN/HEWL post-photoexcitation

FIGURE 1: Mass spectra (m/z) for FMN/HEWL before (a) and after (b) a typical PF MARY experiment on a static sample. The sample was illuminated for 30 min using a 405 nm diode laser operating at 350 mW.

Appendix D: Tables of hyperfine parameters

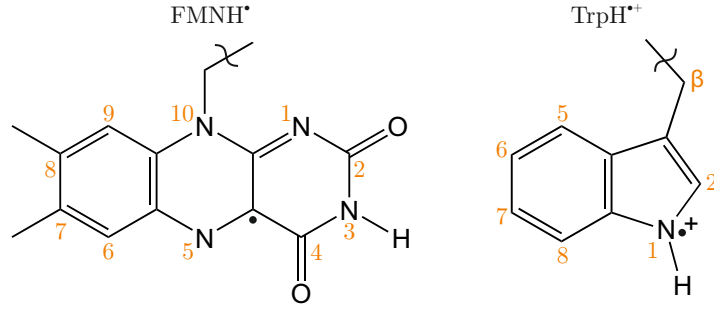


FIGURE 2: Chemical structures for FMNH• and TrpH•+.

The DFT-derived hyperfine parameters in Tables 1 (FMNH•), 2 (FMN•-), 5 (Asc•-), 6 (AscH•) and 7 (AscH2•+) were obtained by Dr. Daniel Kattnig. B3LYP/6-311+G(d,p)//EPR-III.

The experimentally-derived hyperfine parameters in Tables 3 (Trp•) and 4 (TrpH•+) were obtained by EPR and taken from [137] and [138], respectively.

TABLE 1: Table of hyperfine parameters for the neutral FMNH• radical derived from DFT calculations.

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
N ₅	1×1	0.499
N ₁₀	1×1	0.320
H ₅	$1 \times 1/2$	-0.896
H ₆	$1 \times 1/2$	-0.153
H(N ₁₀ -CH ₂)	$2 \times 1/2$	0.250
H(C8 α ₁₀ -CH ₃)	$3 \times 1/2$	0.315
H _{Rest}	$1 \times 1/2$	0.092

TABLE 2: Table of hyperfine parameters for the anionic FMN^{•−} radical derived from DFT calculations.

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
N ₅	1×1	0.611
N ₁₀	1×1	0.260
H ₅	$1 \times 1/2$	−0.896
H ₆	$1 \times 1/2$	−0.319
H(N ₁₀ −CH ₂)	$2 \times 1/2$	0.188
H(C8 α ₁₀ −CH ₃)	$3 \times 1/2$	0.440
H _{Rest}	$1 \times 1/2$	0.235

TABLE 3: Table of hyperfine parameters for the neutral Trp[•] radical derived from EPR [137].

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
N ₁	1×1	0.44
H ₂	$1 \times 1/2$	—
H ₅	$1 \times 1/2$	−0.58
H ₆	$1 \times 1/2$	—
H ₇	$1 \times 1/2$	−0.44
H ₈	$1 \times 1/2$	—
H _{β1}	$1 \times 1/2$	≤ 0.02
H _{β2}	$1 \times 1/2$	1.60

Using the hyperfine values in Tables 1, 2, 3 and 4, the Weller estimates using Equations 2.11 and 2.12 are as follows:

- $B_{1/2}(\text{FMNH}^{\bullet} \text{Trp}^{\bullet}) = 2.97 \text{ mT}$
- $B_{1/2}(\text{FMNH}^{\bullet} \text{TrpH}^{\bullet+}) = 2.69 \text{ mT}$
- $B_{1/2}(\text{FMN}^{\bullet-} \text{Trp}^{\bullet}) = 2.93 \text{ mT}$
- $B_{1/2}(\text{FMN}^{\bullet-} \text{TrpH}^{\bullet+}) = 2.64 \text{ mT}$

TABLE 4: Table of hyperfine parameters for the cationic $\text{TrpH}^{\bullet+}$ radical derived from EPR [138].

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
N_1	1×1	0.20
$\text{H}_{\text{N}1}$	$1 \times 1/2$	0.35
H_2	$1 \times 1/2$	0.51
H_5	$1 \times 1/2$	0.54
H_6	$1 \times 1/2$	0.13
H_7	$1 \times 1/2$	0.42
H_8	$1 \times 1/2$	0.06
$\text{H}_{\beta 1}$	$1 \times 1/2$	0.71
$\text{H}_{\beta 2}$	$1 \times 1/2$	1.07

TABLE 5: Table of hyperfine parameters for the anionic $\text{Asc}^{\bullet-}$ radical derived from DFT calculations.

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
H_4	$1 \times 1/2$	-0.256
H_{Rest}	$1 \times 1/2$	0.0176

TABLE 6: Table of hyperfine parameters for the neutral $\text{AscH}^\bullet$ radical derived from DFT calculations.

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
H_2	$1 \times 1/2$	-0.483
H_4	$1 \times 1/2$	-0.101
H_{Rest}	$1 \times 1/2$	0.018

TABLE 7: Table of hyperfine parameters for the cationic $\text{AscH}_2^{\bullet+}$ radical derived from DFT calculations.

Nucleus	No. of spins $\times$ Nuclear spin (I)	a_{iso}/mT
H_2	$1 \times 1/2$	-0.575
H_3	$1 \times 1/2$	-0.405
H_4	$1 \times 1/2$	0.884
H_{Rest}	$1 \times 1/2$	0.0733

Appendix E: SADS for the flavin triplet excited state

Figure 3 contains the species associated difference spectra (SADS) for the a) protonated and b) non-protonated flavin triplet excited state. The spectra were obtained from a one-species target analysis (see Chapter 3) of transient absorption (TA) data recorded for samples of a) 100 μM FMN (aq, pH 3 formate buffer, $I_c = 0.1$ M) and b) 100 μM FMN (aq, pH 7 phosphate buffer, $I_c = 0.1$ M). The experimental arrangement for the TA measurements is discussed in Chapter 3.

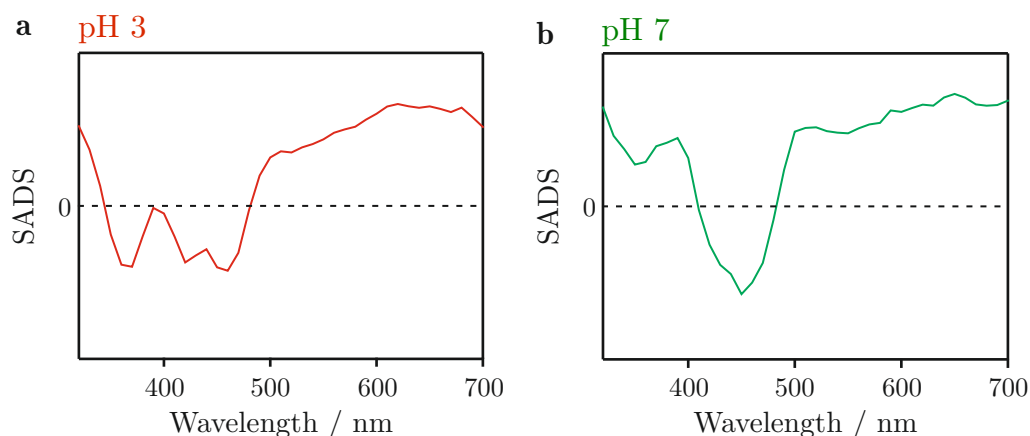


FIGURE 3: SADS for a) ${}^3\text{FMNH}^{+*}$ and b) ${}^3\text{FMN}^*$ as obtained by target analysis on TA data recorded for samples of 100 μM FMN at pH 3 and 7, respectively.

The flavin triplet lifetime was determined to be 2.5 μs at pH 3 and 3.4 μs at pH 7.

Appendix F: Concentration dependence for the relative proportions of singlet- and triplet-born RPs in FMN/ascorbic acid photoreactions

The quantum yields for the formation of singlet- and triplet-born radical pairs are obtained from:

$$\Phi_{\text{S(RP)}} = \frac{k_{\text{Q(S)}}[\text{Q}]}{k_{\text{S(0)}} + k_{\text{Q(S)}}[\text{Q}]} \quad (9)$$

$$\Phi_{\text{T(RP)}} = \frac{k_{\text{ISC}}}{k_{\text{S(0)}} + k_{\text{Q(S)}}[\text{Q}]} \cdot \frac{k_{\text{Q(T)}}[\text{Q}]}{k_{\text{T(0)}} + k_{\text{Q(T)}}[\text{Q}]} \quad (10)$$

where all of the rate constant parameters are defined for ambient conditions (*i.e.* room temperature and atmosphere). $k_{\text{S(0)}}$ is the effective first order rate constant for decay of the flavin singlet excited state by pathways which do not involve quenching by Q (in Chapter 3, ascorbic acid); it is simply $k_{\text{S(0)}} = 1/\tau_{\text{S(0)}}$ with $\tau_{\text{S(0)}}$ being the lifetime of the flavin singlet excited state in the absence of Q. Due to the lack of available data at

pH 3, $\tau_{S(0)}$ is assumed to be independent of pH, with a value of 4.6 ns as determined by Holzer et al. from studies of FMN only at pH 7 (phosphate buffer) [34]. The rate constants for quenching of the flavin singlet excited state by ascorbic acid are: $k_{Q(S)} = 3.0 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (pH 3); and $k_{Q(S)} = 6.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (pH 7). These values have been calculated using the Stern-Volmer constants determined in Chapter 3 ($14 \text{ M}^{-1}\text{s}^{-1}$ at pH 3; and $31 \text{ M}^{-1}\text{s}^{-1}$ at pH 7) and assuming $\tau_{S(0)} = 4.6 \text{ ns}$ as above. The intersystem crossing rate constant for FMN at both pH 3 and 7, lacking known pH-dependent values, is assigned to be $k_{ISC} = 1.3 \times 10^8 \text{ s}^{-1}$ as derived from a flavin triplet quantum yield of 0.6 stated by Heelis and Müller for FMN under neutral conditions [139]. Heelis et al. also obtained the rate constants for quenching of the flavin triplet excited state by ascorbic acid as: $k_{Q(T)} = 1.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ at pH 3; and $k_{Q(T)} = 1.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ at pH 7 [108]. The rate constants for decay of the flavin triplet state has been determined by studies of 100 μM FMN only at pH 3 and 7 using the TA arrangement in Chapter 3 (also see Appendix 3): $k_{T(0)} = 3 \times 10^5 \text{ s}^{-1}$ at pH 3; and $k_{T(0)} = 4 \times 10^5 \text{ s}^{-1}$ at pH 7.

Using the parameters above for varying ascorbic acid concentration, the concentration-dependent probability that the radical pair is singlet- or triplet-born can be obtained from $\Phi_{S(RP)}/(\Phi_{S(RP)} + \Phi_{T(RP)})$ and $\Phi_{T(RP)}/(\Phi_{S(RP)} + \Phi_{T(RP)})$, respectively.

For pH 3, the singlet-born RP probability goes from 0.05 to 0.87 upon increasing the ascorbic acid concentration from 2 to 300 mM. At pH 7, the probability increases from 0.11 to 0.94.

Appendix G: PF MARY plots for flavin/ascorbic acid intermolecular RPs at pH 4

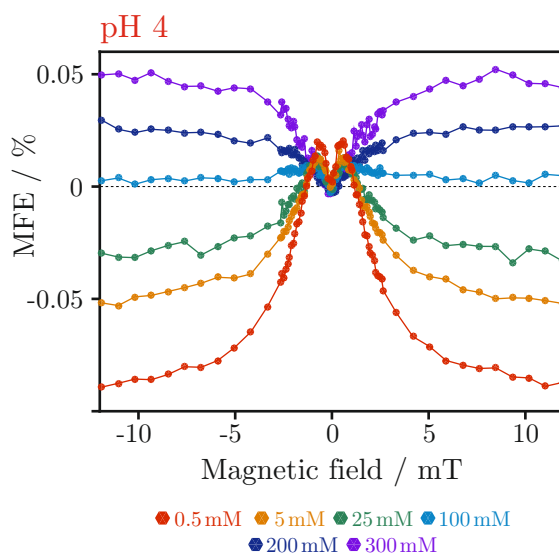


FIGURE 4: PF MARY plots for 10 μ M FMN with AsCH₂ concentration varying from 0.5–300 mM for solutions of pH 4. The pH of solutions were adjusted by the addition of NaOH and HCl; the ionic strength was unregulated for these measurements.

Appendix H: Theoretical calculations for the magnetosensitivity of the flavin/ascorbic acid RP

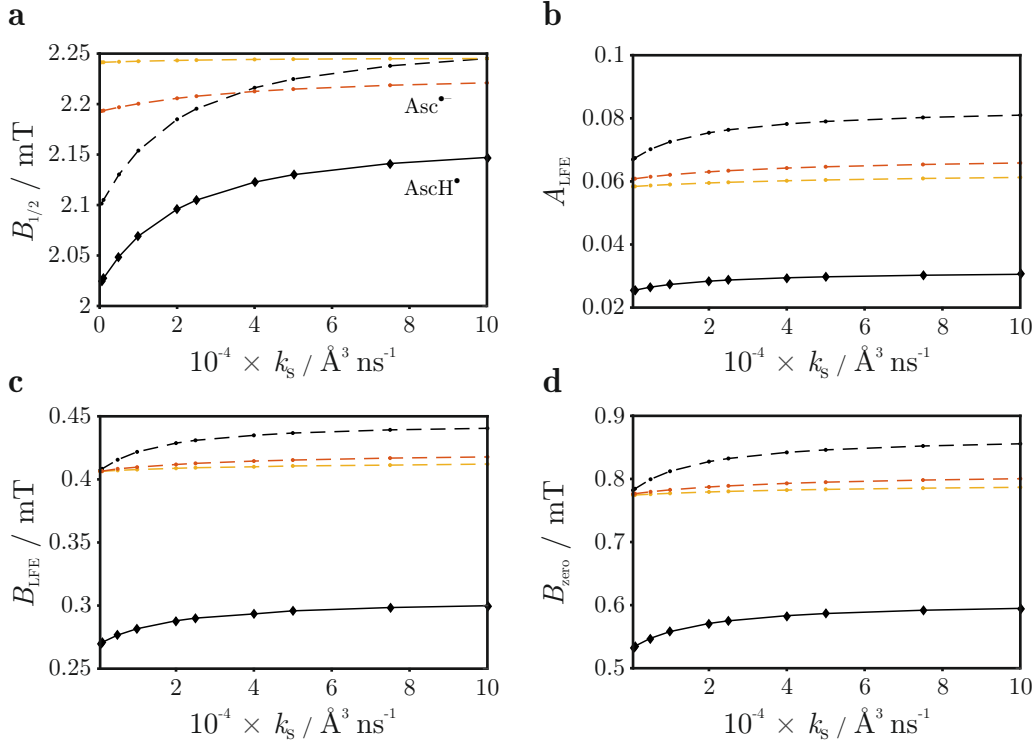


FIGURE 5: Comparison of the characteristic shape parameters for the MFEs arising from the $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$ (dashed lines) and $(\text{FMN}^{\bullet-}\text{AscH}^{\bullet})$ (solid black line) radical pairs. For $(\text{FMN}^{\bullet-}\text{Asc}^{\bullet-})$, the spin dynamics of the radical pair is evaluated with consideration of repulsive Coulomb interactions (yellow: $z_1 z_2 = 3$, orange: $z_1 z_2 = 2$) and the freely diffusing scenario (black: $z_1 z_2 = 0$). Only the latter case is considered for $(\text{FMN}^{\bullet-}\text{AscH}^{\bullet})$ due to the zero net charge of $\text{AscH}^{\bullet}$. The hyperfine parameters used for the calculations are given in Appendix 3. Simulation parameters: $j = 0$, $\tau_{\text{sc}} = 500 \text{ ns}$, $r_c = 7 \text{ \AA}$, $D = 129 \text{ \AA}^2 \text{ ns}^{-1}$, $\sigma = 7 \text{ \AA}$.

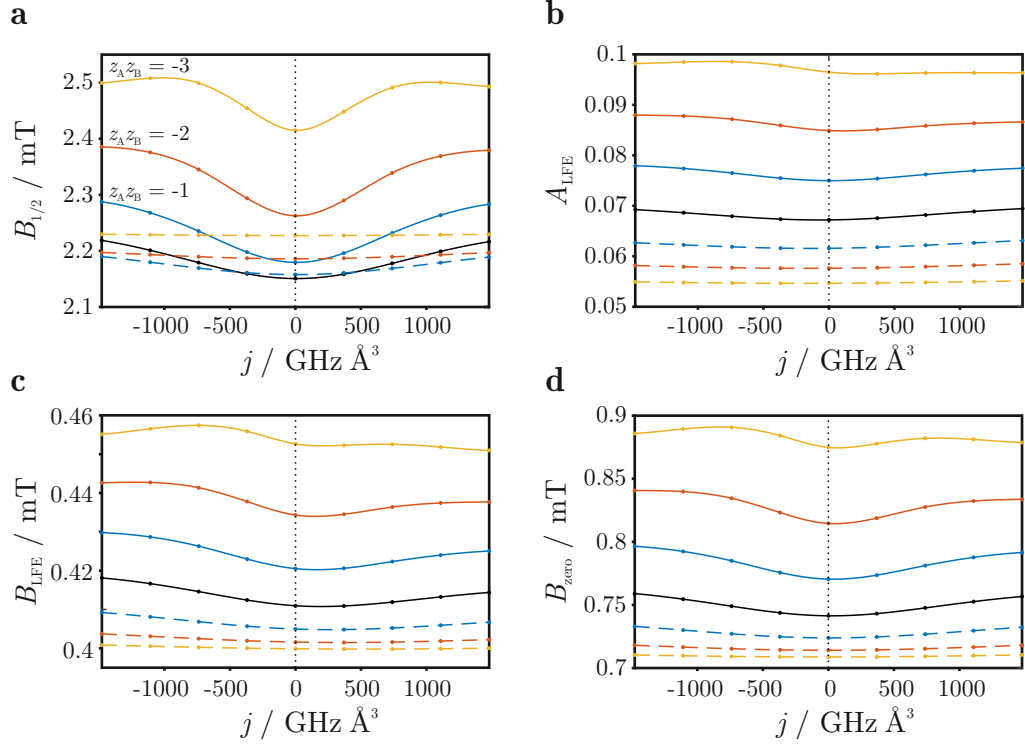


FIGURE 6: Dependence of the MFE shape parameters for the diffusing ($\text{FMNH}^\bullet \text{Asc}^{\bullet -}$)-pair on the strength of the contact exchange interaction and the charges of the radicals. Solid lines correspond to evaluation of the spin dynamics of the radical pair subject to attractive (yellow: $z_1 z_2 = -3$, orange: $z_1 z_2 = -2$, blue: $z_1 z_2 = -1$) or no Coulomb interactions (black); dashed lines profile for repulsive interactions (colours as for the attractive interactions but with the positive sign of $z_1 z_2$). The hyperfine parameters used for the calculations are given in Appendix 3. Additional parameters: $k_S = 1.6 \times 10^4 \text{\AA}^3 \text{ns}^{-1}$, $\tau_{\text{sc}} = 500 \text{ns}$, $r_c = 7 \text{\AA}$, $D = 129 \text{\AA}^2 \text{ns}^{-1}$, $\sigma = 7 \text{\AA}$. The shape parameters are independent of the initial spin configuration.

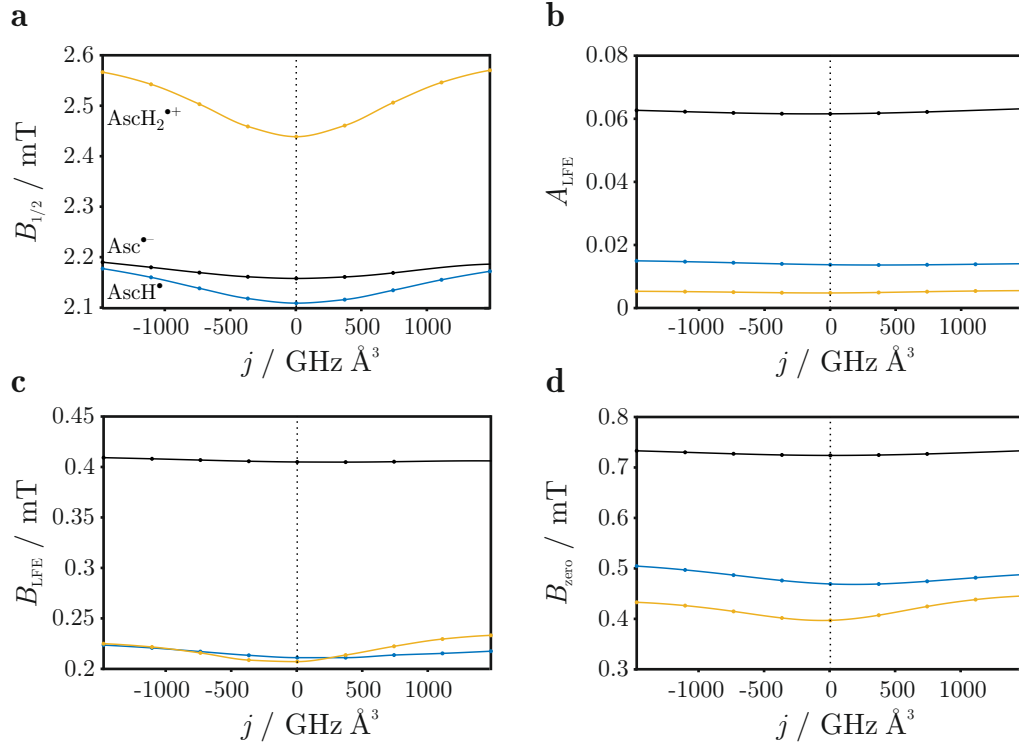


FIGURE 7: Comparison of the j -dependent shape parameters of the magnetic field effect originating from the diffusing radical pairs: (FMNH•Asc•⁻) ($z_1 z_2 = 1$; black lines), (FMNH•AscH•) ($z_1 z_2 = 0$; blue) and (FMNH•AscH₂•⁺) ($z_1 z_2 = -1$; yellow). The charges reflect the equilibrium protonation state of the FMN radical at pH 3. $r_c = 7 \text{ \AA}$, $D = 129 \text{ \AA}^2 \text{ ns}^{-1}$, $\sigma = 7 \text{ \AA}$, $\tau_{\text{sc}} = 500 \text{ ns}$. The hyperfine parameters used for the calculations are given in Appendix 3.

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