

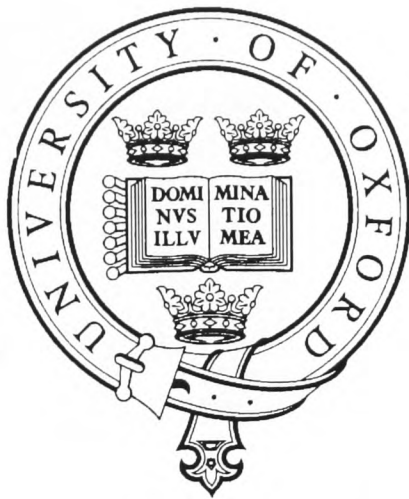


Photo-CIDNP and Protein Folding

A thesis submitted for the degree of
Doctor of Philosophy
by

Charles E. Lyon

Michaelmas Term 1999

Magdalen College
Oxford University



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The work described in this thesis is concerned with the development of new applications of the photo-CIDNP (photochemically induced dynamic nuclear polarization) technique to aspects of protein structure and folding.

Chapters 1 and 2 are introductory chapters; Chapter 1 describes the theoretical basis of the CIDNP phenomenon in terms of the underlying spin chemistry of the radical pair mechanism, while Chapter 2 presents the apparatus, photosensitizer and pulse sequences used, along with some important experimental considerations.

Chapter 3 describes how ^{15}N CIDNP can be used to probe the accessibility of tryptophan side-chains in both native and denatured states of proteins. The polarization of indole nitrogens in uniformly ^{15}N labeled protein is detected in a two-dimensional ^{15}N - ^1H NMR heteronuclear correlation experiment.

Chapter 4 describes two new techniques offering considerable improvements in the quality of photo-CIDNP spectra of proteins. Both focus on the problem of progressive photo-degradation of the flavin dye and in both cases a larger number of scans can be accumulated before the flavin is exhausted than would otherwise be possible.

In Chapter 5, the potential of stopped-flow photo-CIDNP spectroscopy for the study of protein folding is explored. Rapid dilution of denatured protein into a buffer solution is used to initiate a refolding process which is followed using short laser pulses to generate ^1H CIDNP in the side-chains of exposed aromatic residues.

In Chapter 6, the field dependence of amino acid photo-CIDNP intensities is investigated using a stopped-flow CIDNP device that allows sample irradiation over a range of magnetic fields (0.1-7 T) within the bore of a 9.4 T NMR magnet and rapid transfer into the NMR tube for detection.

Finally, in Chapter 7 two photo-CIDNP techniques that probe the exposure of aromatic residues in partially folded states are described. Both involve transfer of polarization to the native state for detection. One approach achieves this kinetically by rapid refolding, and the other involves monitoring exchange cross peaks in a two-dimensional CIDNP spectrum under conditions where the two states are interconverting.

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Chapter 1

Photo-CIDNP and protein folding

The theoretical basis of the chemically induced dynamic nuclear polarization (CIDNP) phenomenon is discussed in terms of the underlying spin chemistry of the so-called radical pair mechanism. Some of the important features of the *cyclic* mechanism involved in photo-CIDNP of amino acids are described, and a brief introduction to protein applications of the photo-CIDNP technique with particular emphasis on protein folding is presented.

1.1 Chemically induced dynamic nuclear polarization

The CIDNP phenomenon is manifested by enhanced emission or absorption in the nuclear magnetic resonance (NMR) signals of products from free radical reactions and was first observed by Bargon and Fischer [1] and independently by Ward and Lawler [2] in 1967. Early theories [3–5] were based on dynamic nuclear polarization (hence the name CIDNP) however, they failed to explain both the magnitude of the detected enhancements, and the occurrence of both emission and absorption lines (the multiplet effect) in many polarized spectra. An alternative explanation that could account for these experimental observations was therefore proposed by Closs [6] and independently by Kaptein and Oosterhoff [7] in 1969 which placed the emphasis on the ability of magnetic nuclei to alter the electronic spin state of a radical pair and hence modulate its reactivity. This theoretical approach subsequently became known as the radical pair mechanism and has led to the application of CIDNP to mechanistic problems in organic free radical chemistry [8]. Of particular importance in the context of this thesis was the development by Kaptein [9, 10] in 1978 of a photo-CIDNP technique in which polarization is generated in reversible photochemical reactions between an excited dye and certain aromatic amino acids on the surface of a protein. The technique has since been exploited for the structural infor-

mation it provides on protein surfaces [11, 12] and the aim of the work described in this thesis has been to examine and develop applications of the technique to aspects of protein folding.

Before addressing the radical pair theory itself, the spin chemistry of radical pairs will be described and the nuclear spin dependence of the mixing between singlet and triplet electronic states that lies at the heart of the radical pair mechanism will be discussed in terms of the vector model.

1.1.1 The spin chemistry of radical pairs

Radical pairs can be classified according to their electronic spin state. Radical pairs that result from the random encounter of two free radicals in solution will have electron spins oriented in a random manner and are known as uncorrelated radical pairs. Radical pairs formed thermally or photochemically from a molecular precursor, under conservation of the total spin angular momentum, will have electron spins mutually oriented in a given way and are known as spin-correlated radical pairs. The total electron spin quantum number S is obtained by coupling the individual electron spin quantum numbers s_1 and s_2 from the two component radicals using the Clebsch-Gordan series:

$$S = |s_1 - s_2|, |s_1 - s_2| + 1, \dots, s_1 + s_2 = 0, 1 \text{ when } s_1 = s_2 = 1/2 \quad (1.1)$$

Hence if the electron spins are anti-parallel, the radical pair has a total spin quantum number $S=0$ resulting in a *singlet* state with overall magnetic quantum number $M_S=0$ labeled S. If the electron spins are parallel, the radical pair has a total spin quantum number $S=1$ resulting in three *triplet* states with overall magnetic quantum numbers $M_S = -1, 0, 1$ labeled T_-, T_0 and T_+ respectively. The spin parts of the singlet and triplet electronic wavefunctions of a radical pair may be written:

$$\begin{aligned} S &= \frac{1}{\sqrt{2}}(\alpha_1\beta_2 - \beta_1\alpha_2) \\ T_- &= \beta_1\beta_2 \\ T_0 &= \frac{1}{\sqrt{2}}(\alpha_1\beta_2 + \beta_1\alpha_2) \\ T_+ &= \alpha_1\alpha_2 \end{aligned} \quad (1.2)$$

where α and β denote respectively the $m_S=+1/2$ and $-1/2$ states of the electrons on the component radicals. In the liquid state the radical pair and its component radicals tumble rapidly ($\sim 10^{11}$ Hz for free amino acids and $\sim 10^9$ Hz for proteins), anisotropic terms of the spin Hamiltonian are therefore averaged to zero and the spin Hamiltonian required to describe a radical pair needs to contain only isotropic terms. In particular, the electron Zeeman interaction $\hat{H}_{S.B_0}$, the Heisenberg or exchange

interaction $\hat{H}_{S,S}$ and the isotropic component of the hyperfine interaction $\hat{H}_{I,S}$ need to be included:

$$\hat{H} = \hat{H}_{S,B_0} + \hat{H}_{S,S} + \hat{H}_{I,S} \quad (1.3)$$

the spin Hamiltonian can therefore be written (in angular frequency units) as:

$$\hat{H} = (g_1 \hat{S}_{1z} + g_2 \hat{S}_{2z}) B_0 \frac{\mu_B}{\hbar} - J(r) \left(\frac{1}{2} + 2\hat{S}_1 \cdot \hat{S}_2 \right) + \sum_i A_i \hat{I}_i \cdot \hat{S}_1 + \sum_j A_j \hat{I}_j \cdot \hat{S}_2 \quad (1.4)$$

where, g_1 and g_2 are the g -factors of radical 1 and 2; $\hat{S}_{1z}$ and $\hat{S}_{2z}$ are the z -components of the electron spin angular momentum operators $\hat{S}_1$ and $\hat{S}_2$ of the unpaired electron on radical 1 and 2; B_0 is the magnetic field strength; μ_B is the Bohr magneton of the free electron; $\hbar = h/2\pi$ where h is the Planck constant; $J(r)$ represents the magnitude of the distance dependent exchange interaction; A_i and A_j are hyperfine coupling (hfc) constants of nuclei i and j on radical 1 and 2, respectively; and $\hat{I}_i$ and $\hat{I}_j$ are nuclear spin angular momentum operators of nuclei i and j on radical 1 and 2 respectively. $J(r)$ is usually assumed to decrease exponentially with increasing electron separation r , and can normally be neglected for separations greater than 1 nm. The representation of the spin Hamiltonian in the S, T₋, T₀ and T₊ basis set is therefore given (in angular frequency units) by:

$$\begin{pmatrix} |T_+\rangle & |S\rangle & |T_0\rangle & |T_-\rangle \\ \omega - J(r) & 0 & 0 & 0 \\ 0 & J(r) & Q & 0 \\ 0 & Q & -J(r) & 0 \\ 0 & 0 & 0 & -\omega - J(r) \end{pmatrix} \quad (1.5)$$

with

$$\omega = \frac{1}{2}(\omega_1 + \omega_2), \quad Q = \frac{1}{2}(\omega_1 - \omega_2), \quad (1.6)$$

and

$$\omega_1 = g_1 B_0 \frac{\mu_B}{\hbar} + \sum_i A_i m_i, \quad \omega_2 = g_2 B_0 \frac{\mu_B}{\hbar} + \sum_j A_j m_j \quad (1.7)$$

where m_i and m_j are the magnetic quantum numbers of nuclei i and j on radical 1 and 2, respectively. In the absence of an applied magnetic field and any magnetic nuclei, the three triplet levels are degenerate at all separations, whilst the singlet and triplet levels only become degenerate at large separations when the exchange interaction is negligible. In the presence of an applied magnetic field the degeneracy of the triplet states is lifted. This is illustrated in Fig.1.1 where the energies of the singlet and triplet states at a given applied magnetic field strength in the absence of any magnetic nuclei are shown as a function of the electron separation r . When the interactions mixing electronic states are much weaker than the energy difference separating them, the extent of mixing between states is amongst other things inversely proportional to this energy difference and hence, in the presence of strong

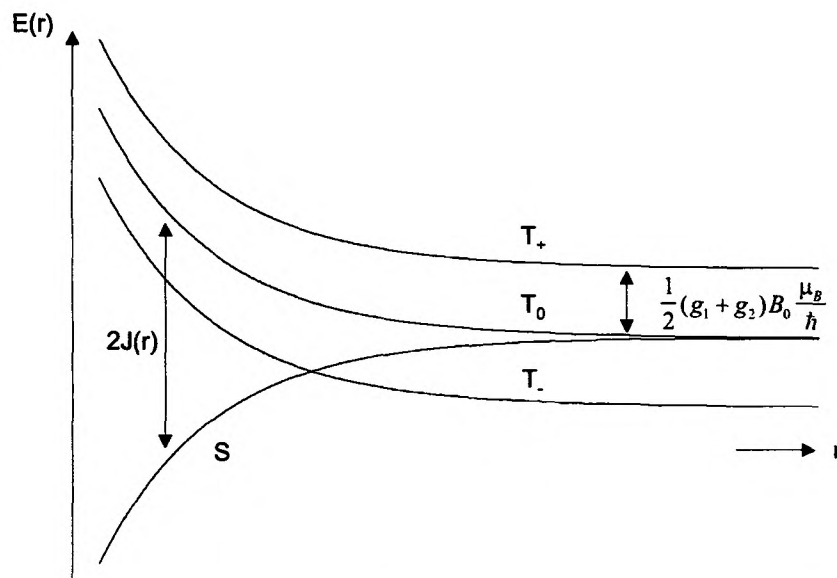


Figure 1.1: Energies of singlet and triplet states of a radical pair in a given magnetic field as a function of the electron separation r .

magnetic fields (> 0.1 T), the T_- and T_+ states are so far removed from the singlet state that mixing is restricted to the S and T_0 states and even then can only occur at sufficiently large electron separations.

Vector model of S- T_0 mixing

The mixing between S and T_0 states is most simply described using a vector model. Within such a semiclassical picture the magnetic moments associated with the electron spins s_1 and s_2 of the component radicals of a radical pair (say $R_1^{\bullet-}$ and $R_2^{\bullet+}$) can be represented by vectors precessing about the direction of the applied magnetic field B_0 as shown in Fig.1.2. In this representation the S and T_0 states differ sim-

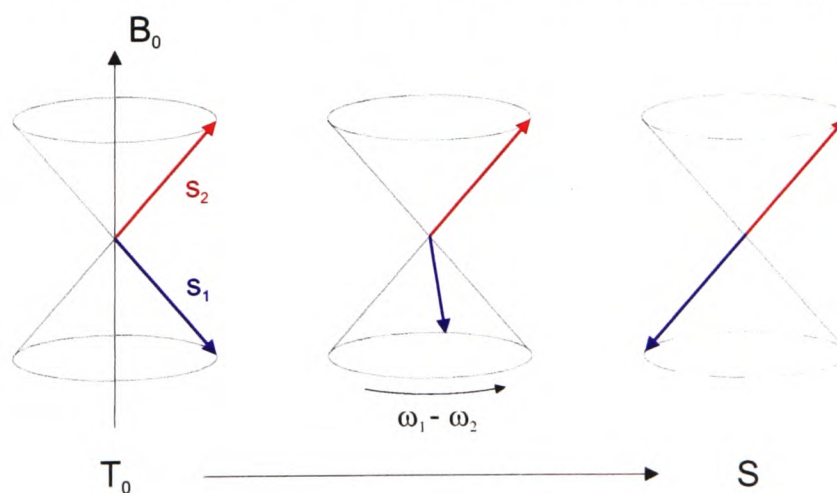


Figure 1.2: Vector model for S- T_0 mixing.

ply in the phase difference between the two vectors s_1 and s_2 , and the precession frequencies of the two vectors are given respectively by ω_1 and ω_2 of Eqn.1.7. As shown schematically in Fig.1.2, conversion between S and T_0 states will therefore occur at a frequency determined by the difference in precession frequencies $\omega_1 - \omega_2$. In the absence of any hyperfine interactions, ω_1 and ω_2 are determined solely by the

g -factors of the two radicals (Eqn.1.7) and hence, S-T₀ mixing is then simply driven by the Zeeman interaction.

Nuclear spin dependence of S-T₀ mixing

The effect of adding a single proton with a positive hyperfine coupling constant on one of the radicals is illustrated in Fig.1.3. The electron spin vector s_1 representing

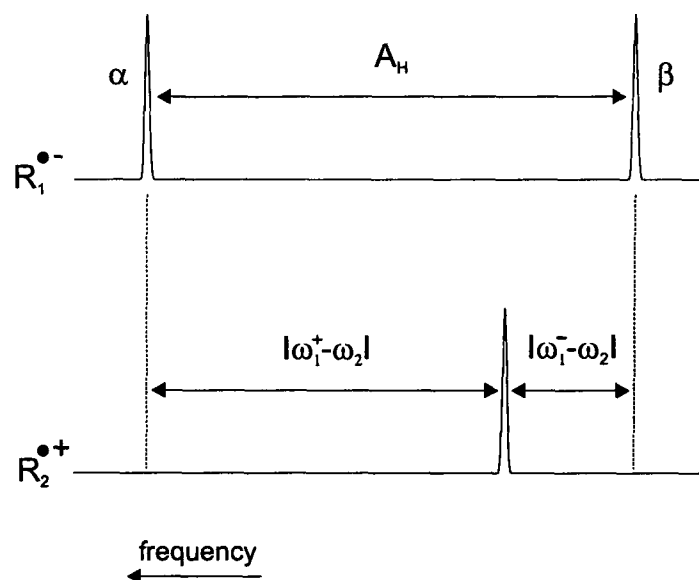


Figure 1.3: Theoretical EPR spectra of the free radicals $R_1^{\bullet-}$ and $R_2^{\bullet+}$ in the presence of a single proton with hyperfine coupling constant $A_H > 0$ on $R_1^{\bullet-}$. α and β denote respectively the $m_I = +\frac{1}{2}$ and $-\frac{1}{2}$ spin states of the proton on $R_1^{\bullet-}$.

$R_1^{\bullet-}$ now has two components (s_1^+ and s_1^-) whose precession rates depend on the orientation of the nuclear spin. From Eqn.1.7, the EPR resonance frequencies are:

$$\omega_1^{\pm} = g_1 B_0 \frac{\mu_B}{\hbar} \pm \frac{1}{2} A_H, \quad \omega_2 = g_2 B_0 \frac{\mu_B}{\hbar} \quad (1.8)$$

in which the $+$ and $-$ signs denote the α and β nuclear spin states respectively. The difference in precession rates for the two pairs of vectors (i.e. s_1^+, s_2 and s_1^-, s_2) is therefore given by:

$$\Delta\omega^{\pm} = \omega_1^{\pm} - \omega_2 = (g_1 - g_2) B_0 \frac{\mu_B}{\hbar} \pm \frac{1}{2} A_H \quad (1.9)$$

hence, as illustrated in Fig.1.3 the rate of S-T₀ mixing will now depend on the nuclear spin state and for $\Delta g > 0$, $A_H > 0$, will be greatest when the proton on $R_1^{\bullet-}$ is in the α state. In general, the rate of S-T₀ mixing will be greatest for the α state when Δg and A_H are of the same sign and for the β state when they are of opposite sign.

1.1.2 The radical pair mechanism

In order to understand how the nuclear spin dependence of S-T₀ mixing leads to the CIDNP phenomenon, consider the reaction scheme in Fig.1.4. A triplet spin-correlated radical pair is initially formed by an electron abstraction reaction between

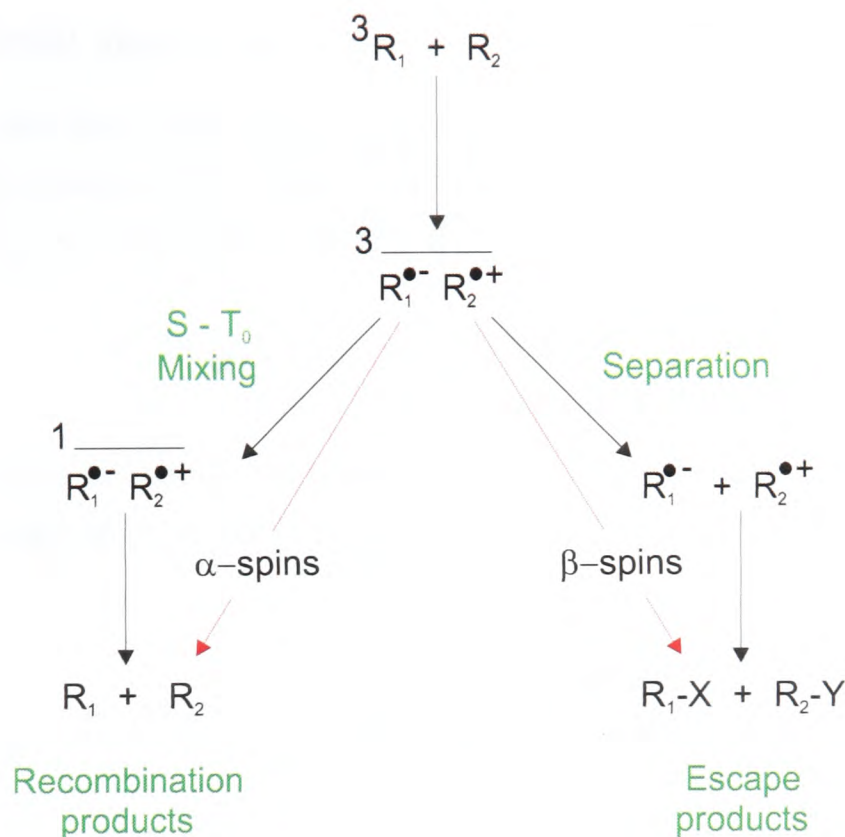


Figure 1.4: A schematic of the radical pair mechanism. S-T₀ mixing is faster when the proton on $R_1^{\bullet-}$ is in the α state (Fig.1.3) and hence a nuclear spin sorting process occurs. The overbar is used to indicate a spin-correlated radical pair.

the triplet excited R_1 and ground state R_2 . The triplet radical pair then has two possible fates. The constituent radicals can either diffuse apart and get scavenged at a later stage to form escape products, here R_1-X and R_2-Y or react via back electron transfer to give recombination products (i.e. the original R_1 and R_2). The recombination probability of a radical pair depends strongly on the electronic spin state of the pair, with reaction usually only possible from a singlet state. Hence as shown in Fig.1.4 the triplet radical pair must first convert to a singlet state in order to proceed via the recombination route. If singlet-triplet mixing (i.e. S-T₀ mixing) is faster when a proton on $R_1^{\bullet-}$ is in the α state as shown in Fig.1.3 then a nuclear spin sorting process occurs with triplet radical pairs containing a spin- α proton more likely to cross over to a singlet state, and then recombine and triplet pairs possessing a spin- β proton having a greater probability of diffusing apart, and ending up as escape products. As a consequence of this nuclear spin selective chemistry, recombination products will contain an excess of spin- α protons and hence an absorptive NMR enhancement and conversely escape products will possess an excess of spin- β protons and therefore an emissive NMR enhancement.

In an analogous manner, when S-T₀ mixing is faster for radical pairs possessing spin- β protons (i.e. Δg and A_H of opposite sign, Fig.1.3) or for an initial radical pair formed from a singlet precursor, the situation is simply reversed with emission in recombination products and absorption in escape products.

Kaptein's CIDNP sign rules

These observations have been conveniently summarized in a multiplicative sign rule [13] in which the phase of the enhancement (i.e. absorption or emission) for a given nucleus i is given by the product of four signs:

$$\Gamma_{net}(i) = \mu \cdot \epsilon \cdot \Delta g \cdot A_i = \begin{cases} + & A \\ - & E \end{cases} \quad (1.10)$$

where A_i is the sign of the hfc for nucleus i ; Δg is the sign of $g_1 - g_2$ where g_1 is the g factor of the radical carrying nucleus i ; and the other two quantities are defined by:

$$\begin{aligned} \mu &= \begin{cases} + & \text{for a triplet precursor} \\ - & \text{for a singlet precursor} \end{cases} \\ \epsilon &= \begin{cases} + & \text{for recombination products} \\ - & \text{for escape products} \end{cases} \end{aligned} \quad (1.11)$$

Note that this sign rule only applies for CIDNP spectra recorded at high field where the hyperfine interactions are small in comparison to $\Delta g B_0 \mu_B / \hbar$ and therefore every multiplet component for a given nucleus has the same polarization. This type of CIDNP is known as the *net effect*. At fields much lower than those used in the majority of this thesis or for small Δg values, a *multiplet effect*, in which simultaneous emission and absorption occurs within the multiplet of a given nucleus, is often observed in conjunction with the *net effect* and will be described in slightly more detail in Chapter 6.

The concept of secondary recombination

The discussion has so far largely ignored both the dynamic nature of radical pairs and the effects of the distance dependent exchange interaction. As was described in Section 1.1.1 and was shown schematically in Fig.1.1, the S and T₀ states of a radical pair only become degenerate once the component radicals have separated by a few nm and hence S-T₀ mixing is precluded within the initially formed primary pair. For recombination to occur from a triplet precursor, the component radicals must therefore first diffuse apart (typically only a few molecular diameters) before re-encountering to form a so-called secondary radical pair. Re-encounter times (10⁻¹⁰-10⁻⁷ s) are such that singlet-triplet mixing (10⁻⁹-10⁻⁸ s) can take place while the radicals are separated and experience a negligible exchange interaction. In Chapter 6, the relative timescales of the spin dynamics and the motional behaviour of the component radicals will be described in greater detail within the quantitative framework of the diffusion model [14–16].

It is also worth noting at this stage that electron spin relaxation times are typically of the order of 10^{-6} s and hence loss of spin correlation between the two radicals of the pair does not interfere with the somewhat faster processes involved in generating CIDNP. In addition, once CIDNP has been generated within the *diamagnetic* products of the radical pair reactions the window for observation is significantly extended through slow nuclear spin relaxation (10^{-1} -10 s).

1.2 Photo-CIDNP of amino acids

The basic photo-CIDNP experiment as applied to amino acids or proteins involves adding a small amount of a photosensitizer (e.g. a flavin dye) to an amino acid or protein sample [10–12]. Spectra are then recorded *with* and *without* prior irradiation of the sample on an NMR spectrometer that has been specially adapted to allow light from a suitable light source (e.g. an argon ion laser) to pass into the sample within the probe (the experimental considerations will be discussed in greater detail in Chapter 2). As shown in Fig.1.5 the photoexcited sensitizer then reacts reversibly with certain amino acid residues to generate polarization that is most easily detected by subtracting the spectrum obtained without prior laser irradiation from the spectrum obtained with prior laser irradiation. This reaction scheme is essen-

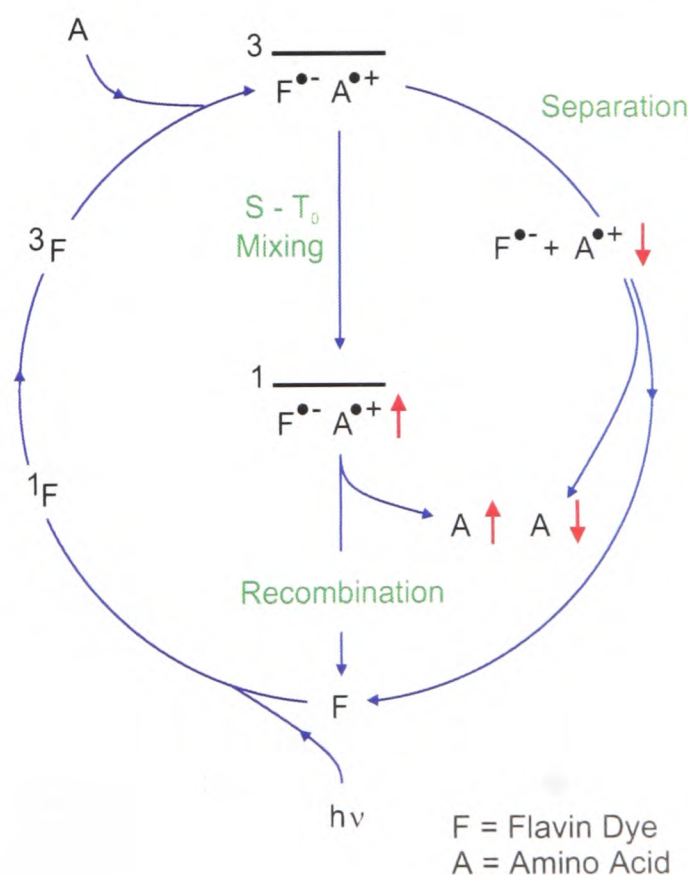


Figure 1.5: A schematic of the *cyclic* radical pair mechanism involved in the production of photo-CIDNP in proteins. An electron transfer mechanism is shown; a similar set of reactions take place if the initial step is hydrogen abstraction. Recombination and escape products are identical and hence the net polarization depends on the amount lost by nuclear spin-lattice relaxation in the longer lived escape radicals.

tially identical to that described in Section 1.1.2, except that unlike in Fig.1.4 where recombination and escape products were physically different (e.g. R_1 and $R_1\text{-X}$), amino acid radicals that escape from the radical pair are no longer scavenged, but react with independently formed dye radicals to regenerate the original amino acid and the flavin in its ground state. The cyclic nature of the mechanism is vital in ensuring that when applied to proteins, polarization is observed in the intact protein rather than in a chemically modified form, however it also introduces a number of new features to the radical pair mechanism that will be discussed in the following section.

1.2.1 Recombination cancellation

When recombination and escape products are identical one would expect cancellation of the equal and opposite polarizations that proceed via the recombination and escape routes, and one might therefore be inclined to wonder how CIDNP can possibly be generated within such cyclic reaction schemes. Fortunately, *complete* cancellation does not occur because product formation via the escape route tends to be slower than via the recombination route (typically 10^{-4} vs. 10^{-7} s), and hence polarization in the longer lived escaped radicals decays to a greater extent via nuclear spin relaxation [17]. As a consequence, the *net* recombination polarization is observed experimentally and its magnitude depends sensitively on the relative timescales of nuclear spin relaxation (typically 10^{-4} s) and the lifetime of escaped radicals. Nuclear spin relaxation in free radicals occurs predominantly via the dipole coupling to the unpaired electron and hence, relaxation rates are related to the electron spin density at the nucleus. If the lifetimes of escaped radicals are short, nuclei with large hyperfine coupling constants will therefore lose less recombination polarization through cancellation than nuclei with small hyperfine interactions [18].

1.2.2 Exchange cancellation

A second cancellation route in cyclic reactions arises if the escaped radicals are able to undergo rapid degenerate electron transfer reactions with their diamagnetic counterparts:



Exchange effectively shortens the lifetime of escaped radicals and therefore competes with nuclear spin relaxation:



The CIDNP intensity for a given proton on amino acid A obtained in the presence of exchange is therefore given by:

$$I_A = p_A \frac{T_{1A}^{-1}}{k_A^{ex}[A] + T_{1A}^{-1}} \quad (1.14)$$

where p_A is the polarization generated per radical pair in the absence of exchange; k_A^{ex} is the rate constant for electron exchange; and T_{1A} is the nuclear spin-lattice relaxation time in the escaped radical. The rate constant k_A^{ex} is typically of the order of 10^7 - 10^8 mol⁻¹ dm³ s⁻¹ [19, 20] and hence for concentrations of A in the range 10^{-3} - 10^{-2} mol dm⁻³ exchange will occur on a timescale (10^{-6} - 10^{-4} s) that competes efficiently with nuclear spin relaxation (10^{-4} s). It is worth noting that the equivalent hydrogen atom transfer of the protonated radical:



is much slower (10^5 - 10^6 mol⁻¹ dm³ s⁻¹); hence CIDNP intensities for amino acids may vary considerably with pH [12, 19].

The large size of proteins makes translational diffusion slow thereby reducing both the recombination and exchange rates. Nuclear spin-lattice relaxation rates on the other hand are broadly similar and as a consequence, whilst both recombination and exchange cancellation play an important role in determining the CIDNP intensities of polarized nuclei within *free* amino acids, they play only a relatively minor role within the bulkier proteins [12].

1.2.3 Amino acids

When using one of the flavin dyes as a photosensitizer, only three of the twenty commonly occurring amino acids can normally be polarized, namely tyrosine, histidine and tryptophan (methionine also reacts with flavins but exhibits very weak CIDNP at high magnetic fields and will therefore be discussed separately in Chapter 6). In the following sections, the photo-CIDNP spectra of these three “CIDNP active” aromatic amino acids obtained with flavin mononucleotide (FMN) will be presented and the reaction mechanisms involved in generating radical pairs discussed.

Tyrosine

Figure 1.6 compares the NMR and photo-CIDNP spectra obtained for tyrosine using FMN as photosensitizer and an argon ion laser as light source (Chapter 2). A strong emissive enhancement is observed for the 3,5 protons along with weaker absorptive enhancements for the 2,6 and β -CH₂ protons. The lack of polarization for the α -CH proton suggests that it is too far removed from the unpaired electron spin density on the ring of the tyrosine radical to experience an appreciable hyperfine interaction. The reaction between tyrosine and the excited triplet state of the flavin is thought to occur via transfer of the phenolic hydrogen atom to give a neutral pair comprising a tyrosyl radical ($g=2.0041$ [21]) and a flavosemiquinone radical ($g=2.0030$ [22]):



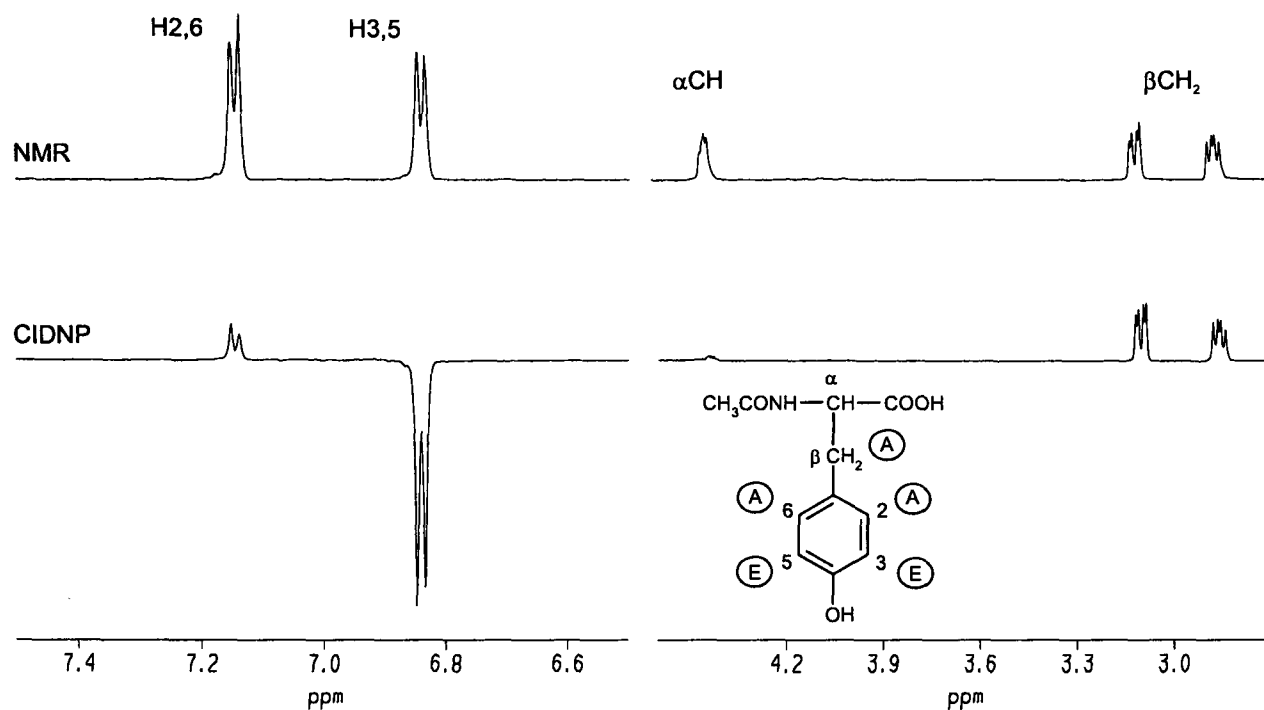


Figure 1.6: A comparison of the 600 MHz ^1H NMR and photo-CIDNP spectra of tyrosine (4 mM, pH 4.5). Both spectra were averaged over 16 scans and the photo-CIDNP spectrum was obtained with 100 ms 4 W laser flashes.

The hyperfine coupling constants for the tyrosyl radical have been measured [21] and are $A_{3,5} = -6.15$ G, $A_{2,6} = +1.5$ G and $A_{\text{CH}_2} = +7.7$ G. Application of the CIDNP sign rule (Eqn.1.10) to the 3,5 protons gives:

$$\Gamma_{\text{net}}(3, 5) = \mu \cdot \epsilon \cdot \Delta g \cdot A_{3,5} = + \cdot + \cdot + \cdot - = - (E) \quad (1.17)$$

confirming the involvement of the triplet state of the flavin. Evidence for a hydrogen abstraction mechanism is based on a number of experimental observations [12] most notably the lack of CIDNP in O-methyltyrosine in which the abstractable phenolic hydrogen has been replaced by a methyl group [11].

Histidine

Under similar experimental considerations, histidine (Fig.1.7) shows absorptive enhancements for the ring 2 and 4 protons and emission for the β -CH₂ protons. A hydrogen abstraction mechanism similar to that for tyrosine is believed to occur and is supported by similar experimental evidence to that just described for tyrosine [12]. The polarizations are opposite to those observed for tyrosine suggesting that $\Delta g < 0$ and hence that the g -value of the neutral histidine radical is lower than that of the flavosemiquinone radical ($g=2.0030$).

Tryptophan

As shown in Fig.1.8, tryptophan exhibits absorptive enhancements for the ring 2, 4 and 6 protons and emission for the β -CH₂ protons. In contrast to both tyrosine and histidine, reaction between excited flavin and tryptophan is thought to proceed via an electron abstraction mechanism to give a radical ion pair comprising a tryptophan

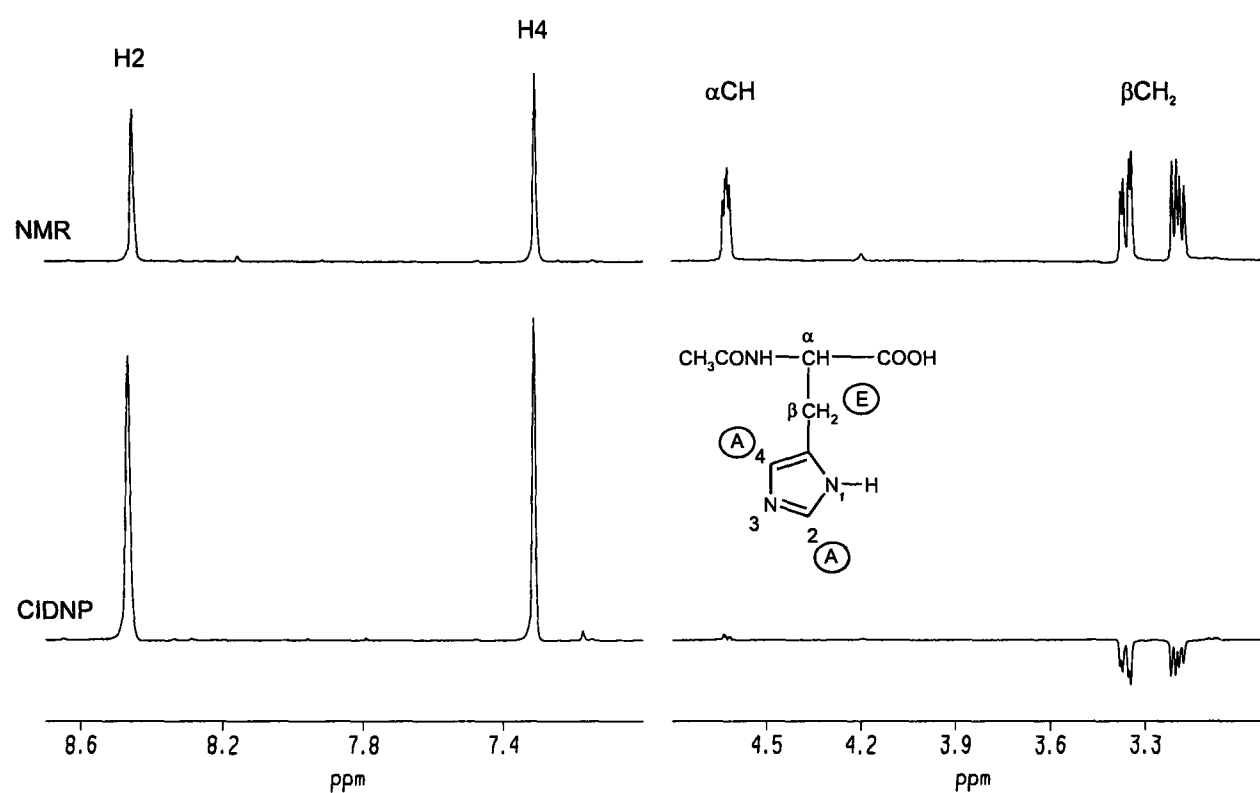


Figure 1.7: A comparison of the 600 MHz ^1H NMR and photo-CIDNP spectra of histidine (10 mM, pH 7). Both spectra were averaged over 16 scans and the photo-CIDNP spectrum was obtained with 100 ms 4 W laser flashes.

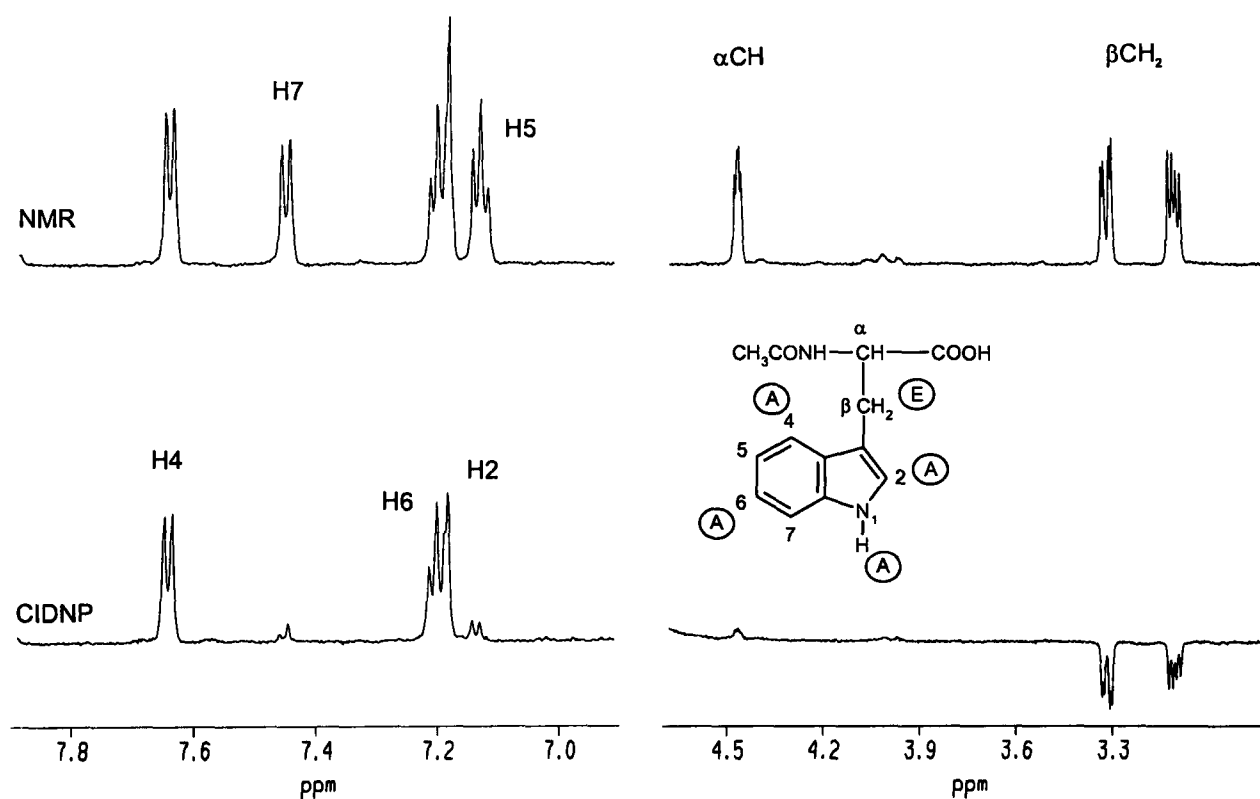


Figure 1.8: A comparison of the 600 MHz ^1H NMR and photo-CIDNP spectra of tryptophan (3 mM, pH 8). Both spectra were averaged over 16 scans and the photo-CIDNP spectrum was obtained with 100 ms 4 W laser flashes.

cation radical and a flavosemiquinone anion ($g=2.0034$ [23]):



The polarizations are again opposite to those observed for tyrosine suggesting that $\Delta g < 0$ and hence that the g -value of the tryptophan radical cation is lower than that of the flavosemiquinone anion ($g=2.0034$). The lack of polarization for both the 5 and 7 ring protons suggests negligible spin densities at these positions in the radical cation. Evidence for an electron abstraction mechanism is based amongst other things [12], on the observation of CIDNP in 1-N-methyltryptophan [11, 24] and on the absorptive enhancement observed for the indole NH proton in both H_2O [25] and dimethylsulphoxide [24] that is consistent with its position on a tryptophan radical cation formed by electron transfer ($\Delta g < 0$, $A_{\text{NH}} < 0$).

1.2.4 Cross-polarization

The photo-CIDNP spectra shown in Fig.1.6, 1.7 and 1.8 for tyrosine, histidine and tryptophan respectively exhibit enhanced absorption and emission for nuclei that experience hyperfine interactions within their radical pair precursors. Dipolar cross-relaxation (i.e. the nuclear Overhauser effect or NOE [26]) can transfer these direct polarizations to other nuclei in the molecule [18, 27, 28]. By analogy with the NOE, the extent of transfer depends on both the internuclear distances and the rates of molecular tumbling [18, 26]. Hence whilst for rapidly tumbling molecules (e.g. free amino acids) transfer is more likely to occur via $\Delta m_I = \pm 2$ relaxation processes and hence with inversion of phase (e.g. emission to absorption), for the slower tumbling proteins, transfer tends to occur via $\Delta m_I = 0$ relaxation processes and hence with retention of phase. The principal intraresidue cross-relaxation pathways observed for the three amino acids have been illustrated for reference in Fig.1.9. Note that it is

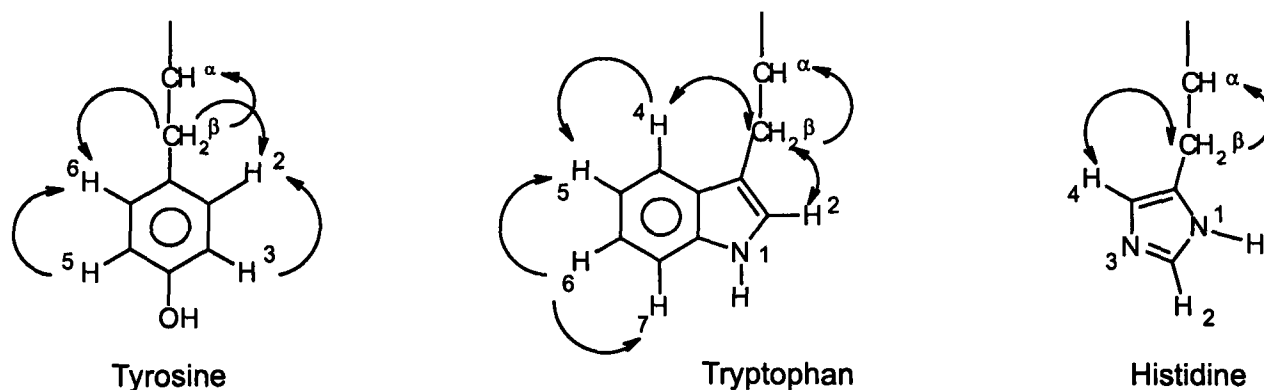


Figure 1.9: Cross-relaxation pathways in tyrosine, tryptophan and histidine sidechains. Note that transfer of polarization occurs with inversion of phase in free amino acids and with retention of phase in proteins.

usually possible to distinguish directly and indirectly polarized peaks by comparing the time dependence of CIDNP, as enhancements due to cross-polarization tend to

increase with both the length of the laser flash used to generate CIDNP and the delay separating the generation and detection of polarization [18].

1.2.5 Competition effects

When more than one side-chain is able to react with excited flavin dye (e.g. a binary mixture of free histidine and tryptophan amino acids), the CIDNP intensities observed for either amino acid type are likely to depend sensitively on their ability to compete for the small concentrations of excited triplet flavin:



In addition, triplet flavins are also quenched by fluorescence and/or reaction with O_2 :



and hence overall, the CIDNP intensity of a proton in an amino acid A in a binary mixture of amino acids A and B in the absence of degenerate exchange (Section 1.2.2, Eqn.1.14) is determined by a competition between these three processes:

$$I_A = p_A \frac{k_A[A]}{k_q + k_A[A] + k_B[B]} \quad (1.22)$$

where p_A is the polarization produced per radical pair in the absence of any competing mechanisms; k_A and k_B are the second order rate constants for reaction of A and B with ${}^3\text{F}$ respectively; and k_q is the (pseudo-) first order rate constant for decay of ${}^3\text{F}$ by fluorescence and/or quenching by molecular oxygen. Second order rate constants for reaction of the three amino acids with excited flavin are of the order of 10^8 - $10^9 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ [29, 30] and hence with amino acid concentrations in the range 10^{-3} - $10^{-2} \text{ mol dm}^{-3}$ reaction occurs on a timescale (10^{-7} - 10^{-5} s) that competes efficiently with quenching via fluorescence and/or by molecular oxygen (10^{-6} s [19]).

The relative magnitudes of the second order rate constants for all three aromatic amino acids have been estimated by both transient absorption [29] and binary photo-CIDNP competition experiments [30]. In both cases, the order of reaction rate constants was found to be $k_{Trp} > k_{Tyr} \gg k_{His}$. As a consequence great care must be taken in interpreting CIDNP intensities in particular for histidine under conditions where changes in the relative accessibility and hence reactivity of amino acid residues other than the one under observation are likely to occur (e.g. in protein unfolding).

1.3 Photo-CIDNP as a probe of protein surfaces

For a given amino acid residue to exhibit a CIDNP enhancement it must be able to react with excited flavin molecules and when applied to proteins, the photo-CIDNP technique can therefore be used to probe the surface exposure of tryptophan, tyrosine and histidine residues [9–12]. To illustrate this, Fig.1.10 compares the NMR

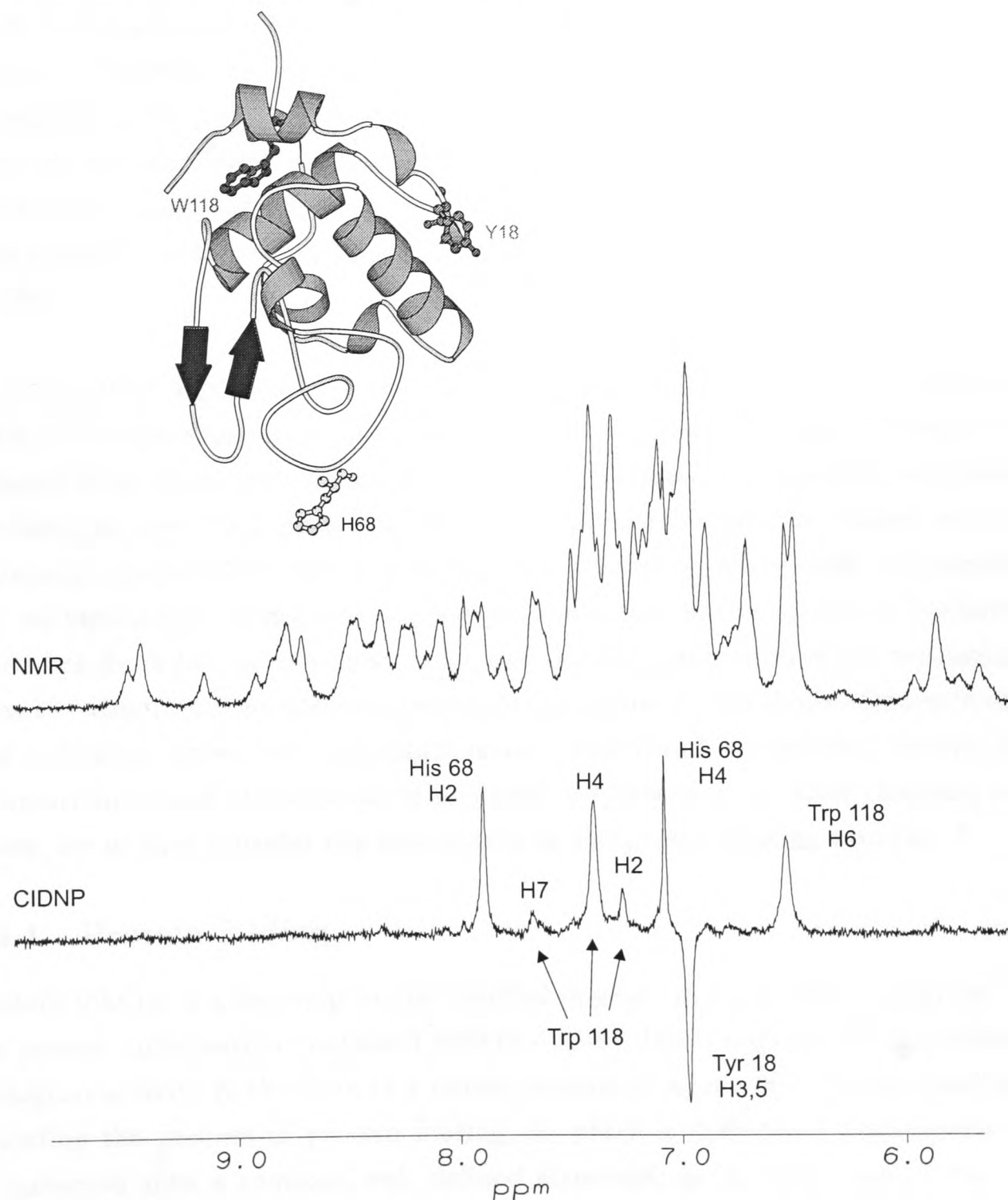


Figure 1.10: A comparison of the 600 MHz ^1H NMR and photo-CIDNP spectra of the protein bovine α -lactalbumin in its native state (1.5 mM, pH 7). The NMR spectrum was averaged over 64 scans whilst the photo-CIDNP spectrum was obtained with 16 laser flashes (100 ms, 4 W). The inset shown the crystal structure of bovine α -lactalbumin and the three polarized aromatic residues.

and photo-CIDNP spectra of the protein bovine α -lactalbumin in its native state. Of the four tryptophans, four tyrosines and three histidines only three residues are polarized, namely Trp 118, Tyr 18 and His 68 [31]. The protein amino acid spectra

are essentially the same as for the free amino acids except for small chemical shifts due to differences in chemical environment within the folded protein. The crystal structure of bovine α -lactalbumin is also shown and confirms that the polarized residues lie on the surface of the native protein structure.

The ability to probe the surface of a protein in such a site specific manner has led to the application of the photo-CIDNP technique in a variety of contexts, the majority of which have been centered around monitoring changes in the accessibility of residues in the native state caused by the addition of some form of surface perturbant [11, 12]. Examples include protein-ligand, protein-micelle, protein-nucleic acid and protein-protein interactions and in this context, the technique has most recently been applied to the protein-oligosaccharide interactions found within glycoproteins [32–36].

An alternative application of the photo-CIDNP technique that has received relatively little attention, involves monitoring the potentially dramatic changes in the accessibilities of the side chains of tryptophan, tyrosine and histidine residues that are likely to occur as a protein unfolds from its native state [12]. Based on such an approach, photo-CIDNP has the potential of providing site specific information on the conformations adopted by a given protein as the native structure is disrupted. The work described in this thesis is focused on this application of the technique and aims to extend and develop experimental approaches to the study of protein folding and unfolding under both equilibrium and “real-time” conditions. Before briefly summarizing some of the applications that are described in later chapters of this thesis, let us first consider the importance of the protein folding process.

1.3.1 Protein folding

Protein folding is a key step in the “central dogma” of molecular biology by which the genetic information contained within a given DNA sequence is converted into biological activity in the form of a native protein structure [37]. Understanding and modeling the process of protein folding, in which a disordered polypeptide chain is converted into a compact well defined structure, is therefore one of the major challenges of modern structural biology, and as of yet remains unsolved. A greater understanding of the complex relationship between the amino acid sequence and the formation of tertiary structure (i.e. the so-called “protein folding problem”) would not only open up the possibility of genetically designing specific proteins it would also aid in our understanding of diseases associated with protein misfolding such as Alzheimer’s, cystic fibrosis and the spongiform encephalopathies [38].

In 1968, Levinthal [39] noted that for an average protein to find the correct fold

from a denatured state would take on the order of a million years if the folding process were to occur via a random search of conformational space. Proteins typically fold on a time scale of seconds and hence at an early stage, it became apparent that pathways must somehow be involved in directing the search for the native structure. A description of the folding process therefore requires a clear definition of the pathways of folding and of both the intermediate and transition states that may occur along these pathways.

Although folding in the cell is a highly complex process involving a cascade of helper proteins [40], it is clear that proteins may also fold efficiently and correctly in isolation [41], for example by transferring a protein from denaturing conditions to an environment in which the native conformation is favoured. Studies of the molecular basis of protein folding have therefore appropriately been initiated *in vitro*, where biophysical techniques capable of providing detailed structural information can be used most readily and where folding of molecules can be examined in isolation. The inherent complexity of protein structures and hence of the folding process has led to the application of a whole host of biophysical techniques each capable of probing different aspects of the native structure [42]. In this context, nuclear magnetic resonance has played a particularly important role through its ability to provide site specific information at the level of individual nuclei [43].

The experimental approaches developed so far can be divided into those that involve monitoring and comparing the structural changes that occur as a protein is unfolded or folded under equilibrium conditions (e.g. a thermal titration) and the more direct approach that involves monitoring changes that occur in “real-time” as a protein is refolded or unfolded under non-equilibrium conditions (e.g. by rapid dilution from a high concentration of denaturant). By comparing the structural changes detected under the same conditions by a variety of techniques a global picture can be formed in terms of formation of hydrophobic cores, secondary structure and tertiary contacts [44, 45]. Of particular importance in this context has been the search under both equilibrium and non-equilibrium conditions for non-cooperative transitions in which states with structures that differ from either the initial and final states of folding are detected [46, 47].

Although the protein folding problem as such has not yet been solved, a number of advances have been made towards describing the sequence of events that occur for a variety of systems. For example by combining data obtained from circular dichroism, tryptophan fluorescence and hydrogen exchange labeling experiments, the folding of the protein hen lysozyme has been shown to occur in a series of distinct steps and to involve multiple parallel pathways [48].

1.3.2 Photo-CIDNP and protein folding

Whilst assigning CIDNP peaks to specific residues in the well resolved spectrum of native proteins is relatively simple, it becomes increasingly difficult as a protein becomes less structured and the differences in chemical environment that are responsible for the chemical shift dispersion within a given residue type (e.g. tyrosine) become less pronounced. The few reported photo-CIDNP studies of protein denaturation [49–58] have relied on the poor resolution of one-dimensional ^1H spectra and have thus so far been limited to a somewhat qualitative description of changes in the relative exposure of different residue *types* (e.g. Trp and Tyr) rather than of specific residues. In an attempt to quantify the exposures of the different residue types relative to those expected for a fully unfolded protein, comparisons of the relative CIDNP intensities obtained in the denatured protein to those obtained with mixtures of free amino acids have been used [52, 58]. However, such an approach is complicated by some of the differences between small amino acids and bulky proteins described in Section 1.2.1 and 1.2.2. In Chapter 3, a two-dimensional photo-CIDNP ^{15}N - ^1H heteronuclear correlation experiment that takes advantage of the greater chemical shift dispersion available from the heavier ^{15}N nuclei [59] is described and opens up the possibility of both assigning and unambiguously quantifying the relative accessibilities of exposed tryptophan residues in non native states. In Chapter 7, two alternative techniques that take advantage of the resolution of the native spectrum by transferring polarization generated within a partially folded state to its native state for detection, and hence assignment, will be presented. The possibility of observing protein folding in “real-time” using photo-CIDNP has recently been demonstrated by Hore and Winder [60] and in Chapter 5, new experimental and theoretical developments of the technique will be discussed along with its application to the refolding of the single tryptophan mutants of a small protein. Finally, on a more practical level, in Chapter 4 two new techniques that prolong the lifetime of samples by counteracting the effects of dye photodegradation are presented and in Chapter 6, in an attempt to maximize CIDNP intensities, the magnetic field dependence of amino acid polarizations is discussed.

Chapter 2

Experimental procedures

To obtain a photo-CIDNP spectrum one requires an NMR spectrometer, a light source, a means of coupling light into the NMR probe, a suitable photosensitizer and an appropriate radiofrequency pulse sequence. The apparatus, photosensitizer and pulse sequences used for photo-CIDNP experiments in this thesis, along with some important experimental considerations are described. In particular, a series of preliminary tests used to optimize the coupling of laser light into the NMR sample are presented.

2.1 Nuclear magnetic resonance spectrometer

Spectra described in this thesis were recorded on a home-built spectrometer operating at 600 MHz (Chapters 3, 4, 5, 6 and 7) and a Varian XL-400 spectrometer operating at 400 MHz (Chapters 6 and 7). The probe-head for the 600 MHz spectrometer consisted of two double tuned Helmholtz coils (^1H , ^2H and ^{13}C , ^{15}N) for triple resonance detection and was also equipped with triple axis pulsed field gradients (65 G cm^{-1}). ^{19}F NMR and photo-CIDNP spectra (Chapter 5) were obtained by retuning the ^1H coil to 564 MHz. A Peltier semiconductor device was used to control the temperature within the range 5-75 °C [61]. The probe-head for the 400 MHz spectrometer consisted of a single double tuned Helmholtz coil (^1H , ^2H). All spectra were processed using Felix software (Hare Associates).

2.2 Light source and coupling method

2.2.1 Argon ion laser

All experiments were performed using a continuous wave Spectra-Physics 2016-05 argon ion laser as a light source operating in multi-line mode with a maximum output of 5 W that produces a series of wavelengths in the blue-green region, principally at 488 and 514 nm. The highly collimated beam, roughly two millimeters in diameter

was gated into 50-500 ms pulses via a mechanical shutter (model 200 FNC, NM Laser Products) controlled by the output from one of the spectrometer spare ports.

2.2.2 Optical fibre and coaxial insert

The laser beam was focused onto a 1 mm diameter optical fibre (model F-MMC, Newport) using Newport F-915T launcher optics and then guided into the NMR sample from above by mounting the opposite end of the fibre in a coaxial insert (model WGS-5BL, Wilmad) fitted into the 5 mm sample tube (model 528-PP, Wilmad) as shown in Fig.2.1. Such an approach has previously been described [62] and although

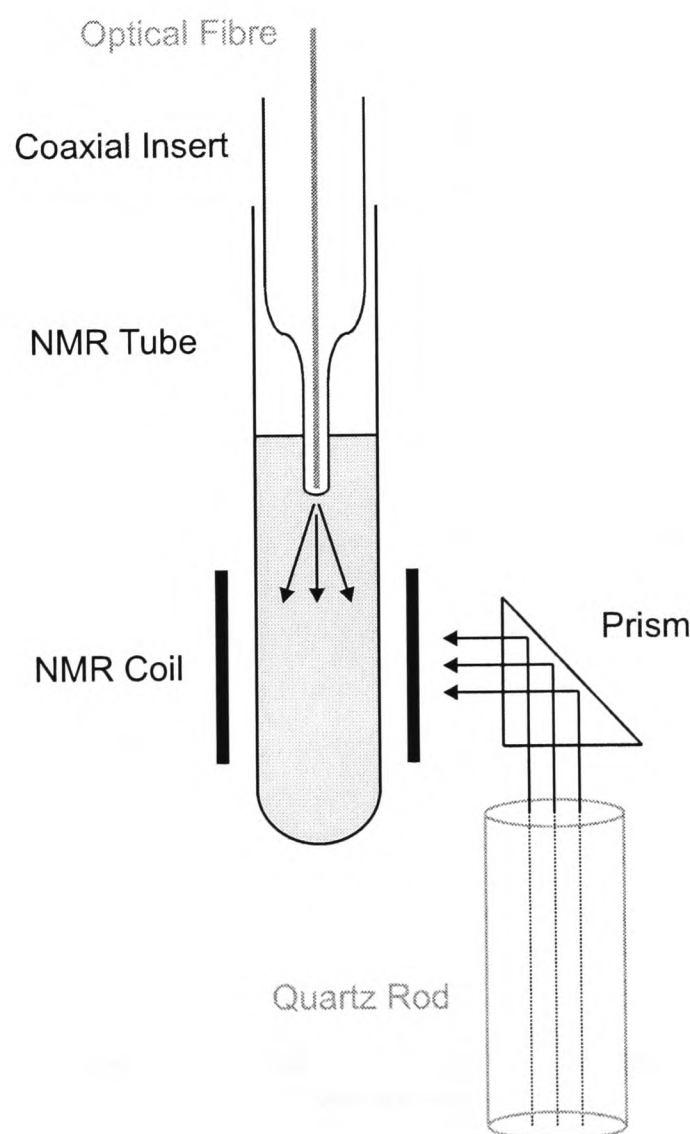


Figure 2.1: A comparison of two popular methods used to couple laser light into an NMR probe. Experiments described in this thesis were performed using the optical fibre and coaxial insert.

it precludes sample spinning it has a number of advantages over one of the popular alternative methods also shown in Fig.2.1, that involves guiding light using an off-centre cylindrical quartz rod and prism, running from the base of the NMR probe up to the sample (with coated mirrors to direct light from the laser onto the lower end of the rod) [11, 24, 63]. In particular, whilst small modifications to commercial NMR probes are necessary to accommodate a quartz rod and prism, the optical fibre is non-intrusive and can therefore be used on any available spectrometer. This opened

up the possibility of using pulse field gradients and of performing heteronuclear photo-CIDNP experiments on the state-of-the-art 600 MHz spectrometer described in Section 2.1. In addition, whilst experiments performed with modified CIDNP probes are particularly sensitive to misalignment of the laser beam at the base of the quartz rod and are typically only 40% efficient [20], 70% efficient coupling of laser light into the sample via an optical fibre and coaxial insert was achieved quite straightforwardly.

2.3 Photosensitizer

The dyes most commonly used in the photo-CIDNP studies of proteins are flavins [11, 12] with structures based on the yellow tricyclic isoalloxazine core. Flavins are involved in a number of biological redox processes and have hence been studied extensively [64, 65]. The flavin derivative used in all experiments described in this thesis was flavin mononucleotide (FMN). The UV/Visible absorption spectrum in neutral aqueous solution and chemical structure of FMN are shown in Fig.2.2. The

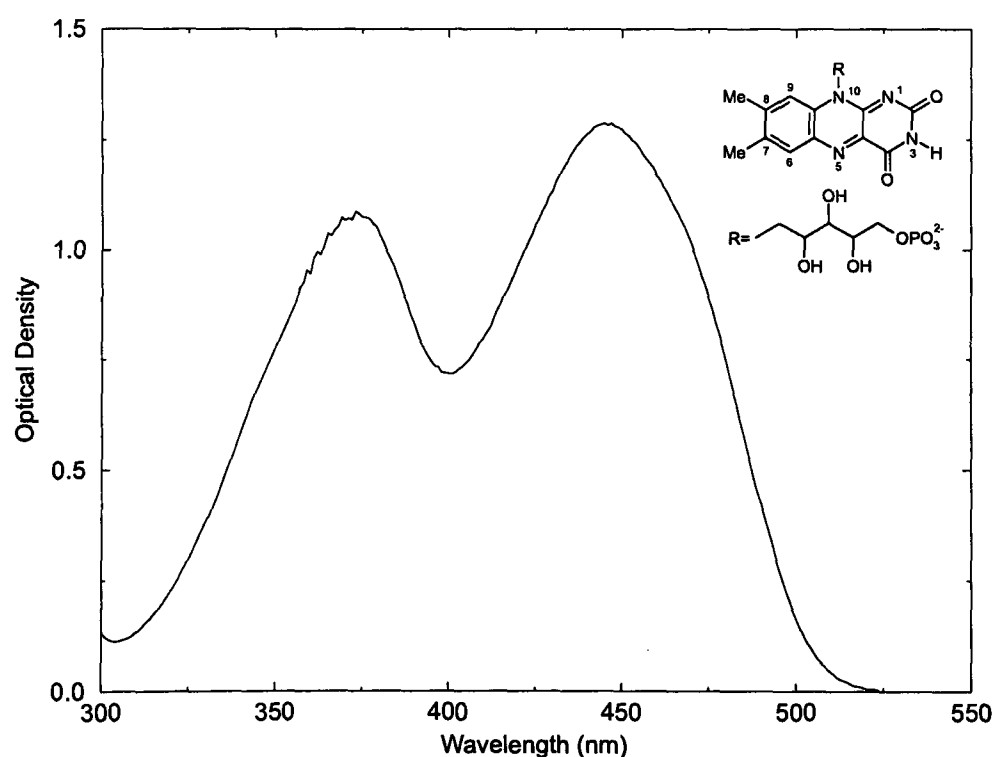


Figure 2.2: UV/Visible absorption spectrum and structure of FMN (0.05 mM, pH 7). Spectrum was obtained using a Cary 3 spectrophotometer and 1 cm path length cell.

absorption spectrum is characterized by bands in the near-UV at 375 nm and in the visible region at 450 nm ($\epsilon > 10^4 \text{ mol dm}^{-3} \text{ cm}^{-1}$). FMN also has an appreciable absorption at the Ar^+ wavelength 488 nm that leads efficiently to an excited triplet state [66] via intersystem crossing from an excited singlet state (quantum yield ~ 0.5). As described in Chapter 1, triplet excited flavin molecules react efficiently with the amino acids tryptophan, tyrosine, histidine and methionine (Chapter 6) via electron or hydrogen abstraction to yield a pair of radicals. Triplet excited flavins are also quenched by O_2 [67], secondary and tertiary amines [68, 69] as well as thiols

[9]. The photochemistry of flavins, in particular the photodegradation of oxidized flavin molecules will be discussed in more detail in Chapter 4.

2.4 Preliminary tests

By placing the tip of the coaxial insert 4 mm above the top of the NMR coil (Fig.2.1) no loss in field homogeneity was observed and hence normal linewidths were easily obtained. In order to optimize the experimental conditions with such an experimental setup the dependence of tyrosine (4 mM, pH 4.5) CIDNP intensity on flavin concentration was measured and is shown in Fig.2.3a. The drop off at higher flavin

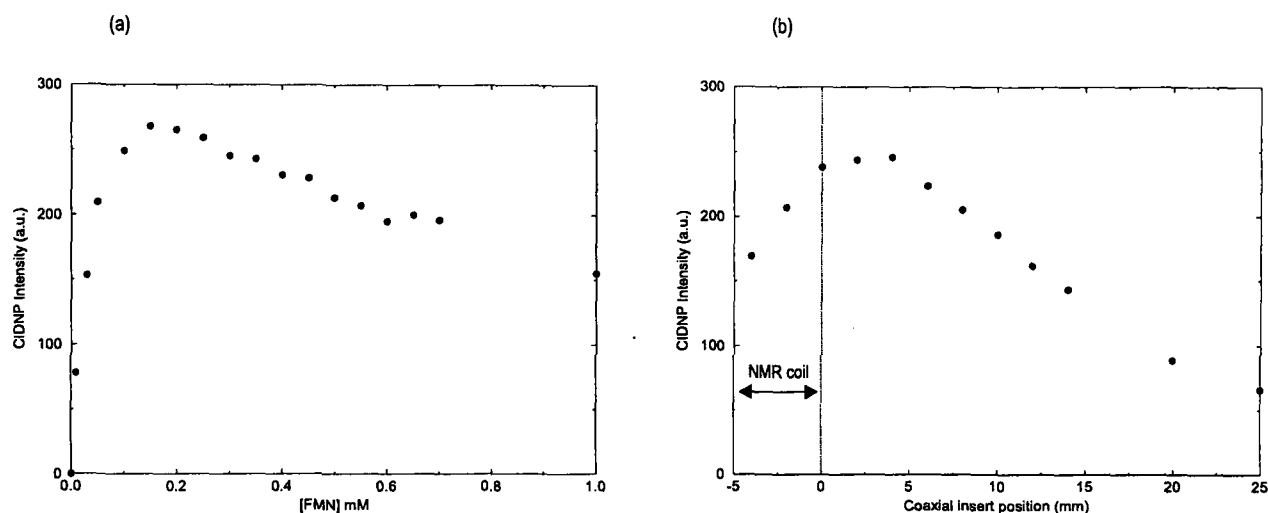


Figure 2.3: Dependence of the tyrosine (4 mM, pH 4.5) H3,5 photo-CIDNP intensity on (a) the flavin concentration and (b) the position of the tip of the coaxial insert relative to the top of the NMR coil (FMN 0.2 mM).

concentrations is presumably associated with the increased optical density which limits the amount of light reaching the sensitive coil region. The optimum concentration of 0.2 mM was used throughout this thesis. In order to assess any potential loss in CIDNP signal caused by the 4 mm “dead volume” separating the fibre and NMR coil, the dependence of tyrosine (4 mM, pH 4.5, FMN 0.2 mM) CIDNP intensity was also measured as a function of the distance separating the tip of the coaxial insert and the top of the NMR coil and is shown in Fig.2.3b. Note that in this case, peak heights and not integrals were measured in order to reflect the true benefit in terms of signal to noise ratios. No gain in signal is apparent when the insert tip is placed closer to the NMR coil (the use of higher flavin concentrations was not explored) and hence a 4 mm separation was used throughout this thesis.

2.5 Pulse sequences

2.5.1 Difference spectroscopy

The simplest way to observe photo-CIDNP effects is by difference spectroscopy [70]. As shown in Fig.2.4, this involves acquiring free induction decays with (“light”) and

without (“dark”) a prior laser flash in an interleaved fashion and then subtracting the two to yield a difference spectrum containing resonances with non-Boltzmann populations. A short delay $\Delta=5$ ms was placed between the light and r.f. pulse

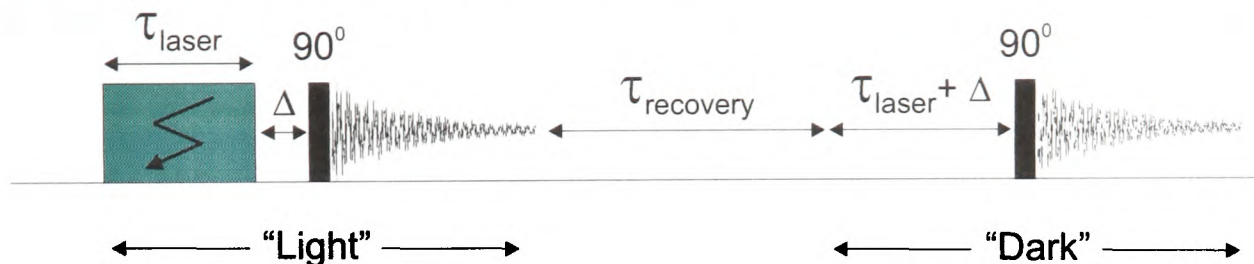


Figure 2.4: The basic pulse sequence for photo-CIDNP *difference* spectroscopy. Typically, $\tau_{laser}=100-500$ ms, $\Delta=5$ ms and $\tau_{recovery}=10$ s

to allow time for radicals to recombine and hence avoid paramagnetic broadening [71]. Difference spectroscopy highlights the features that change between the “light” and “dark” spectra, but does not provide new information and hence the signal-to-noise ratio found in these two spectra is degraded by a factor of $\sqrt{2}$ in the difference spectrum [72]. In addition, sample heating during acquisition of the “light” spectrum can lead to small chemical shifts and thus subtraction artifacts in the difference spectrum.

2.5.2 Presaturation methods

An alternative method that avoids both of these features involves presaturating the whole spectrum prior to the light flash [70] as shown in Fig.2.5. This was achieved

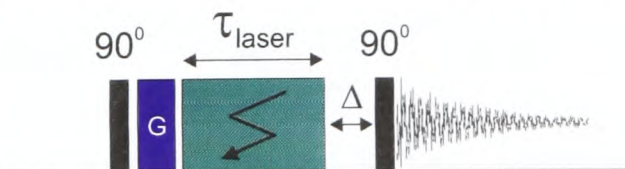


Figure 2.5: The basic pulse sequence for photo-CIDNP *presaturation* spectroscopy. Shown here is the presaturation sequence used on the 600 MHz spectrometer that involves a 90° r.f. pulse and short (<5 ms) gradient (G) pulse. Typically, $\tau_{laser}=50-100$ ms and $\Delta=5$ ms.

using a 90° r.f. pulse and short (<5 ms) gradient (G) pulse [73] on the 600 MHz spectrometer (Fig.2.5) and a series of 90° r.f. pulses of random phase with an interpulse delay determined by a converging geometric series [70] on the 400 MHz spectrometer. The former sequence is complete in approximately 10 ms as opposed to 1 s for the latter and is hence particularly attractive when a short delay between spectra is required (Chapter 5). Both methods effectively suppress background signals by several orders of magnitude and hence the spectrum recorded after a short laser flash only contains CIDNP signals. During the laser flash Boltzmann polarization will recover by spin-lattice relaxation and hence for the technique to be successful the length of the laser flash must be small compared with T_1 . Subtraction artifacts in spectra obtained by difference spectroscopy can be reduced by presaturation and hence the

two techniques are frequently combined under conditions of fast spin-lattice relaxation. To illustrate this, Figure 2.6 compares the aromatic and aliphatic regions of the light, dark and difference spectra of the protein hen lysozyme denatured in 10 M urea obtained *with* presaturation and a 100 ms laser flash. The presence of

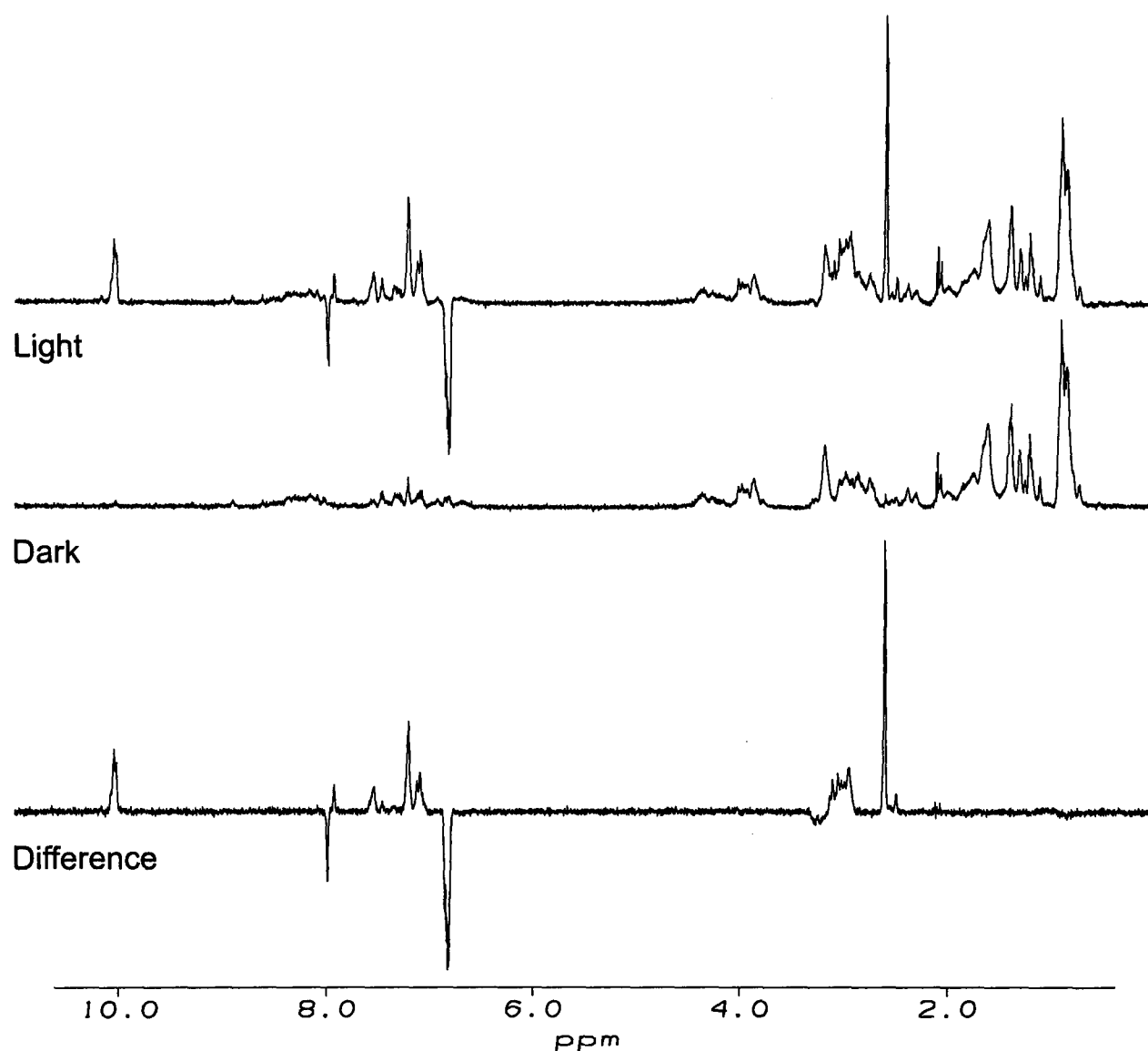


Figure 2.6: A comparison of the light, dark and difference spectra of hen lysozyme denatured in 10 M urea, obtained *with* presaturation and a 100 ms laser flash.

geminal protons in methylene and methyl groups leads to fast proton spin-lattice relaxation as compared to aromatic protons, and hence, whilst in the aromatic region a spectrum essentially free of background Boltzmann signals is obtained by simple presaturation (i.e. the light spectrum), difference spectroscopy is required in order to obtain the same result in the aliphatic region.

2.5.3 Solvent suppression

In order to minimize dynamic range problems caused by large solvent signals [74], all samples (except those in Chapter 3) were prepared in D₂O and solvent suppression steps were included in all pulse sequences. A jump and return sequence (Fig.2.7a) that leaves the water magnetization along the *z* axis while exciting spins at different chemical shifts into the *xy* plane [74, 75] and a double pulse field gradient spin echo

sequence (Fig.2.7b) that leads to selective dephasing of the water signal [76] were used on the 400 and 600 MHz spectrometers respectively. The latter is far more

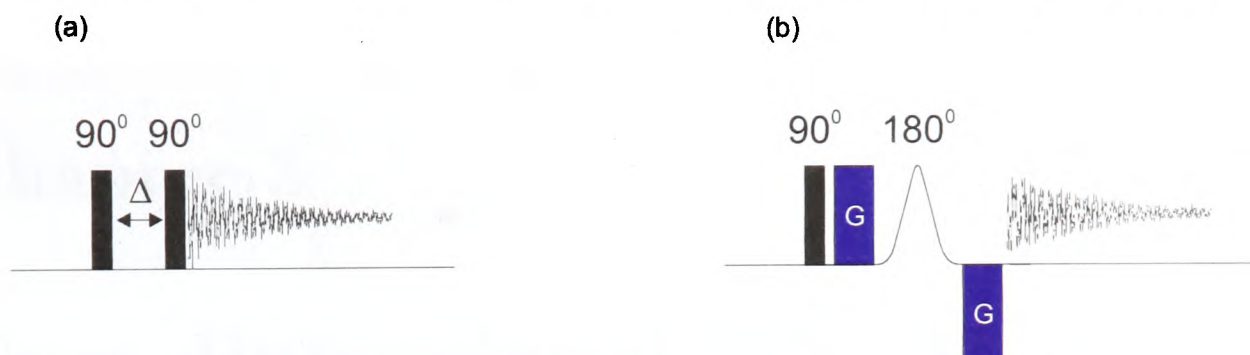


Figure 2.7: Solvent suppression sequences. (a) Jump and Return used on the 400 MHz spectrometer ($\Delta \sim 250 \mu\text{s}$) and (b) Double Pulse Field Gradient Spin Echo (DPFGSE) used on the 600 MHz spectrometer (note that the sequence shown here is a slightly simplified version of the actual pulse sequence used [76]). In both cases the carrier is placed at the solvent frequency.

efficient and unlike the jump and return sequence leads to uniform excitation over a wide range of offsets.

2.6 Materials

The deuterated solvents used in this work were deuterium oxide (99.9%, D) from Apollo Scientific Ltd. and 2,2,2-trifluoroethanol- d_3 (99%, D) from Cambridge Isotope Laboratories. $^{15}\text{N}_2$ labeled tryptophan (96-99%, ^{15}N) (Chapter 3) was ordered from Cambridge Isotope Laboratories, urea- d_4 (98%, D) and sodium cacodylate from Acros Organics and hydrogen peroxide (35% (v/v)) from Lancaster Synthesis. ^{15}N labeled hen lysozyme (Chapter 3) was kindly provided by Dr.A.Spencer of the Institute for Food Research, Norwich, England and the wild-type and single tryptophan mutants of histidine-containing phosphocarrier protein (Chapter 5) were the generous gift of Prof.G.T.Robillard of the University of Groningen, The Netherlands. All other chemicals mentioned were from Sigma-Aldrich-Fluka. All chemicals were used without further purification. Guanidinium- d_6 deuteriochloride was prepared by repeated dissolution in D_2O and lyophilization.

A Corning 240 pH meter with a glass electrode, calibrated with standard pH 4, pH 7 and pH 10 buffers, was used for all pH measurements. All pH values are direct readings and have not been corrected for the deuterium isotope effect. When a buffer was not being used, pH adjustments were made by adding small amounts of concentrated DCl and NaOD.

Chapter 3

Two-dimensional ^{15}N - ^1H photo-CIDNP

The use of ^{15}N CIDNP to probe the accessibility of tryptophan side-chains in native and denatured proteins is demonstrated. The polarization of indole nitrogens in uniformly ^{15}N labeled hen lysozyme is detected in a two-dimensional ^{15}N - ^1H NMR heteronuclear correlation experiment. While only two tryptophans are polarizable in the native state, all six appear in the spectrum of lysozyme denatured in 10 M urea, indicating partial exposure to solvent and involvement in local regions of structure.

3.1 Introduction

The first stage of any structural or dynamic protein NMR study involves assigning individual resonances to the correct residues within the primary sequence. Under native conditions, proteins adopt a well defined rigid fold. The free energy well in which they reside is steep and the ensemble of conformations they can adopt is therefore relatively limited. Under such conditions, each residue of a given type experiences a particular chemical environment which depends on its position within the overall protein fold. The chemical shifts observed for each residue type are therefore well dispersed and as a consequence, complete assignment of ^1H NMR spectra of proteins smaller than ~ 15 kDa, is usually possible using homonuclear 2D methods alone. However, as proteins get larger the number of resonances increases causing peaks to overlap. In this context, the development of heteronuclear 2D and 3D NMR techniques using ^2H , ^{13}C and ^{15}N labeled proteins has had a major impact enabling extensive site-specific characterization of proteins up to ~ 30 kDa [77, 78].

There has been considerable interest recently in using NMR to investigate the structures and dynamics of *denatured* proteins because of their relationship to kinetic intermediates in protein folding and aggregation [79, 80]. These studies are also

hampered by low spectral resolution which now arises from the conformational heterogeneity and complex dynamical nature of such partially folded species.

The term “denatured protein” is used to cover all non native protein states, from the highly unfolded states found at high temperatures and high concentrations of denaturant to the more structured molten globule states that are sometimes observed under milder denaturing conditions. The free energy landscape under denatured conditions is flatter than under native conditions. This allows a far wider ensemble of conformers to be sampled and if the interconversion between these conformers is fast on the NMR timescale, the observed NMR parameters will be averaged [81, 82]. In the limit of a completely unstructured random coil in which, on average, only random interactions of side-chains are present and no single conformation is significantly favoured, all chemical shift dispersion is lost, and a protein spectrum should resemble that obtained from a mixture of its component amino acids. Fortunately, most denatured proteins seem to possess local regions of residual structure and small deviations from random coil chemical shifts are usually observed. Small denatured proteins and large native proteins therefore pose the same resolution problem and similar techniques to those described above can be applied. For example, by combining the small deviations from random coil chemical shifts in an ^{15}N - ^1H heteronuclear correlation experiment, resonances that cannot be resolved in either the ^{15}N or ^1H dimensions alone can now be resolved and therefore assigned.

In a completely unstructured random coil one would expect all residues to be solvent accessible and therefore able to react with a photo-excited flavin. Photo-CIDNP could therefore provide direct information on residual structure within partially folded proteins at the level of individual residues. Reported photo-CIDNP experiments on proteins appear to have been restricted to 1D and 2D ^1H NMR [10–12] presumably for reasons of sensitivity and convenience. The resolution in such homonuclear experiments essentially limits the technique to native proteins. Several proteins in unfolded or partially folded states have however been studied by 1D ^1H CIDNP, usually to shed light on the structure of the native state [49–57]. A recent photo-CIDNP study of hen lysozyme denatured under various conditions discussed the relative exposure of the six tryptophan and three tyrosine residues by comparing tryptophan and tyrosine CIDNP intensities in the protein to those obtained with appropriate amino acid mixtures [58]. However, this approach relies on the assumption that the factors governing CIDNP intensities in proteins and in amino acids are identical. In addition, discussion is restricted to the general exposure of a given residue type rather than the exposure of individual residues. This chapter aims to improve on this approach by combining photo-CIDNP with a 2D ^{15}N - ^1H heteronuclear correlation experiment thereby opening up the possibility of

discussing the exposure of *individual* residues in partially folded states.

It is worth noting at this stage that ^{15}N CIDNP has been studied previously however in a quite different context. In particular, a number of mechanistic studies in organic chemistry have made use of ^{15}N labeling and CIDNP detection [83, 84]; and more recently, in a series of elegant solid state ^{15}N NMR experiments McDermott and co-workers made use of the Q-blocked photosynthetic reaction centre of *Rhodobacter Sphaeroides* enriched in ^{15}N to artificially create a radical pair involving the bacteriochlorophyll dimer (special pair, P) and the bacteriopheophytin acceptor (I) [85, 86].

The theoretical basis of heteronuclear correlation spectroscopy is first introduced followed by some exploratory experiments carried out on the free ^{15}N labeled tryptophan amino acid. The two-dimensional experiment is then tested on the native state of hen lysozyme, before being applied to the denatured state in 10 M urea.

3.2 ^{15}N - ^1H heteronuclear correlation spectroscopy

A two-dimensional heteronuclear correlation experiment correlates the chemical shifts of two scalar coupled heteronuclei and is therefore similar in principle to a homonuclear COSY experiment. At its simplest level, the heteronuclear experiment involves two chemical shift precession periods (t_1 and t_2) - one on each of the coupled nuclei - separated by an INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) like transfer step between the two nuclei [87]. The gyromagnetic ratio of ^{15}N is very small (~ 0.1 that of ^1H) therefore of the two nuclei ^1H has both a higher Boltzmann population difference (proportional to γ) and a higher detection sensitivity (proportional to $\gamma^{3/2}$). As a consequence, in a conventional NMR experiment greatest sensitivity is obtained when polarization begins and ends on ^1H nuclei. Rather than using a single $\text{N} \rightarrow \text{H}$ polarization transfer, a double polarization transfer of the type $\text{H} \rightarrow \text{N} \rightarrow \text{H}$ is therefore used, and is shown schematically in Fig.3.1a. However, in a photo-CIDNP

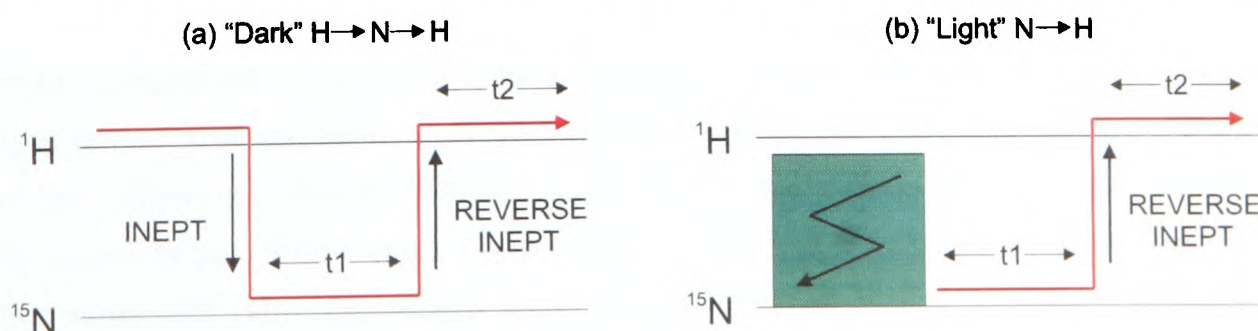


Figure 3.1: Schematic pulse sequences for 2D heteronuclear correlation (a) NMR and (b) photo-CIDNP. Whilst the conventional NMR experiment is most sensitive with a round-trip $\text{H} \rightarrow \text{N} \rightarrow \text{H}$ polarization transfer, laser irradiation in a photo-CIDNP experiment polarizes ^{15}N to such an extent that a single $\text{N} \rightarrow \text{H}$ transfer is preferable.

experiment, the initial polarization on ^1H and ^{15}N is no longer proportional to γ

but rather related to the hyperfine interaction on each nucleus within the radical pair. As will be shown in the following section, ^{15}N is polarized to such an extent by laser irradiation (a ~ 100 fold enhancement was observed for free tryptophan) that the initial transfer from ^1H to ^{15}N is no longer beneficial and a single $\text{N} \rightarrow \text{H}$ polarization transfer is therefore preferable (Fig.3.1b). Such polarization transfer experiments are also known as heteronuclear single-quantum correlation (HSQC) experiments [88]. The actual details of the pulse sequences used are somewhat more complex than the simple picture presented here, in particular it is worth noting that pulse field gradients were used for the selection of coherence pathways [89, 90] and to enhance sensitivity [91, 92].

3.3 ^{15}N labeled Tryptophan

There are three possible types of potentially CIDNP active NH groups within a protein - amides, histidine imidazoles and tryptophan indoles. Amide groups are found within every amino acid residue and form part of the polypeptide backbone. However, they are too distant from the electron spin density in the radicals of tryptophan, tyrosine and histidine to show any CIDNP. The histidine imidazole group may be CIDNP active, but the imidazole NH protons of exposed histidines normally exchange too rapidly to be observed. Spin density calculations for the tryptophan cation radical indicate a significant hyperfine interaction for the indole nitrogen, N1, and its directly bonded proton, H1 [93, 94]. In addition, as was mentioned in Chapter 1, an absorptive enhancement of H1 has been observed in both H_2O and dimethylsulphoxide [24, 25]; exploratory experiments were therefore carried out on the free ^{15}N labeled tryptophan amino acid.

The indole NH proton of tryptophans also exchange with the solvent, although at a somewhat slower rate than the NH protons of histidine imidazoles. Experiments were therefore performed in 95% H_2O /5% D_2O and wherever possible under conditions of pH and temperature that favour a slow rate of exchange (the small amount of D_2O is required to provide a lock signal). Exchange tends to be slowest at low temperatures and in the case of the indole NH proton, it is also slowest around pH 5 [95, 96]. However, the CIDNP intensities of amino acids are also pH and temperature dependent. They tend to increase as the temperature is decreased for free amino acids [20] (the opposite is true for proteins [58, 63]) and the optimum pH for tryptophan CIDNP is 9 (rapid degenerate electron-transfer between tryptophan and its radical cation destroys tryptophan polarization below pH 4 [19]). A compromise pH of 6.3 was therefore used in all exploratory experiments with the free amino acid tryptophan and the temperature was set at 7 °C. Note that in proteins the pH dependence is not as strong (Section 1.2.2) and hence, pH values of 5.2 and 3.6 were

used for the native (Section 3.4) and denatured (Section 3.5) states of hen lysozyme respectively.

The existence of spin density on N1 and H1 in the $\text{Trp}^{\bullet+}$ radical, and the possibility of $\text{N} \leftrightarrow \text{H}$ coherence transfer were confirmed in three ways: by direct detection of the H1 enhancement (Fig.3.2); by transferring the H1 polarization to N1 and back $\text{H} \rightarrow \text{N} \rightarrow \text{H}$ (Fig.3.3a); and by observing the N1 CIDNP by transfer to H1 $\text{N} \rightarrow \text{H}$ (Fig.3.3b). The enhancements in NMR intensity in the light spectrum relative to

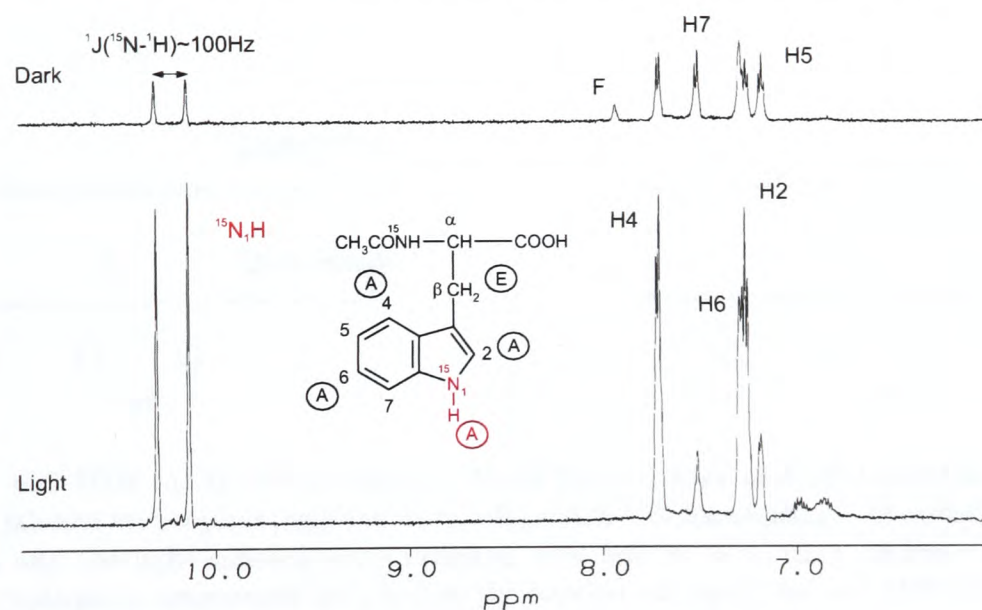


Figure 3.2: 600 MHz ^1H dark and light spectra of the aromatic region of ^{15}N labeled tryptophan, spectra were taken without ^{15}N decoupling in order to reveal the large (~ 100 Hz) indole NH scalar coupling. Both spectra were averaged over 16 scans and the light spectrum was obtained with 500 ms 4 W laser flashes. F denotes a flavin peak. $[\text{Trp}]$ 2 mM, pH 6.3, 7 $^\circ\text{C}$.

the identical experiment without laser irradiation (dark) were 6.5 ± 0.3 , 6.0 ± 0.3 and 100 ± 15 respectively. The much larger enhancement in the $\text{N} \rightarrow \text{H}$ experiment reflects the smaller Boltzmann polarization of ^{15}N and the different hyperfine interactions of N1 and H1. Of the three light spectra, the $\text{N} \rightarrow \text{H}$ experiment had the greatest signal-to-noise ratio (by a factor of two) suggesting that the N1 hyperfine coupling constant in $\text{Trp}^{\bullet+}$ is larger than that of H1. In addition, detection of ^{15}N rather than ^1H CIDNP has the advantage that the $\text{N} \rightarrow \text{H}$ dark spectrum is so weak - less than 1% of the light spectrum (Fig.3.3b) - that subtraction to remove unpolarized peaks, with its $\sqrt{2}$ signal-to-noise penalty, is unnecessary.

As will be discussed in Chapter 4, the photo-CIDNP mechanism is not always perfectly cyclic. In particular, dye exhaustion can lead to a significant loss of polarization [24, 97–99]. Figure 3.4a illustrates this by showing the progressive decay in intensity of the tryptophan indole $\text{N} \rightarrow \text{H}$ light spectrum over 64 successive laser irradiations. This essentially precludes signal averaging and/or phase cycling [100] and sets an upper limit to the number of t_1 increments in a 2D experiment. In addition, significant linebroadening in the indirectly detected dimension of a 2D experiment

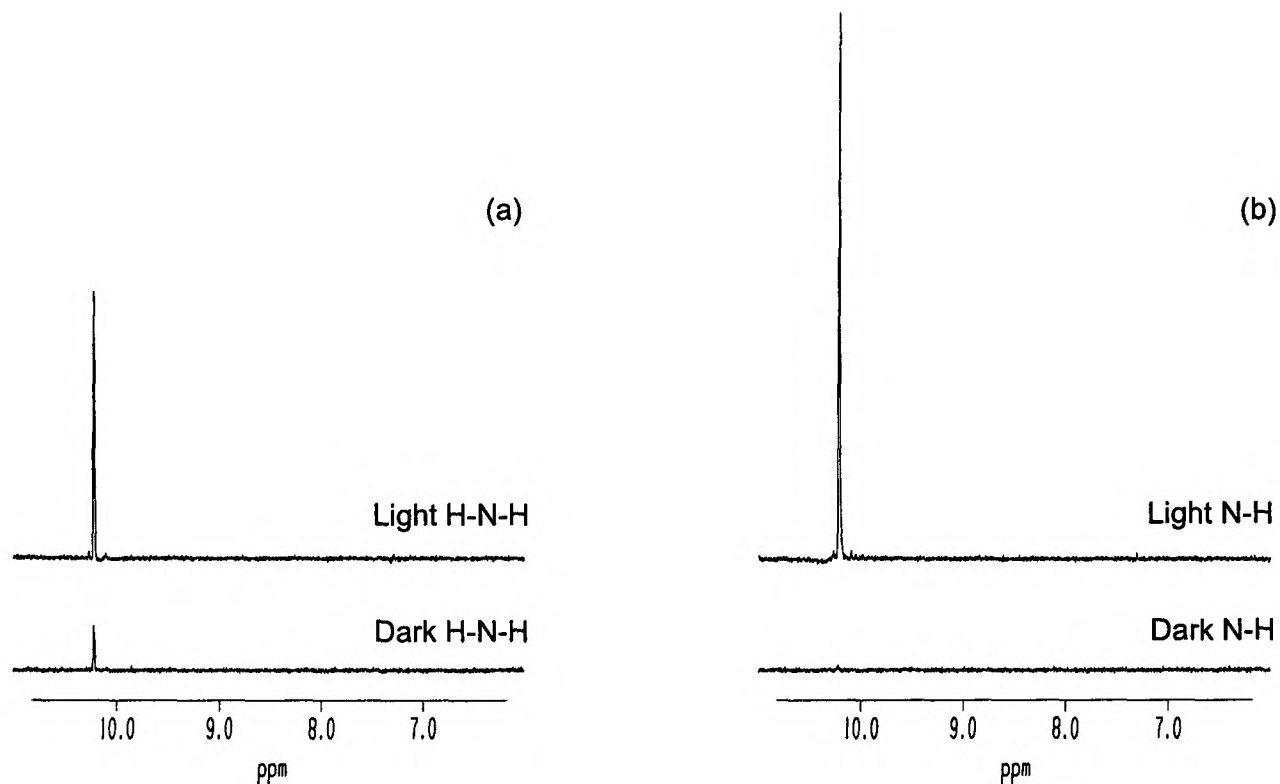


Figure 3.3: 600 MHz (a) H→N→H and (b) N→H filtered dark and light spectra of the aromatic region of ^{15}N labeled tryptophan, spectra were taken with ^{15}N decoupling. All spectra were averaged over 16 scans and the light spectra were obtained with 500 ms 4 W laser flashes. The H2, H4, H5, H6 and H7 tryptophan resonances lack a directly bonded nitrogen and are therefore not observed. [Trp] 2 mM, pH 6.3, 7 °C.

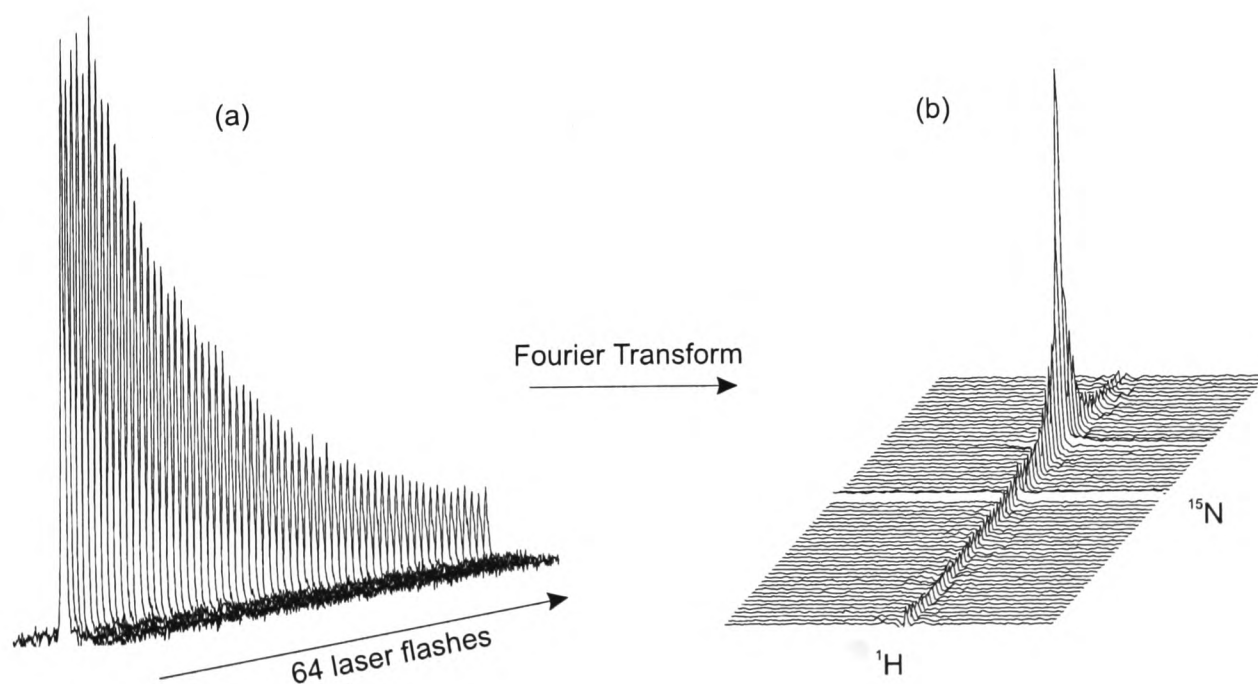


Figure 3.4: (a) Intensity decay of the N→H filtered light spectrum of ^{15}N labeled tryptophan over 64 successive laser irradiations and (b) linebroadening in the indirectly detected dimension of a 2D experiment caused by such a decay. Laser flashes were of 100 ms each at a power of 4 W and separated by a 10 s delay. [Trp] 2 mM, pH 6.3, 7 °C.

(Fig.3.4b) would be expected. As a consequence, all 2D experiments described in the next two sections were carried out with a maximum of 32 complex points in the indirectly detected ^{15}N dimension (i.e. a total of 64 laser irradiations).

3.4 Native Hen Lysozyme

Hen lysozyme was chosen to illustrate the potential of the method for studying proteins. Hen lysozyme is a small monomeric protein of 129 residues (14.6 kDa), found in hen egg white and acts as a glycoside hydrolase. It was the first enzyme to have its three-dimensional structure determined by X-ray crystallography [101] and has been extensively studied by solution state NMR under a variety of conditions [48, 102–118]. The crystal structure is shown in Fig.3.5, and can be divided into two structural domains separated by a deep active site cleft. The α -domain consists

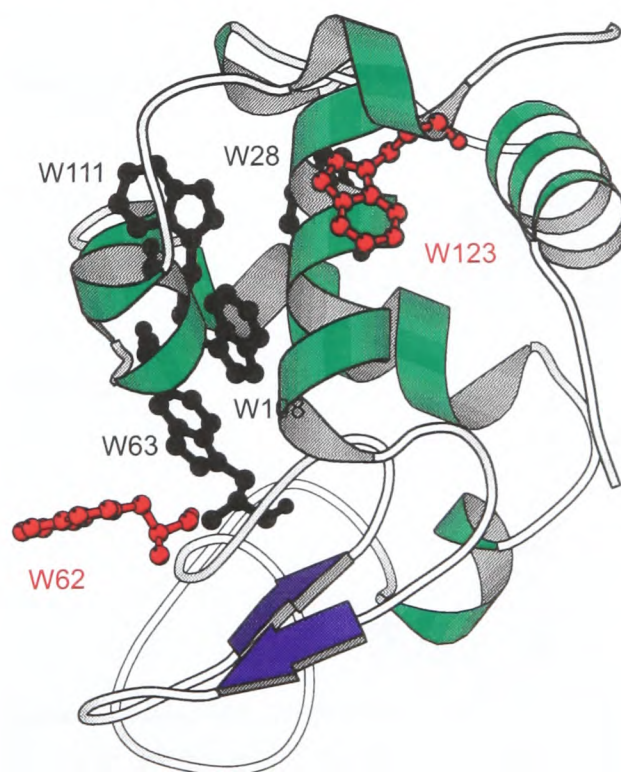


Figure 3.5: Molscript representation of native hen lysozyme. The α -domain is coloured in green and the β -domain in blue. The tryptophan side-chains are drawn in red (exposed) and black (buried).

of four major α -helices; two located towards the C-terminus (88-99, 108-115) and two towards the N-terminus (4-15, 24-36) as well as a short 3_{10} helix (119-124) and two short loops (16-22, 100-107). The β -domain is formed from a triple-stranded antiparallel β -sheet (41-60), another short 3_{10} helix (79-84) and a long loop (61-78). Hen lysozyme also contains four disulphide bridges, 6-127, 30-115, 64-80 and 76-94 the last of which connects the two domains. The disulphide bonds were left intact in all experiments described in this chapter. Hen lysozyme contains ten potentially polarizable residues; three tyrosines (Tyr 20, Tyr 23 and Tyr 53), six tryptophans (Trp 28, Trp 62, Trp 63, Trp 108, Trp 111 and Trp 123) and one histidine (His 15).

The 1D ^1H NMR and photo-CIDNP spectra of the native state taken at pH 5.2 and 40 °C is shown in Figure 3.6. Of the ten potentially polarizable residues, just two of the six tryptophan residues - Trp 62 and to a lesser extent Trp 123 - can be polarized by flavins [119, 120], reflecting their accessibility within the native X-ray structure (Fig.3.5). For such a small native protein (~ 15 kDa) the resolution

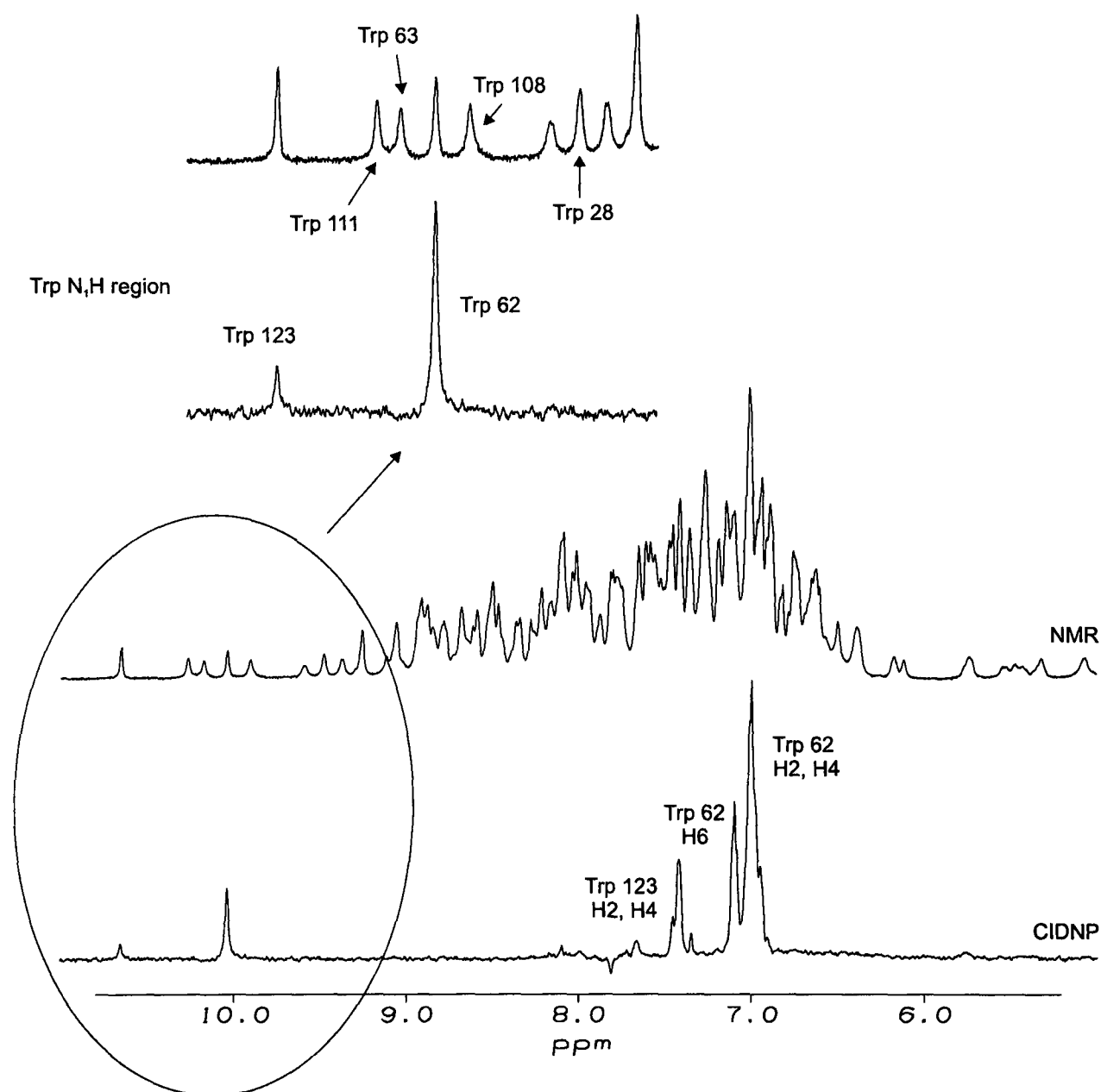


Figure 3.6: 600 MHz ^1H NMR and photo-CIDNP spectra of the aromatic region of native ^{15}N labeled hen lysozyme (1.5 mM, pH 5.2, 40 °C), spectra were taken with ^{15}N decoupling. The resolution in the ^1H dimension is sufficient to resolve both aromatic and indole NH peaks. Assignments were taken from Ref.[104, 119].

in the ^1H dimension is sufficient to resolve both the polarized aromatic and indole NH peaks. The two-dimensional heteronuclear correlation experiment was therefore used as a proof of concept.

Figure 3.7 shows the full H $\rightarrow$ N $\rightarrow$ H dark ^{15}N - ^1H HSQC spectrum of native hen lysozyme. The tryptophan indole NH peaks are well separated from the large number of amide NH peaks that form the bulk of the spectrum. The indole regions of the

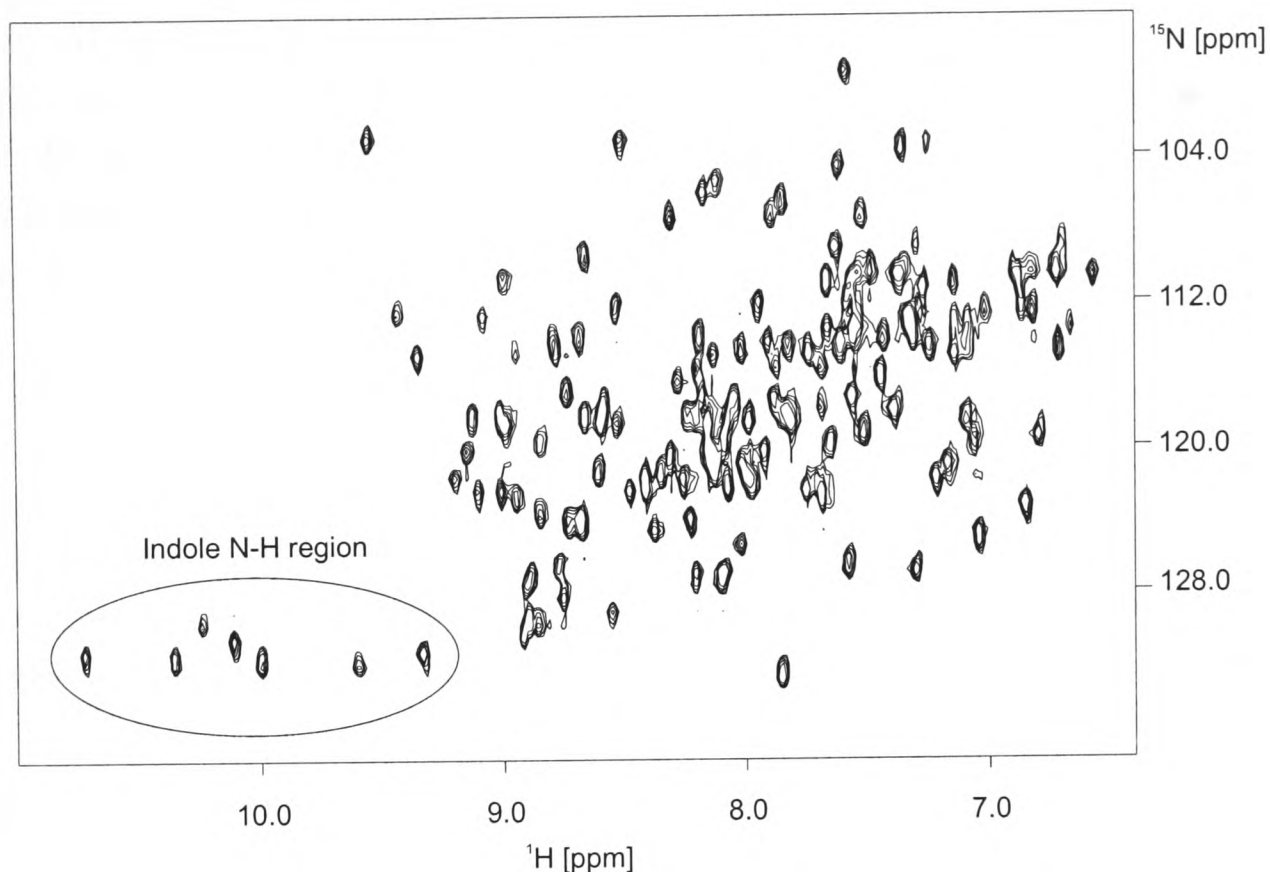


Figure 3.7: 600 MHz 2D ^{15}N - ^1H HSQC spectrum of ^{15}N labeled native hen lysozyme (1.5 mM, pH 5.2, 40 °C) consisting of 32 complex points in the ^{15}N (f_1) dimension, and spectral widths of 2500 Hz and 8000 Hz in the ^{15}N (f_1) and ^1H (f_2) dimensions respectively. The indole peaks are well separated from the crowded amide region.

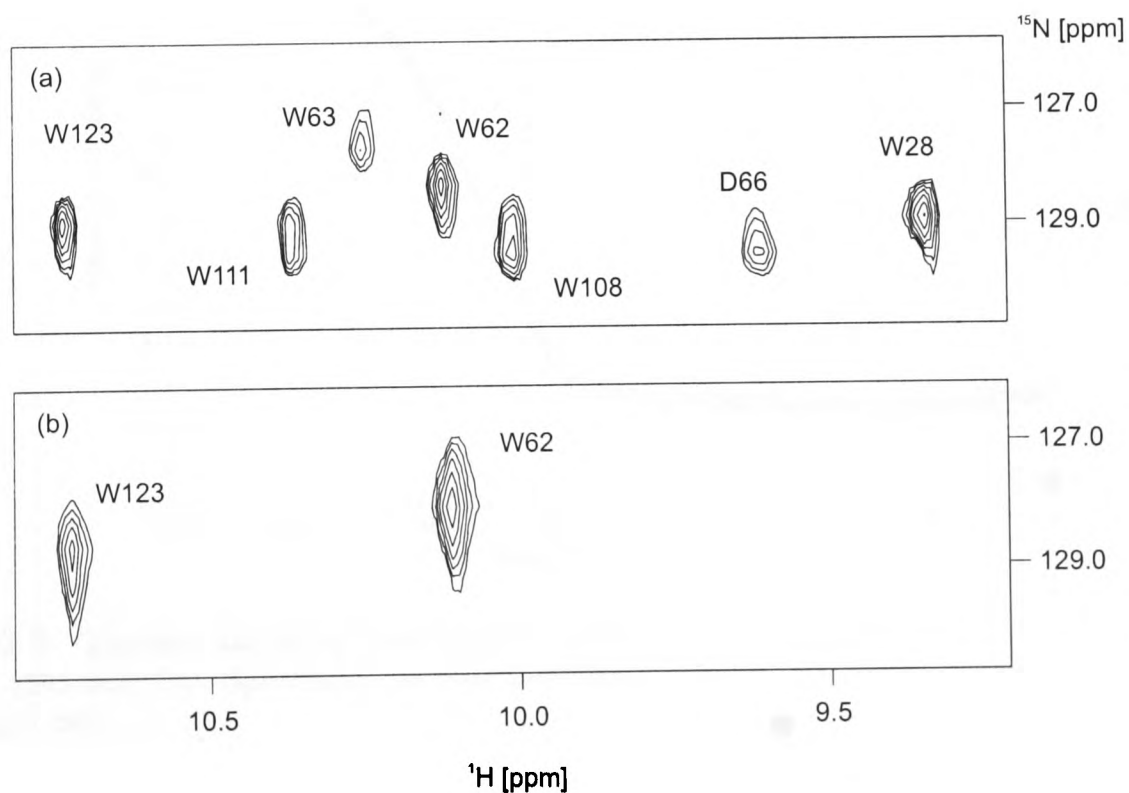


Figure 3.8: Expanded indole NH region of the 600 MHz 2D ^{15}N - ^1H HSQC spectrum of ^{15}N labeled native hen lysozyme (1.5 mM, pH 5.2, 40 °C). (a) Dark H $\rightarrow$ N $\rightarrow$ H spectrum with *four* scans per t_1 increment (expanded from Fig.3.7). (b) Light N $\rightarrow$ H spectrum obtained by preceding the pulse sequence on each scan by a 100 ms pulse of 4 W light with *one* scan per t_1 increment. This experiment was done without the initial H $\rightarrow$ N polarization transfer and so detects the ^{15}N CIDNP. Contour levels are at the same positions in both spectra.

H $\rightarrow$ N $\rightarrow$ H dark and N $\rightarrow$ H light spectra are expanded in Fig.3.8a and b respectively. Whilst the dark spectrum has resonances from all six tryptophans, the light spectrum displays just two peaks (the other four are lost in the noise); as expected from the ^1H spectra (Fig.3.6), these are Trp 62 and Trp 123. The assignments were taken from Ref.[104, 112]; the seventh peak is the amide of Asp 66. The enhancements in the (N $\rightarrow$ H) light spectrum relative to the (H $\rightarrow$ N $\rightarrow$ H) signals detected in the dark, taking into account the different extents of signal averaging in the two experiments, are 4.8 for Trp 62 and 2.9 for Trp 123. The linebroadening observed in the indirectly detected dimension of the N $\rightarrow$ H light spectrum (Fig.3.8b) is a direct consequence of the progressive loss of polarization that occurs upon prolonged irradiation and illustrates the potential for some of the methods described in Chapter 4 that are designed to prolong the lifetime of the sample.

3.5 Denatured Hen Lysozyme

The loss of tertiary structure of hen lysozyme in 10 M urea at pH 3.6 as monitored by near-UV CD [121] as a function of temperature is shown in Figure 3.9. The mid-

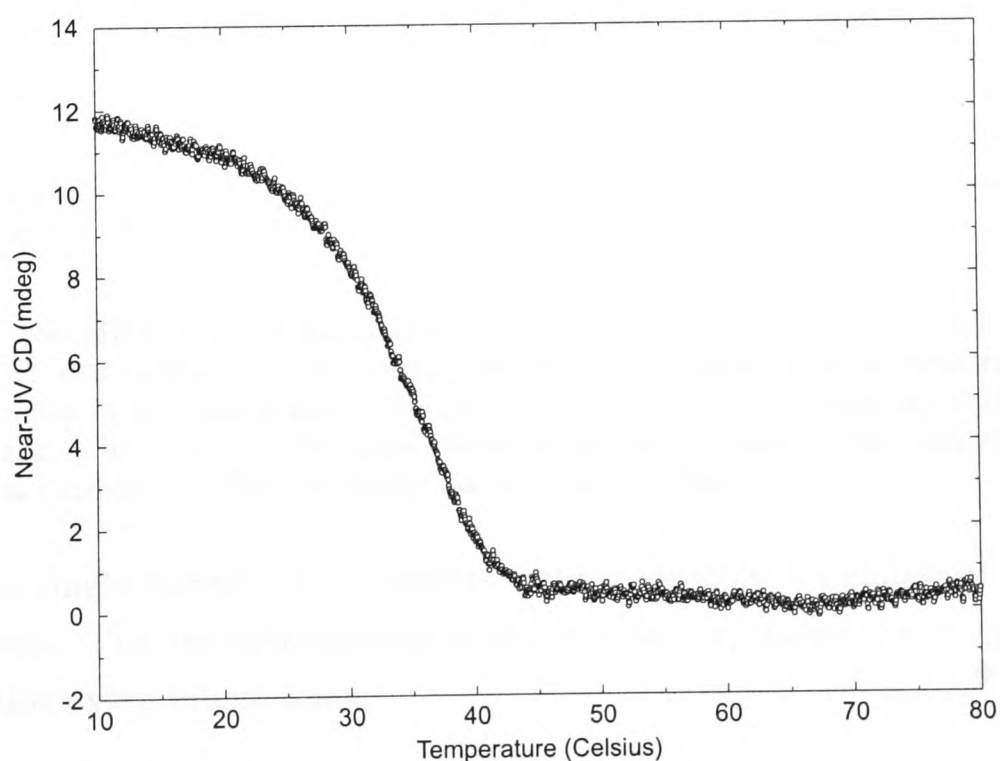


Figure 3.9: Thermal unfolding curve of hen lysozyme (1 mM, 10 M urea, pH 3.6) as judged by near-UV (295 nm) CD. Spectrum was obtained using a Jasco J720 spectropolarimeter and 1 mm path length cell.

point occurs at 35 °C and unfolding is essentially complete by 45 °C. Figure 3.10 shows the 1D ^1H NMR and photo-CIDNP spectra of hen lysozyme denatured in 10 M urea taken at pH 3.6 and 45 °C. Such a denatured state has been shown to have, on average, polarization corresponding to just one exposed tryptophan (in addition to strong enhancements for some or all three tyrosines) [58]. However unlike in the native state, the ^1H spectral resolution is insufficient to determine whether this

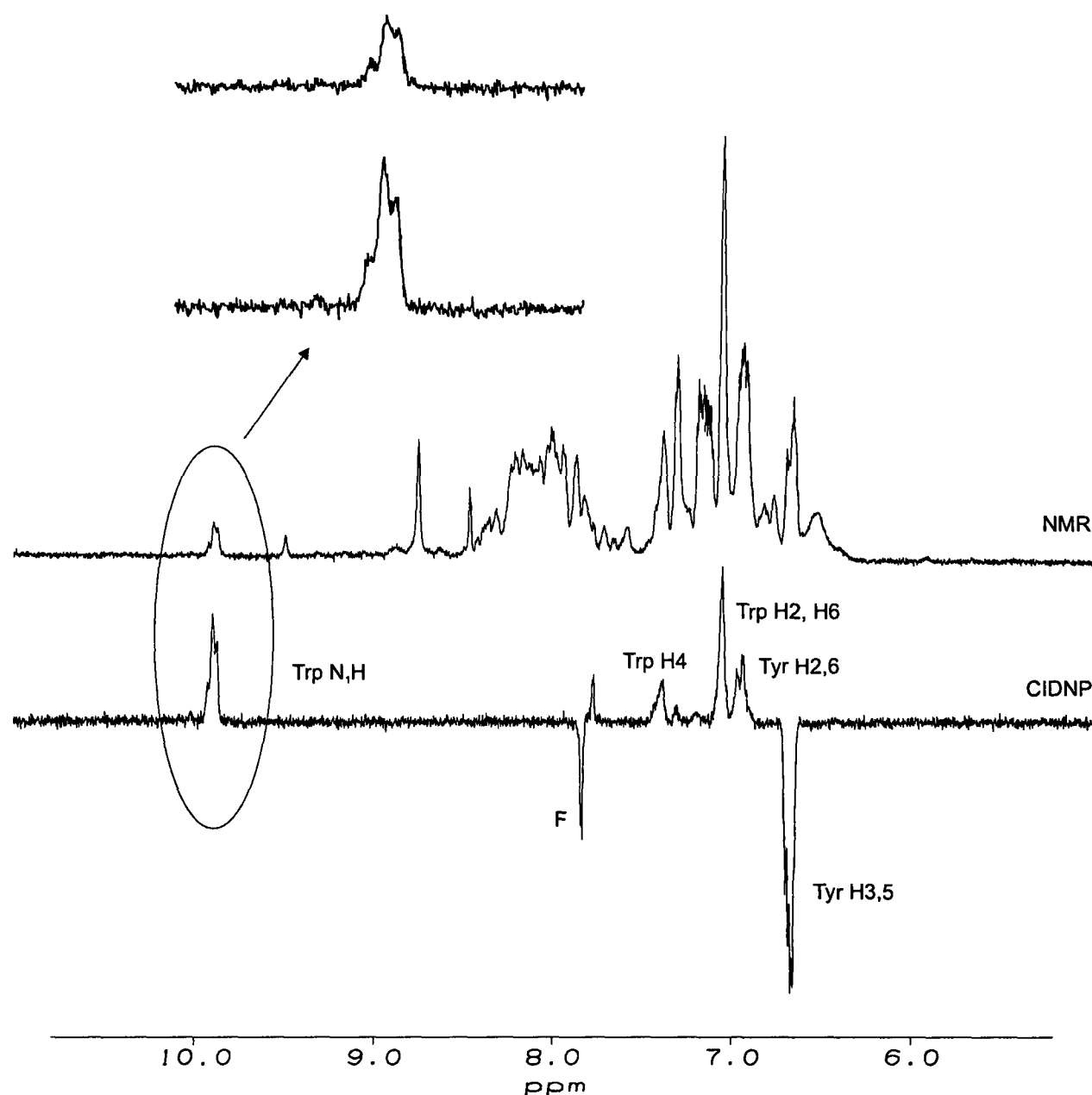


Figure 3.10: 600 MHz ^1H NMR and photo-CIDNP spectra of the aromatic region of ^{15}N labeled hen lysozyme (1 mM, pH 3.6, 45 °C) denatured with 10 M urea, spectra were taken with ^{15}N decoupling. Unlike in the native state, the resolution in the ^1H dimension is no longer sufficient to resolve all aromatic or indole NH peaks. However as the expanded indole region reveals, small deviations from random coil chemical shifts can still just be observed.

arises from a single completely accessible residue, or from a number of partially exposed residues. The two-dimensional heteronuclear experiment was therefore used to provide this extra information.

Figure 3.11 shows the full H $\rightarrow$ N $\rightarrow$ H dark ^{15}N - ^1H HSQC spectrum of lysozyme denatured in 10 M urea. The loss of chemical shift dispersion in both the ^1H and ^{15}N dimensions when compared to the native spectrum (Fig.3.7) is dramatic. However, as the expanded indole region reveals (Fig.3.12a), by placing the ^{15}N (f_1) central carrier frequency on the high field edge of the indole region and reducing the spectral width to 156 Hz, the indole NH peaks can still just be resolved, with four resolved peaks (A-C, E) and a fifth (D) which from its intensity, must correspond to the two remaining tryptophans; the assignments of these peaks to specific residues have not been determined. In the N $\rightarrow$ H light spectrum (Fig.3.12b) all of the tryptophans

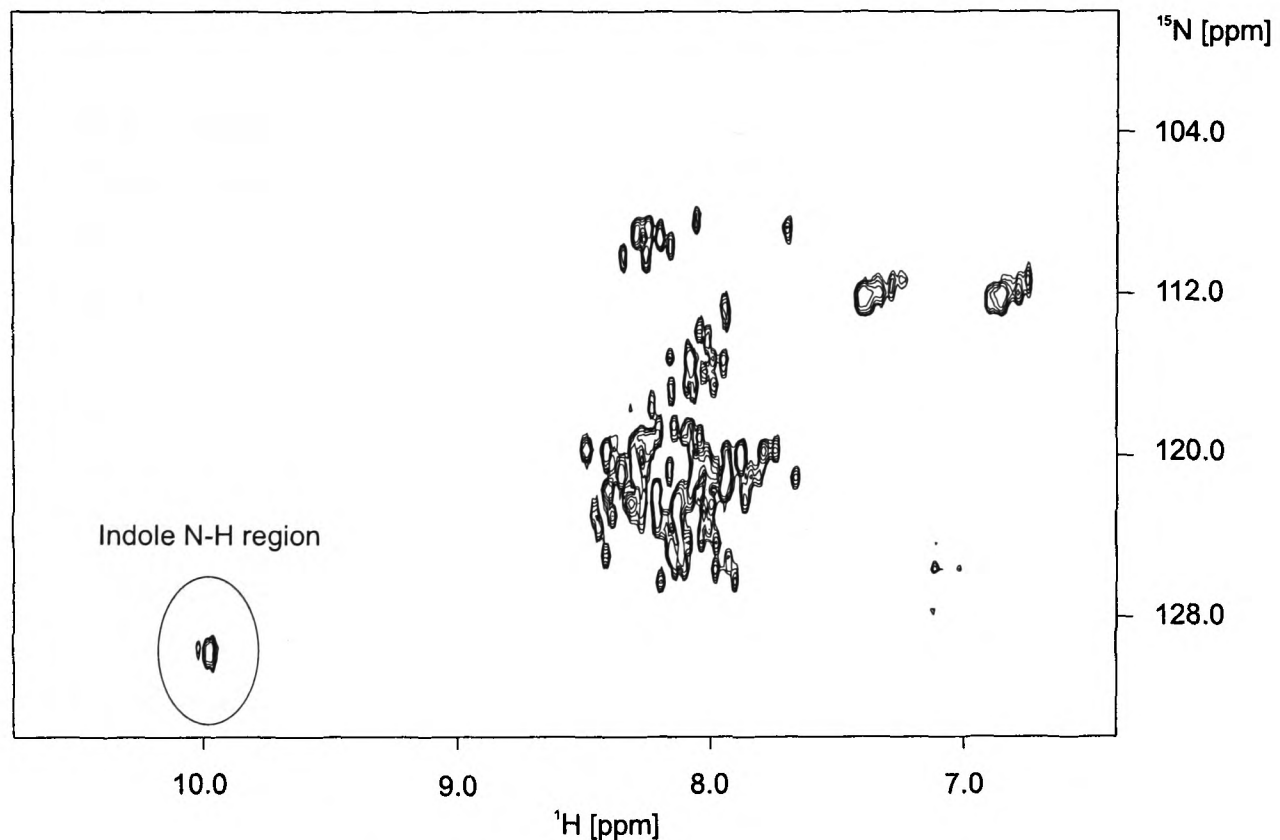


Figure 3.11: 600 MHz 2D ^{15}N - ^1H HSQC spectrum of ^{15}N labeled hen lysozyme (1 mM, pH 3.6, 45 °C) denatured in 10 M urea consisting of 32 complex points in the ^{15}N (f_1) dimension, and spectral widths of 2500 Hz and 8000 Hz in the ^{15}N (f_1) and ^1H (f_2) dimensions respectively. Note the dramatic loss of chemical shift dispersion in both the ^1H and ^{15}N dimensions.

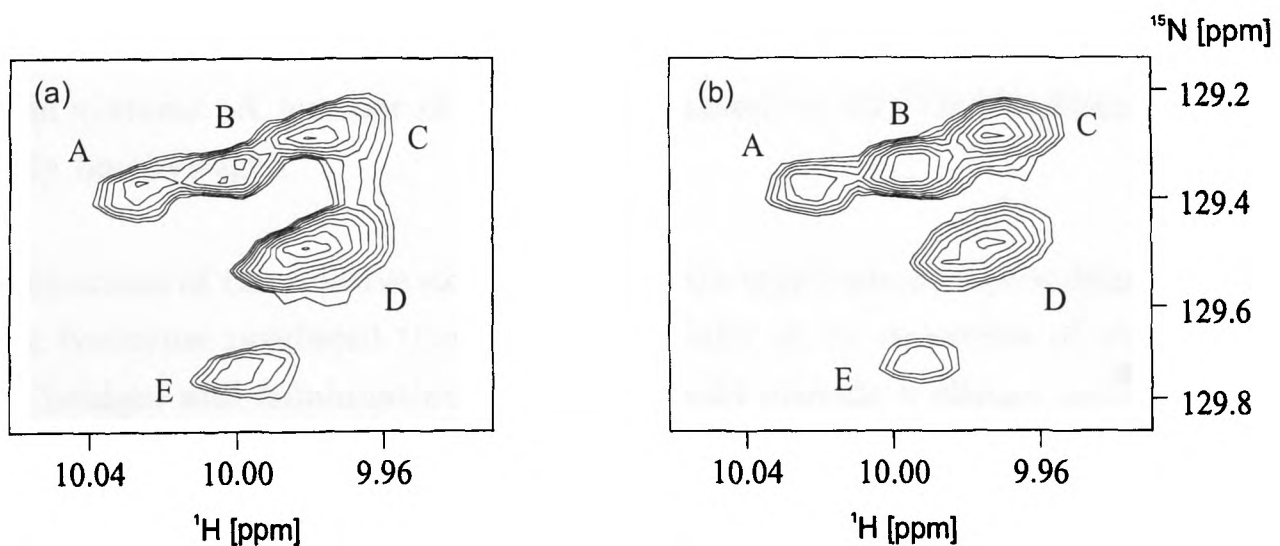


Figure 3.12: Indole NH region of the 600 MHz 2D ^{15}N - ^1H HSQC spectrum of ^{15}N labeled hen lysozyme (1 mM, pH 3.6, 45 °C) denatured in 10 M urea. (a) Dark H $\rightarrow$ N $\rightarrow$ H spectrum with *four* scans per t_1 increment. (b) Light N $\rightarrow$ H spectrum obtained as described in the caption of Fig.3.8 with *one* scan per t_1 increment. Both spectra consist of 32 complex points in the ^{15}N (f_1) dimension and spectral widths of 156 Hz and 7017 Hz in the ^{15}N (f_1) and ^1H (f_2) dimensions respectively. The central carrier frequency was placed at the high field end of the indole region.

are polarized with approximate enhancements (defined as above) of 1.4, 2.0, 1.9, 0.9 and 1.3 for resonances A-E. These enhancements are in theory directly related to the relative reactivity of the individual tryptophans, implying partial exposure of all six side-chains and varying degrees of involvement in local regions of structure. This conclusion is consistent with heteronuclear 3D NMR studies of oxidised and reduced hen lysozyme denatured in 8 M urea at pH 2 in which the most pronounced deviations from random coil predictions (chemical shifts, coupling constants, relaxation rates and NOE's) were found in regions of the protein involving aromatic residues, and the tryptophans in particular [116]. The existence of such structures may be of considerable interest from the point of view of protein folding, as they provide an environment suitable for the construction of elements of secondary structure and limit the number of conformations available to the folding polypeptide chain. In addition, characterization of the states formed in the early stages of the refolding of hen lysozyme by hydrogen exchange [117] and stopped-flow CIDNP [60] experiments both indicate that a compact hydrophobic core involving the tryptophans is a key precursor to the formation of persistent native-like structure during refolding.

3.6 Future work

The combination of photo-CIDNP and two-dimensional ^{15}N - ^1H NMR presented in this chapter suggests that photo-CIDNP of proteins is in no way limited to low resolution 1D and 2D ^1H NMR and can readily be combined with some of the most modern high resolution multidimensional heteronuclear correlation experiments. Such combinations should allow the application of photo-CIDNP to a whole host of new protein systems. A number of applications based on 2D ^{15}N - ^1H photo-CIDNP can already be envisaged.

A comparison of the relative exposure of the six tryptophans in the denatured states of hen lysozyme produced thermally, chemically or by reduction of the four disulphide bridges and combinations thereof, should provide a clearer indication of the apparent extent of unfolding under different denaturing conditions, and assist in our understanding of the underlying forces governing denaturation. Alternatively, by repeating the experiment for a given protein over a range of denaturant conditions (e.g. a urea titration), one could potentially observe the gradual unfolding of different regions of the protein. Such an approach could assist in the detection and characterization of intermediates in non-cooperative unfolding processes [122]. For slowly folding proteins (~ 1 -30 min), it should also be possible to record a series of 2D CIDNP heteronuclear correlation spectra (~ 1 min) as refolding takes place [123], to give insight into changes in side-chain exposure, in addition to the kinetic information available from the line shapes [124].

In Section 3.3, histidine was dismissed as a candidate for ^{15}N - ^1H heteronuclear CIDNP on the basis of the rapid solvent exchange rates observed for exposed imidazole NH groups. An alternative would be to use the small but detectable (~ 10 Hz) two bond coupling that exists between the ring nitrogens and the non-exchanging aromatic protons H2 and H4 [125]. The feasibility of a heteronuclear correlation experiment based on these weak couplings has previously been demonstrated by conventional NMR [126, 127]. The spin density on the ring nitrogens of histidine is likely to be small [128], however, both H2 and H4 exhibit strong CIDNP enhancements [12, 19]. Therefore rather than using a $\text{N} \rightarrow \text{H}$ transfer as with tryptophan, a more conventional round-trip $\text{H} \rightarrow \text{N} \rightarrow \text{H}$ transfer from both H2 and H4 is likely to be preferable.

Chapter 4

Overcoming photo-degradation

Two new techniques offering considerable improvements in the quality of photo-CIDNP spectra of proteins are demonstrated. Both focus on the problem of progressive photo-degradation of the flavin dye. One approach uses rapid addition and removal of protein/flavin solution between light flashes to mix the NMR sample and introduce fresh flavin into the laser irradiated region. The other involves chemical oxidation of photo-reduced flavin by the addition of hydrogen peroxide. In both cases a larger number of scans can be accumulated before the flavin is exhausted than would otherwise be possible.

4.1 Introduction

The photochemical reactions used to produce CIDNP in proteins are cyclic, so that polarization is observed in the intact protein rather than a chemically modified form, but they are not *perfectly* so. A serious problem encountered in CIDNP experiments that require prolonged laser irradiation such as the two-dimensional ^{15}N - ^1H heteronuclear correlation experiment presented in Chapter 3 is the progressive decay in polarization generated by successive light flashes [99]. This is illustrated in Fig.4.1 where the CIDNP spectrum of tyrosine is shown after 1, 32, 64, 128 and 256 laser flashes. Such a dramatic decay in the CIDNP signal causes unwanted linebroadening in the f_1 dimension of a 2D spectrum, reduces the number of 1D spectra that can be acquired from a single sample e.g. during slow protein refolding in a stopped-flow experiment, and limits the potential for improving the signal-to-noise ratio by signal averaging.

An investigation into the reaction of lumiflavin with tryptophan [99] indicated that the gradual loss of polarization is due to both photo-reduction of the flavin (F), which leads to bleaching of the solution (the flavohydroquinone FH_2 absorbs weakly in the visible [64, 129]), and to degradation of the tryptophan. The former is likely

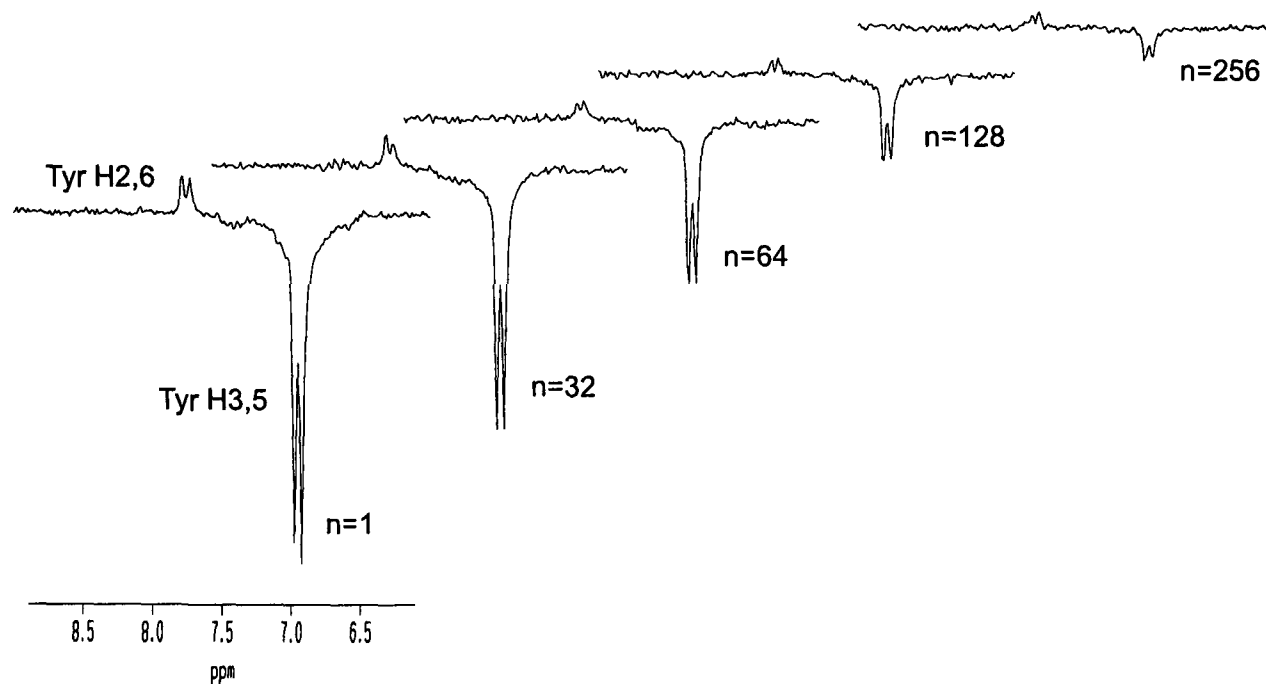


Figure 4.1: A series of 600 MHz ^1H CIDNP spectra showing the H2,6 and H3,5 resonances of tyrosine (4 mM, pH 4.5) after the indicated number of laser flashes. Each spectrum is the result of a single laser flash. Laser flashes were of 100 ms each at a power of 4 W and separated by a 10 s delay.

to be the more serious problem because the CIDNP intensities depend sensitively on the optical density of the sample (Chapter 2), and the concentration of the flavin is usually an order of magnitude smaller than that of polarizable histidine, tyrosine and tryptophan side-chains in a protein. The most probable cause of photo-reduction is the thermodynamically favourable disproportionation of flavosemiquinone radicals ($2\text{FH}^\bullet \rightarrow \text{F} + \text{FH}_2$) [130], as indicated by microsecond time-resolved CIDNP [131]. Flavin photochemistry is however inherently complex, in particular a number of intramolecular and intermolecular photo-reduction, photo-addition and photo-dealkylation mechanisms have been proposed [65, 66]; the predominant reaction pathway chosen depending on factors such as the type of solvent, pH, buffer composition, etc. Further, the primary products may themselves undergo a variety of secondary photolytic and/or thermal reactions leading to a highly complex sample mixture. Hence, by focusing on the primary intermolecular photo-reduction of flavins, it is apparent that only an idealized picture of flavin photochemistry will be presented in this chapter.

Several attempts have been made to overcome the problem. As bleaching occurs predominantly in the irradiated portion of the NMR tube, sample mixing can be used to replenish the flavin [24, 97, 98]. Manipulation of the molecular oxygen concentration is also helpful: O_2 efficiently oxidises FH_2 to F [64], but is consumed in the process; indeed removal of O_2 by degassing accelerates photo-bleaching [99]. However, too high a concentration of O_2 dramatically attenuates the initial polarization, probably by rapid quenching of the photoexcited triplet, ^3F [99]. Hitherto, the most successful technique has been to spin the NMR tube rapidly between flashes so

as to create a vortex in the solution [97, 98]. This re-introduces O_2 and brings fresh flavin into the irradiated region. An alternative approach, in which the solution is flowed slowly through the NMR tube, requires larger quantities of protein and is less straightforward to implement [20].

4.2 Analysis of photo-degradation

In order to characterize the CIDNP decay further, a number of exploratory experiments were performed on amino acid samples. In an attempt to monitor changes in the composition of the sample described in Fig.4.1, a similar experiment shown in Fig.4.2 was carried out in which the NMR intensities (rather than the CIDNP intensities) of tyrosine (4 mM) and FMN (0.2 mM) were measured in a series of spectra taken in between laser flashes. Unlike tyrosine, which is hardly affected by

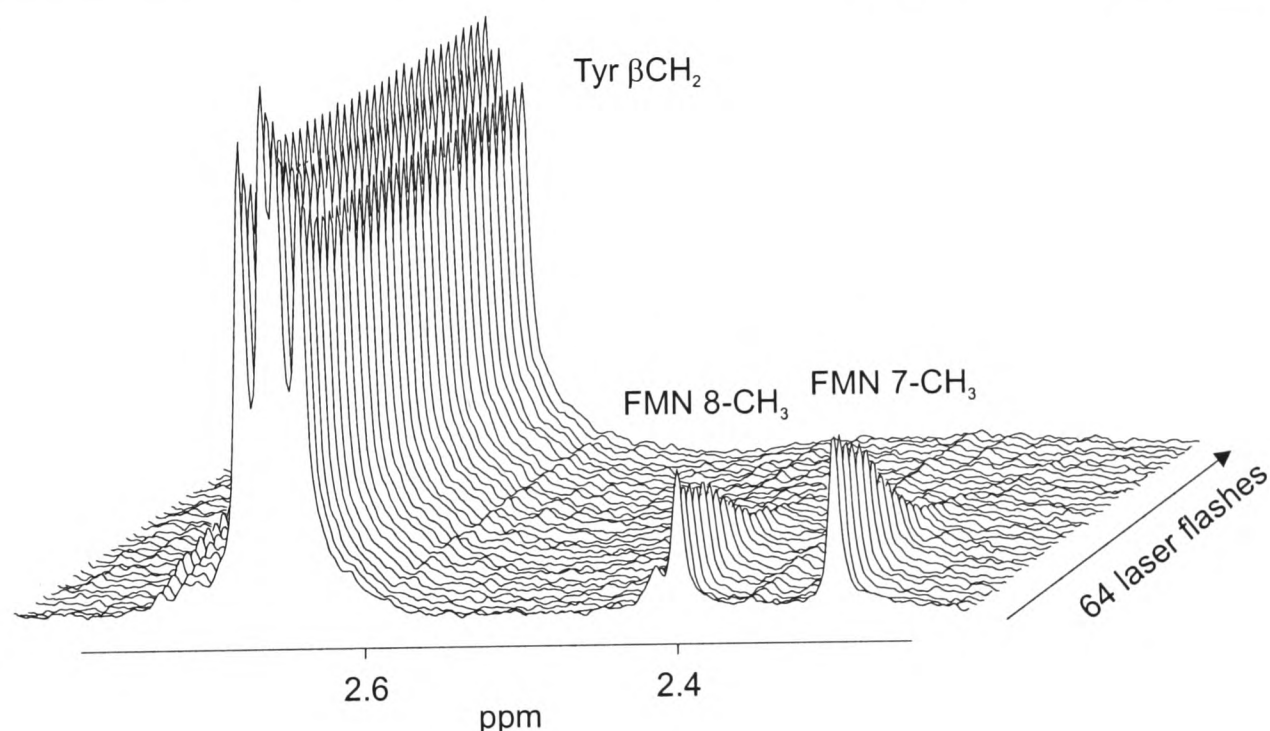


Figure 4.2: A series of 600 MHz 1H NMR spectra recorded under similar conditions to Fig.4.1 showing the 7- CH_3 and 8- CH_3 resonances of FMN (0.2 mM) and one of the β - CH_2 protons of tyrosine (4 mM, pH 4.5). Each spectrum was recorded after a 4 W laser pulse (100 ms) with a long enough delay to allow the CIDNP to relax to the equilibrium polarizations.

the light flashes that precede each scan, the FMN peaks decay rapidly, suggesting that progressive loss of CIDNP is due to photochemical degradation of the flavin rather than the amino acid. Figure 4.3 compares the NMR spectrum obtained (a) after *extensive* irradiation (256×500 ms 4 W pulses) to that of (c) intact FMN and (b) $FMNH_2$ produced chemically by addition of the reducing agent sodium dithionite. The variety of unassigned peaks observed in the irradiated spectrum, apparently due to photo-products of FMN and possibly $FMNH_2$, suggest that the photochemistry is highly complex. This was confirmed by comparing the visible absorption spectra (Fig.4.4) of the three samples described in Fig.4.3. Whilst the irradiated spectrum appears intermediate to that of FMN and $FMNH_2$ over the 400-500 nm

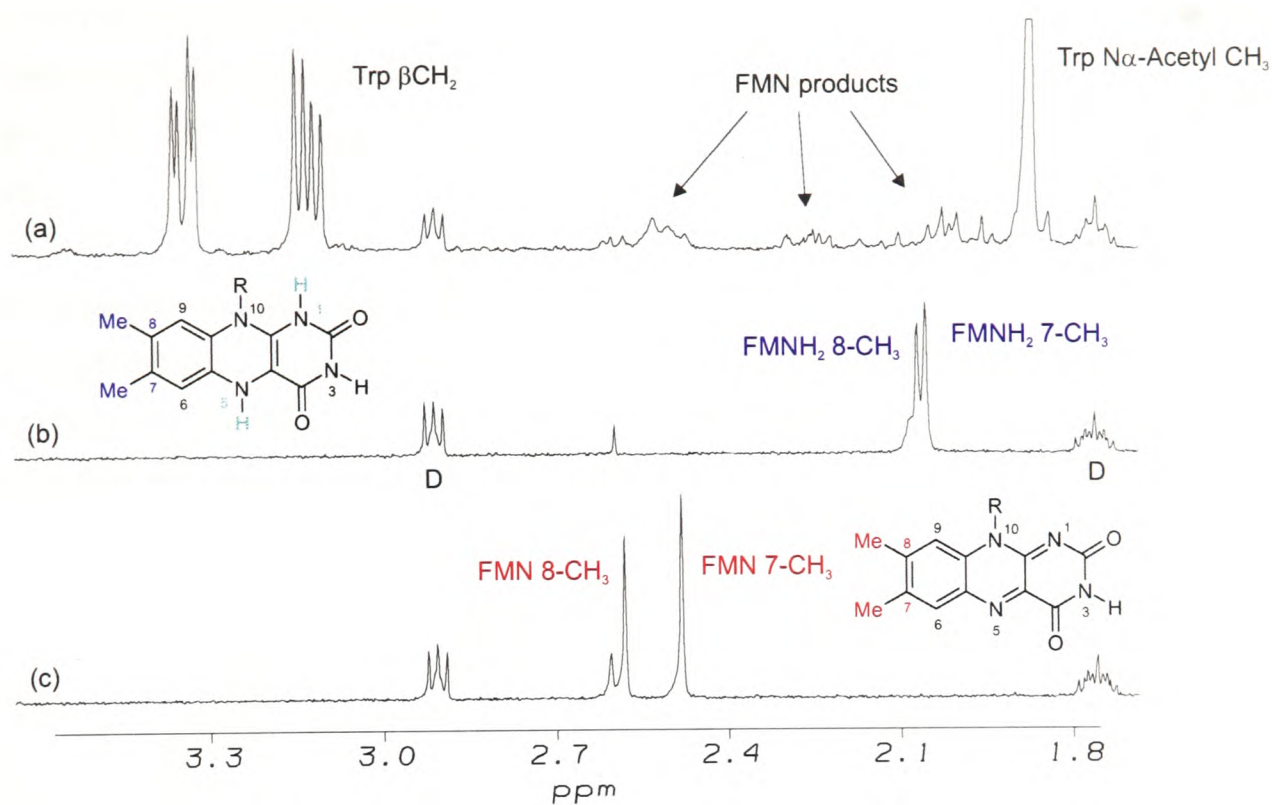


Figure 4.3: 500 MHz ¹H NMR spectra of the aliphatic region of (a) a mixture of tryptophan (2 mM, pH 7) and FMN (0.2 mM) recorded after 256 4 W laser flashes (500 ms), (b) FMNH₂ (0.2 mM, pH 7) produced chemically from FMN by addition of excess sodium dithionite (a reductant) and (c) FMN (0.2 mM, pH 7). The extra protons on FMNH₂ have been highlighted in cyan. Peaks labeled with a D denote resonances from the reference compound DSS. Assignments of flavin peaks were taken from Ref.[132].

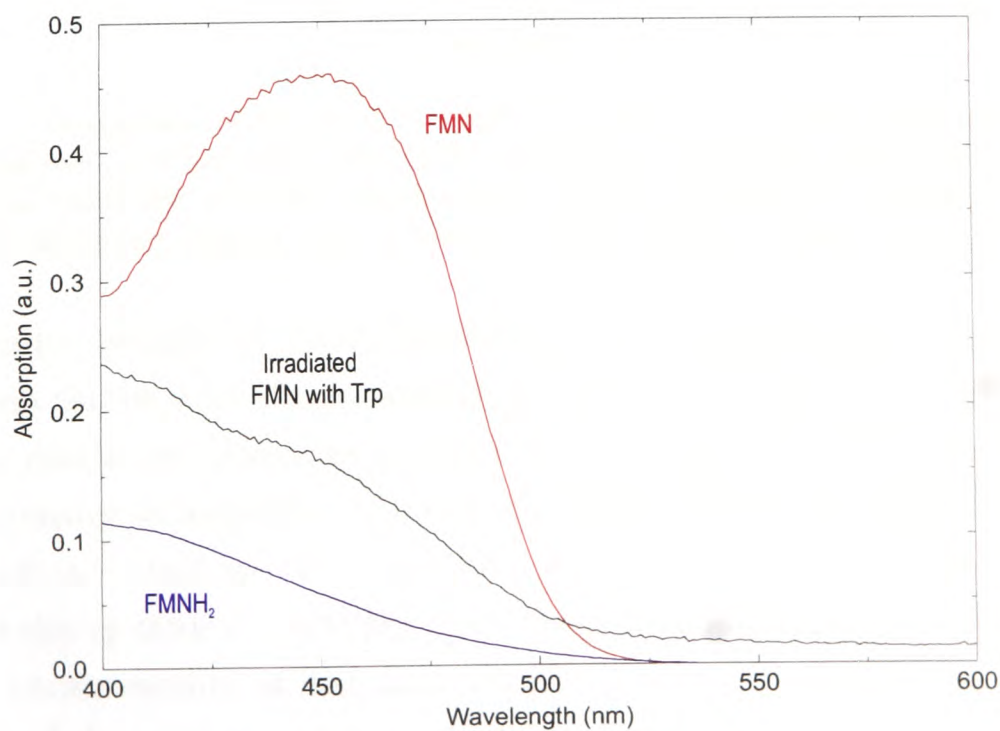


Figure 4.4: Visible absorption spectra (400–600 nm) of the three samples described in Fig.4.3. Note the weak tail that extends at higher wavelengths for the irradiated sample that is absent in both the spectra of the oxidised and reduced forms.

range, a weak tail extends at higher wavelengths that is absent in both the spectra of the oxidised and reduced forms. It is interesting to note that the semi-reduced flavosemiquinone radical shows a strong absorption peak in this higher wavelength range [129, 133], indicating a probable equilibrium between the three flavin redox states.

In an attempt to assess the extent of photo-degradation in the presence of histidine and tryptophan, it was noticed that histidine polarizations decay far more rapidly than those of tyrosine and tryptophan. This is shown in Fig.4.5 where the CIDNP decay rates for the three amino acids are compared under identical conditions. In

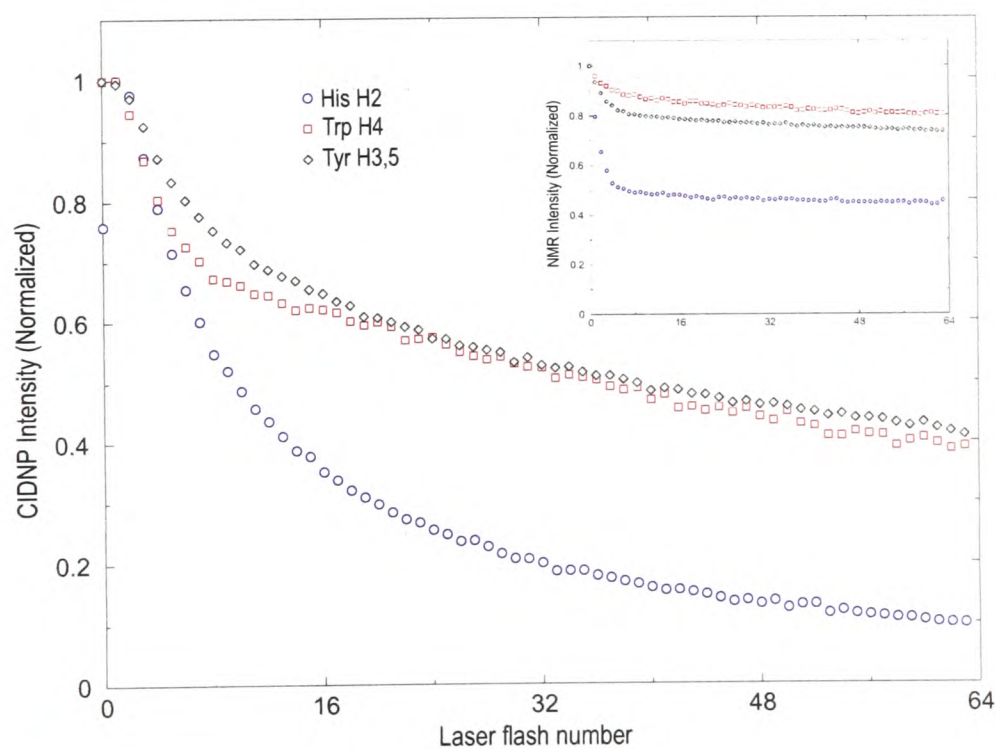


Figure 4.5: A comparison of the decay in CIDNP intensity for tyrosine (black diamonds), tryptophan (red squares) and histidine (blue circles). The inset shows the NMR decay under similar conditions. The rapid loss of NMR intensity and CIDNP polarization for histidine suggests that degradation of the amino acid can also contribute to CIDNP decay. [Amino Acid] 2 mM, pH 7.

order to monitor changes in the composition of the three different samples the amino acid NMR intensities were also measured in a series of spectra taken in between laser flashes. The results are shown as an inset to Fig.4.5 and reveal a dramatic decay in the NMR intensity of histidine. The rate of CIDNP decay was also found to depend sensitively on the histidine concentration, with a 50 mM sample decaying significantly more slowly than a 1 mM sample. These observations are consistent with an irreversible photoreaction of histidine sensitized by triplet flavin, and suggest that degradation of the amino acid can also be to blame in some cases. To assess the extent of photo-damage in a protein, the CIDNP decay of bovine α -lactalbumin was measured. As was described in Chapter 1 (Fig. 1.10), bovine α -lactalbumin possesses one exposed tryptophan, tyrosine and histidine [12, 134] and therefore allows us to compare the relative rates of decay of the three residue types in a protein.

Figure 4.6 shows the CIDNP decay for each residue. Although there is some at-

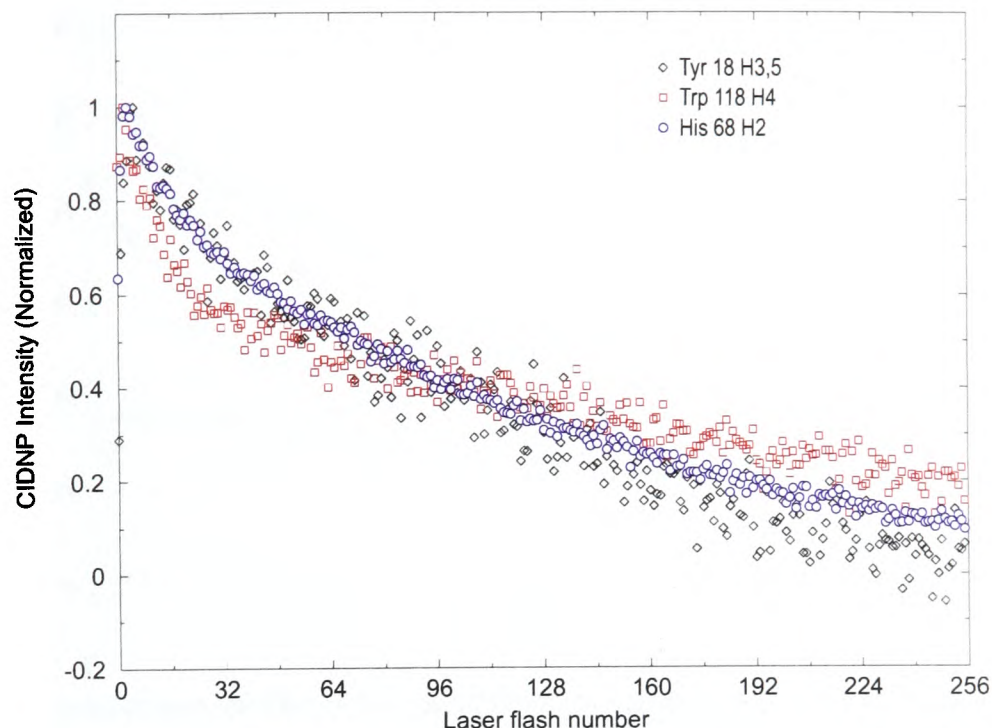


Figure 4.6: A comparison of the decay in CIDNP for Tyr 18 (black diamonds), Trp 118 (red squares) and His 68 (blue circles) in bovine α -lactalbumin (0.6 mM, pH 7, 20 °C). That no change in the relative polarizations of the tyrosine, tryptophan and histidine residues is seen suggests that photo-damage is restricted to FMN.

tenuation in the enhancements, there is no change in the relative intensities of the tryptophan, tyrosine and histidine signals, suggesting that at least in this protein, photo-damage of histidines does not seem to be a significant problem. Note however that *extensive* photolysis of hen lysozyme in the presence of flavin has been shown to cause a significant reduction in its thermal denaturation temperature [63] and photo-damage to exposed tryptophan residues [135].

The loss of polarization per light flash can be attenuated somewhat by using a larger initial FMN concentration (Fig.4.7), but only at the expense of a smaller initial enhancement, associated with the increased optical density which limits the amount of light reaching the coil region. The optimal FMN concentration (0.2 mM) was therefore used throughout. As might be expected, reducing the laser power or the length of the laser flash was also found to slow the rate of CIDNP decay substantially, but again at the expense of a smaller initial polarization. Interestingly, varying the delay in between flashes was found to have only a very minor effect on the rate of CIDNP decay. Increasing the delay from 500 ms to 30 s only led to a 10% intensity gain after 64 laser flashes, this together with the highly localized bleaching of samples, suggests that diffusion of fresh dye into the irradiated region of the NMR tube is very slow. To facilitate comparison, all experiments described in this chapter were performed using laser flashes of 100 ms each at a power of 4 W and separated by a 10 s delay.

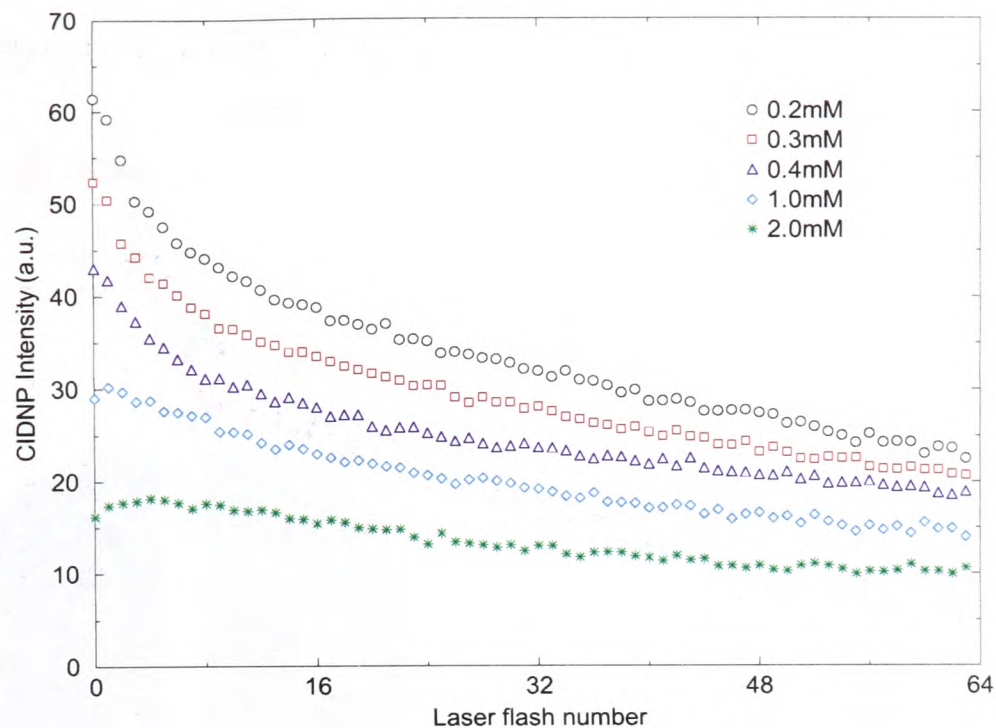


Figure 4.7: A comparison of the decay in CIDNP for tryptophan (2 mM, pH 7) with a range of FMN concentrations. The decay rate is attenuated somewhat by using a larger initial FMN concentration, but only at the expense of a smaller initial enhancement

4.3 Mechanical mixing

Poor diffusion within the NMR tube highlights the potential benefits of mixing the sample in between irradiations. All experiments carried out in this thesis were done on *non-spinning* samples using an optical fibre and coaxial insert to introduce light into the sample. An alternative mixing approach to rapid spinning [97, 98] was therefore devised. The experimental set-up is identical to that used in Chapter 5 for initiating protein folding and is shown Fig.4.8. A thin (0.5 mm internal diameter) PTFE tube is introduced into the sample via a small hole in the coaxial insert and connected to a pneumatically driven syringe (model 2.5MDR-GT, SGE) outside the magnet. The syringe, PTFE transfer line and NMR tube are all filled with the same protein/flavin solution (~ 1.5 ml). The idea is then to inject, as rapidly as possible, typically 300 μ l of solution into 500 μ l in the NMR tube, suck it back again, and wait ~ 10 s before each light flash and signal acquisition. If done sufficiently rapidly (the syringe was driven at a pressure of 10 bar) this efficiently mixes the bleached and oxygen-depleted portion of the sample with the remainder of the solution in the NMR tube, PTFE tubing and syringe. Although somewhat crude, this technique is surprisingly effective, as shown in Fig.4.9. Mixing dramatically prolongs the lifetime of the enhancements of both tyrosine (Fig.4.9a) and the two exposed tryptophan residues in hen lysozyme (Fig.4.9b). Whilst the enhancement drops to half its initial intensity after 32 flashes without mixing, it takes 512 flashes to reach that level with mixing.

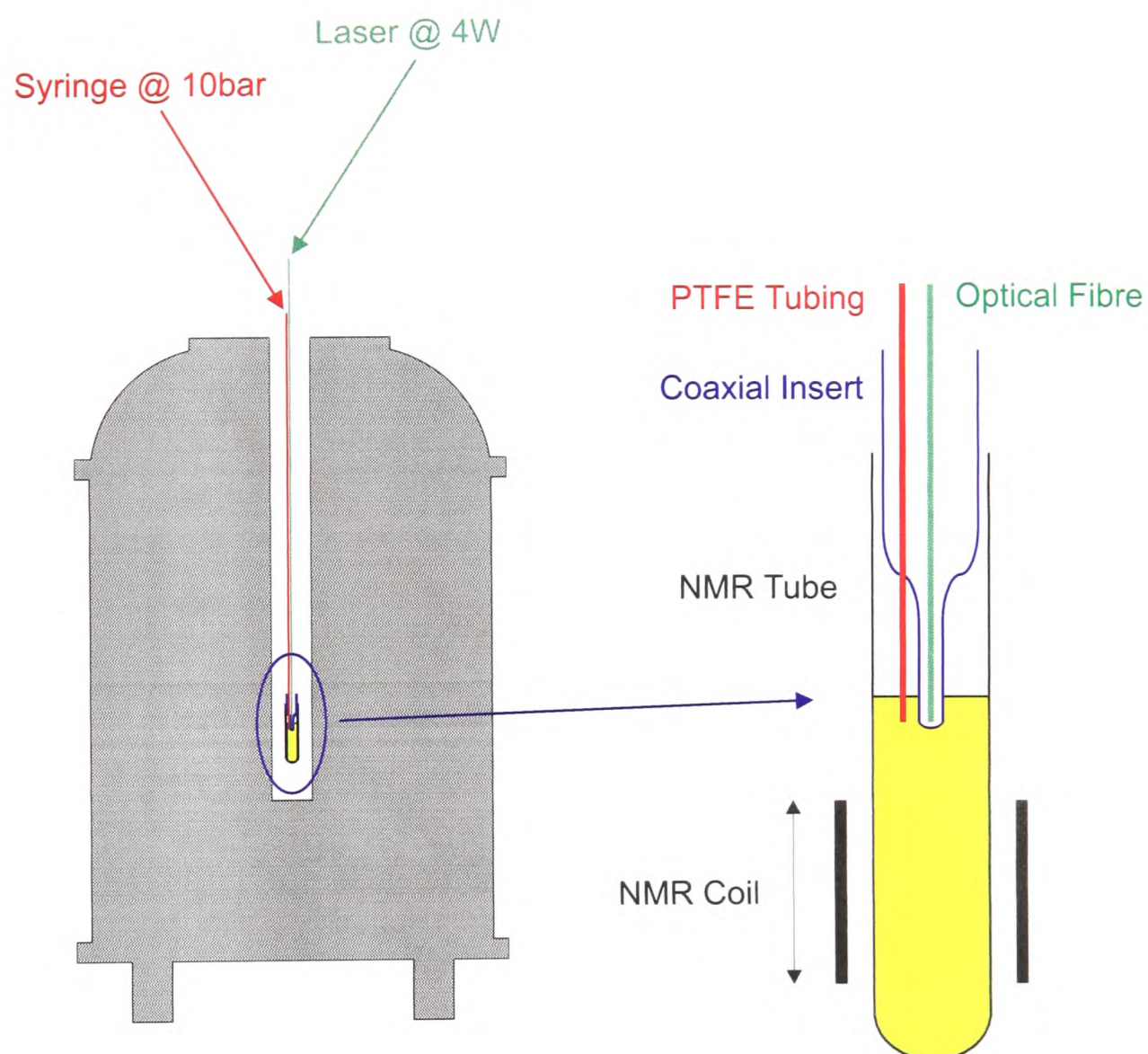


Figure 4.8: The rapid mixing set-up.

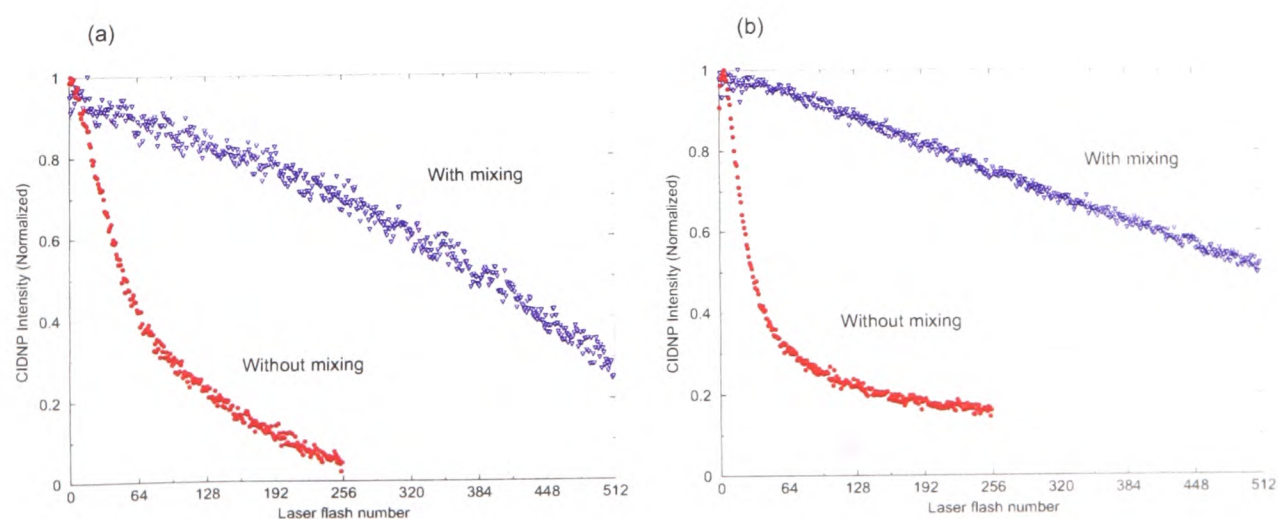


Figure 4.9: (a) CIDNP intensities of the H3,5 protons of tyrosine (4 mM, pH 4.5) with (blue triangles) and without (red circles) mixing of the NMR sample. (b) A similar experiment on hen lysozyme (1.5 mM, pH 5.2) showing the decay of Trp 62 signals. Note the prolonged lifetime of the polarization for the mixed solutions.

4.4 Chemical oxidation

An alternative approach to the problem of FH_2 formation would be to perform the CIDNP experiment in the presence of an oxidising agent. This would either obviate the need for the PTFE tubing, or would allow the injection system to be used for another purpose, e.g. stopped-flow dilution of a chemically denatured protein into a buffer solution for real-time studies of folding as described in Chapter 5. Figure 4.10 shows the effect of 10 mM hydrogen peroxide (an oxidant) and, for contrast, sodium

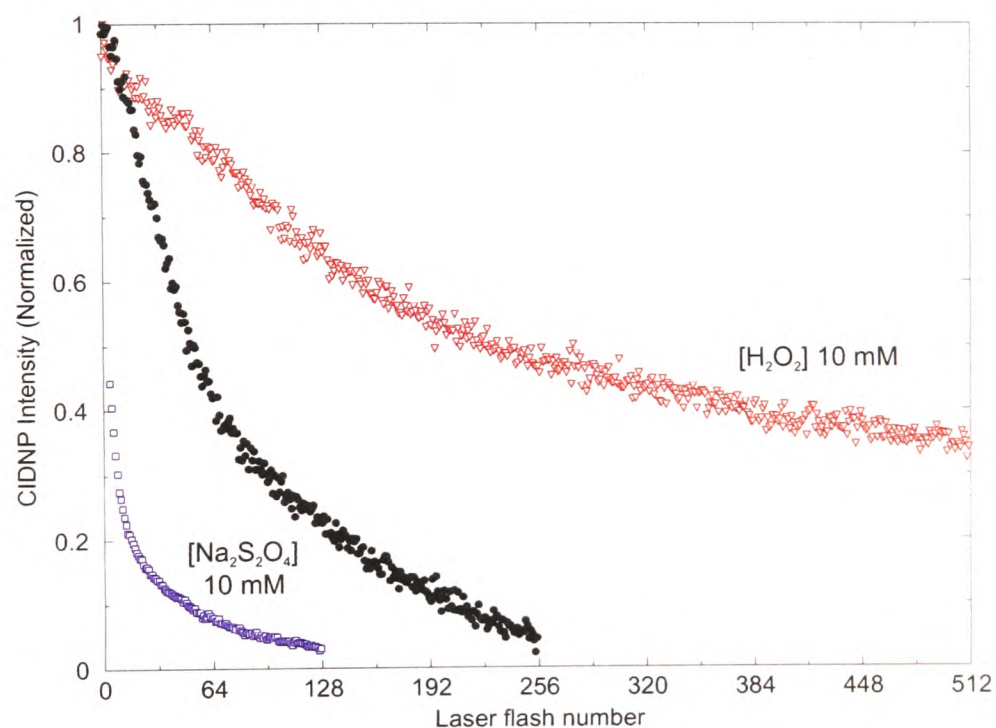


Figure 4.10: CIDNP intensities of the H3,5 protons of tyrosine (4 mM, pH 4.5), with 10 mM hydrogen peroxide (red triangles), 10 mM sodium dithionite (blue squares), and without additives (black circles). Sodium dithionite was only injected into the solution after the first CIDNP spectrum had been acquired.

dithionite (a reductant) on the tyrosine CIDNP decay. As expected the dithionite greatly accelerates the loss of signal, whereas the peroxide has the opposite effect. Sequential NMR spectra, recorded under similar conditions to those in Fig.4.2, but with 10 mM H_2O_2 are shown in Fig.4.11. The deceleration in the disappearance of the FMN peak is marked, suggesting that H_2O_2 present in the sample is effectively oxidising some of the FH_2 back to F. The CIDNP intensity of tyrosine (2 mM, pH 7) after 128 light flashes relative to the initial intensity was measured as a function of H_2O_2 concentration (data not shown); the protection against FMN photo-reduction was found to reach a plateau at 10 mM H_2O_2 . It is interesting to note at this stage that recent experiments carried out on bovine α -lactalbumin used with and without prior purification by simple dialysis found that CIDNP signals decayed substantially faster with unpurified samples [136]; in addition whilst no benefit was observed in the presence of 10 mM H_2O_2 for these samples, a substantial improvement was observed for purified samples. These results suggest that impurities, possibly some of the reducing agents used in protein synthesis, if left unremoved can dramatically

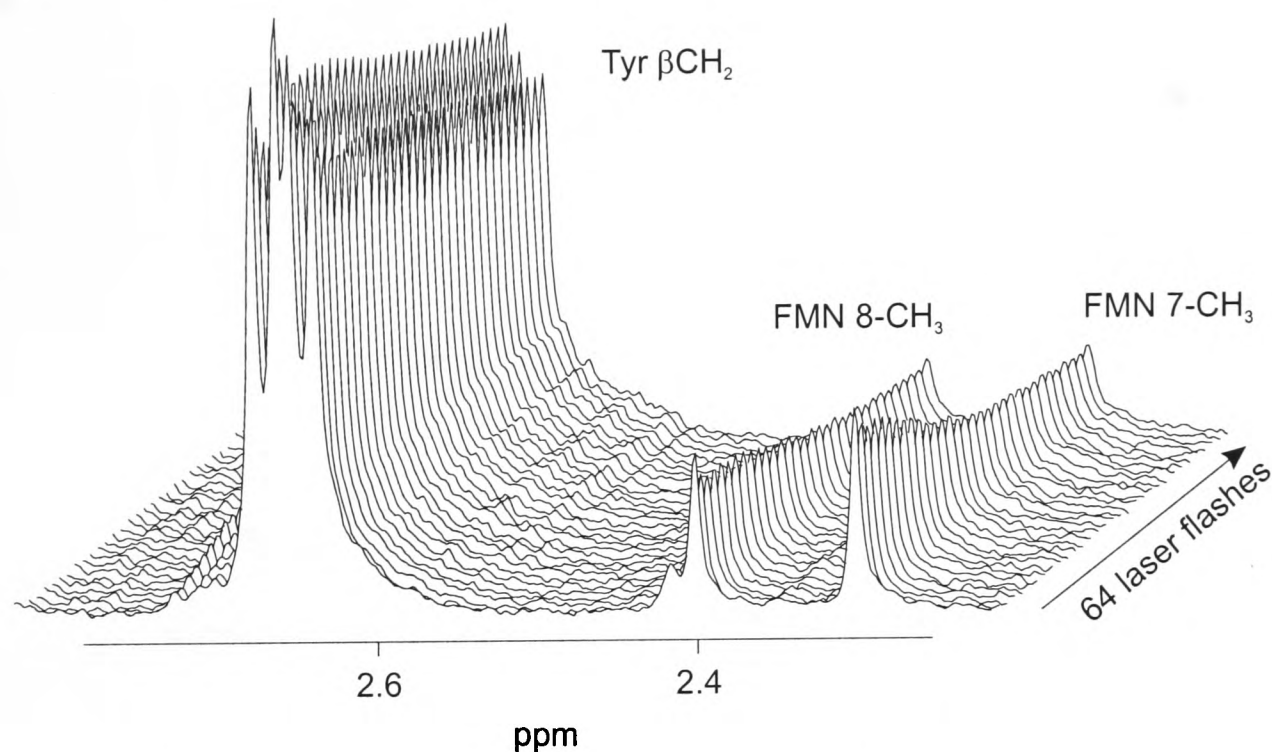


Figure 4.11: A series of 64 NMR spectra recorded under the same conditions as Fig. 4.2 ([Tyr] 4 mM, [FMN] 0.2 mM, pH 4.5) except for the presence of 10 mM hydrogen peroxide. The FMN methyl resonances decay much less rapidly reflecting the re-oxidation of reduced flavin by H_2O_2 .

accelerate photo-degradation in photo-CIDNP experiments.

In order to assess any potential oxidative damage to the protein caused by the presence of 10 mM H_2O_2 a number of spectroscopic controls were run. Native hen lysozyme (1.5 mM, pH 5.2) was used for all experiments and spectra were recorded 24 hours after sample preparation. The amide fingerprint region and indole region of the 2D ^{15}N - ^1H HSQC spectra taken in the presence and absence of 10 mM H_2O_2 were essentially identical (data not shown). Figure 4.12 shows the aromatic region of the 1D ^1H spectra taken as a function of H_2O_2 concentration. No significant changes occur below 1 M, however above this concentration resonances appear to broaden and a dramatic loss of chemical shift dispersion is observed. 1D ^1H CIDNP spectra taken up to 1 M (Fig. 4.13) also appear identical. As a final control, mass spectra taken over a similar range of H_2O_2 concentrations are shown in Fig. 4.14. Below 100 mM, a single mass peak corresponding to the intact hen lysozyme is observed; above 100 mM peaks at $M+16$ and $M+32$ (tentatively assigned to $+O$ and $+O_2$) gradually appear and by 1 M significant broadening indicates substantial damage of the protein. Together these results indicate that the presence of *small* amounts of H_2O_2 seems to have no adverse effect on the protein or the CIDNP, however hen lysozyme is a robust protein, and care should therefore be taken in using H_2O_2 with less stable proteins (e.g. with ribonuclease A the $M+16$ peak was already present at 10 mM H_2O_2) where oxidative damage to surface residues might occur.

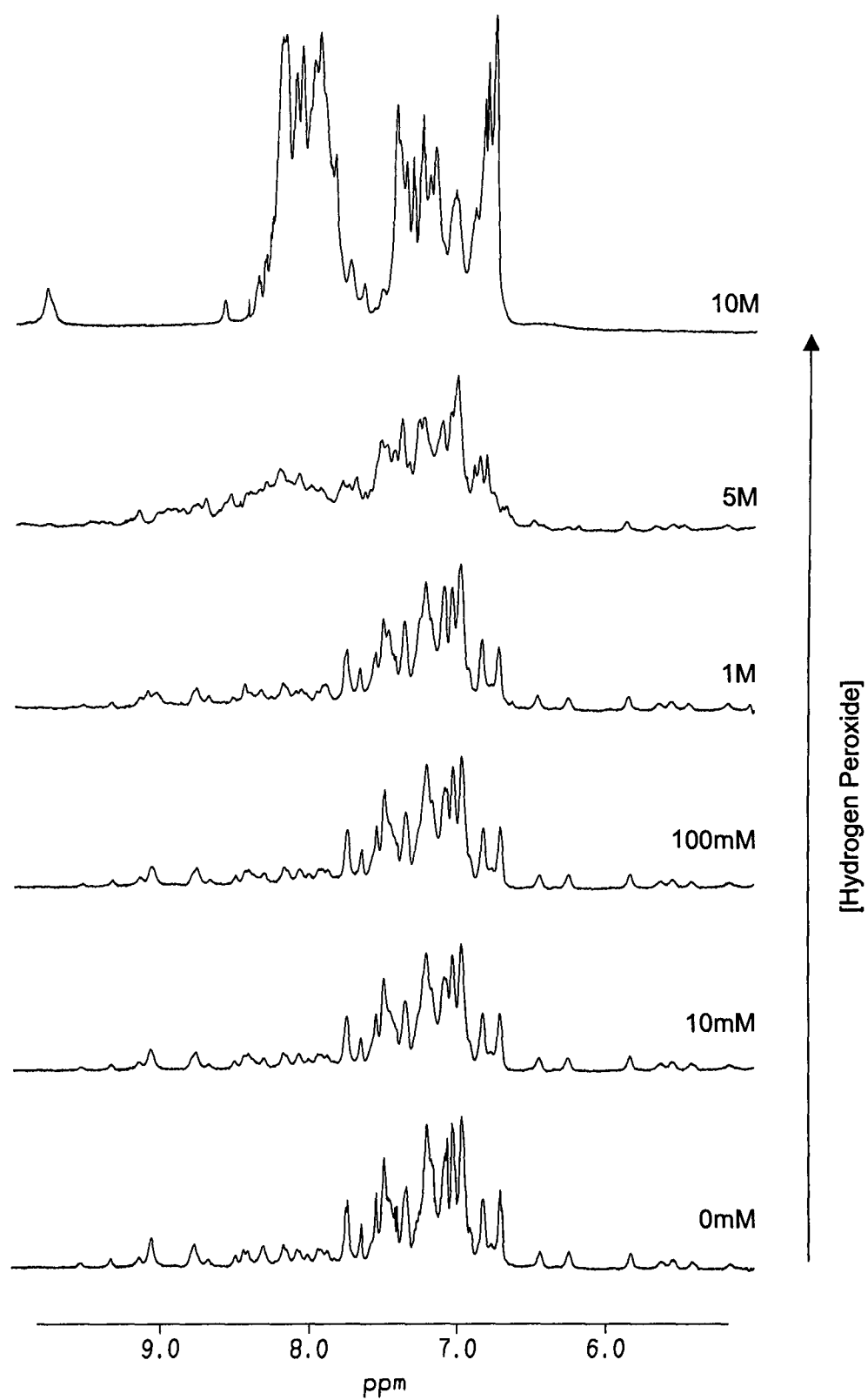


Figure 4.12: Aromatic region of the 600 MHz ^1H NMR spectrum of hen lysozyme (1.5 mM, pH 5.2) taken at the concentrations of H_2O_2 shown. Samples were prepared 24 hours before recording the spectra.

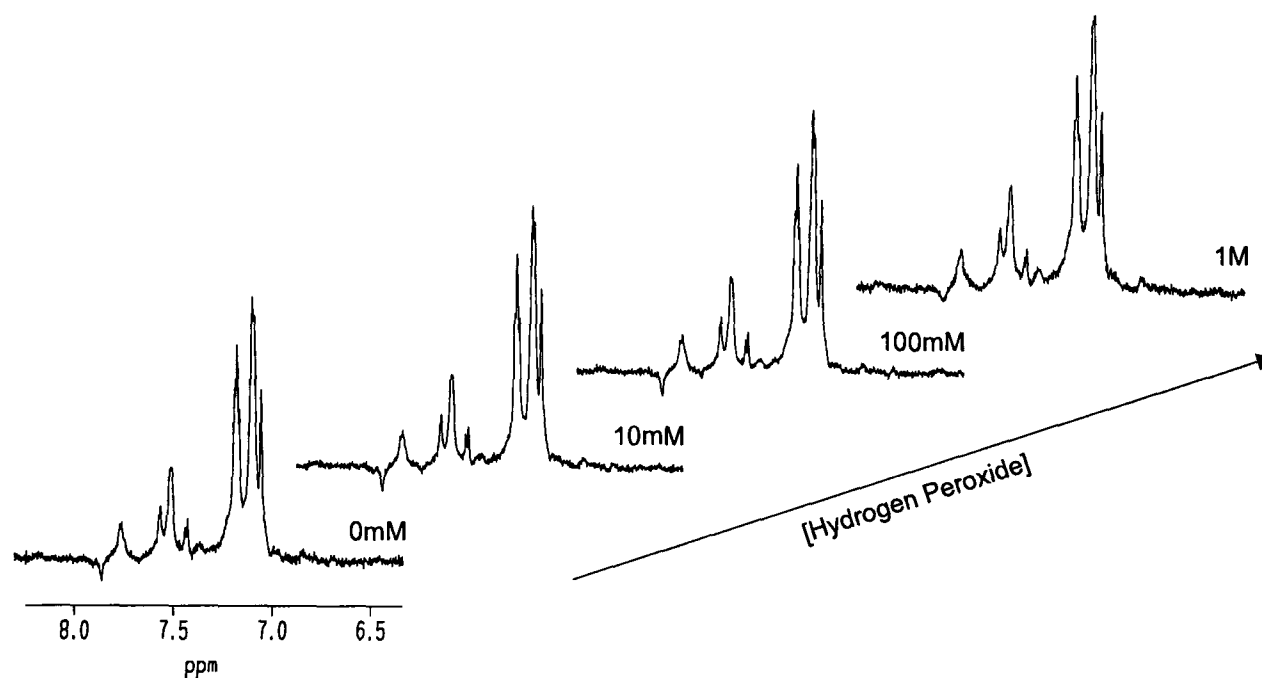


Figure 4.13: 600 MHz ¹H photo-CIDNP spectra of hen lysozyme (1.5 mM, pH 5.2) taken at the concentrations of H₂O₂ shown. Samples were prepared 24 hours before recording the spectra. Spectra up to 1 M appear identical.

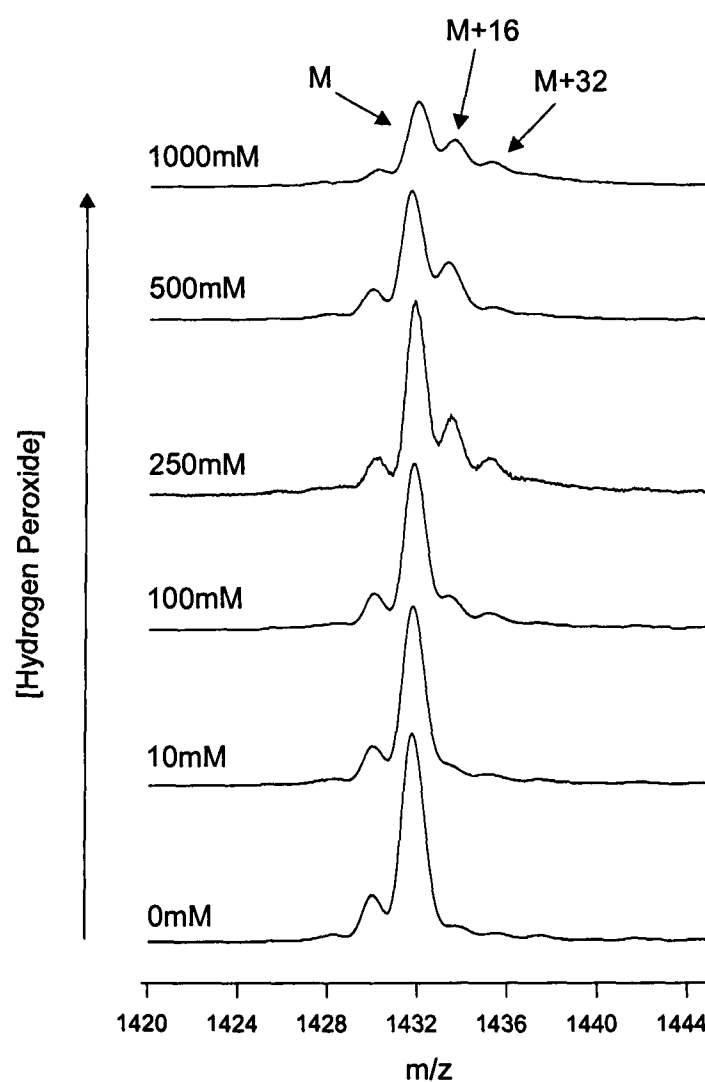


Figure 4.14: Mass spectra of hen lysozyme (30 μM, pH 5.2) as a function of H₂O₂ concentration. The peak shown corresponds to a charge $z=+10$. New peaks at $M+16$ and $M+32$ are observed above 100 mM indicating damage to the protein. Samples were prepared 24 hours before recording the spectra on a Micromass Q-TOF spectrometer. Spectra were kindly recorded by Ms.M.McCammon.

4.5 Applications

4.5.1 Mechanical mixing - Two-dimensional CIDNP spectroscopy

Loss of flavin by photo-reduction presents severe problems for two-dimensional CIDNP spectroscopy [97, 98]. Even with rapid sample spinning between scans, the number of t_1 increments has hitherto been limited to 64 or 128, with just one transient per increment, without f_1 quadrature detection, and with a spectral width in f_1 just wide enough to cover the aromatic region of the ^1H spectrum [97, 98, 120, 137]. The results presented in Section 4.3 for tyrosine (Fig.4.9a) and hen lysozyme (Fig.4.9b) suggest that mechanical mixing might allow up to 512 successive laser flashes in a given 2D CIDNP experiment. Such an increase in the total number of laser flashes could allow signal averaging (e.g. 8×64 or 4×128) or alternatively an increase in the resolution (e.g. 1×512) with or without an increase in the spectral width in f_1 to include the aliphatic region of the ^1H spectrum. To illustrate this, Fig.4.15 compares the aromatic region from 600 MHz CIDNP-NOESY spectra [97, 98] of bovine α -lactalbumin (1.5 mM, pH 7.4, 65 °C) obtained with and without mechanical mixing. Spectra were taken with 128 increments in t_1 , one transient per increment, *with* quadrature detection (i.e. 256 laser flashes in total) and no folding in the f_1

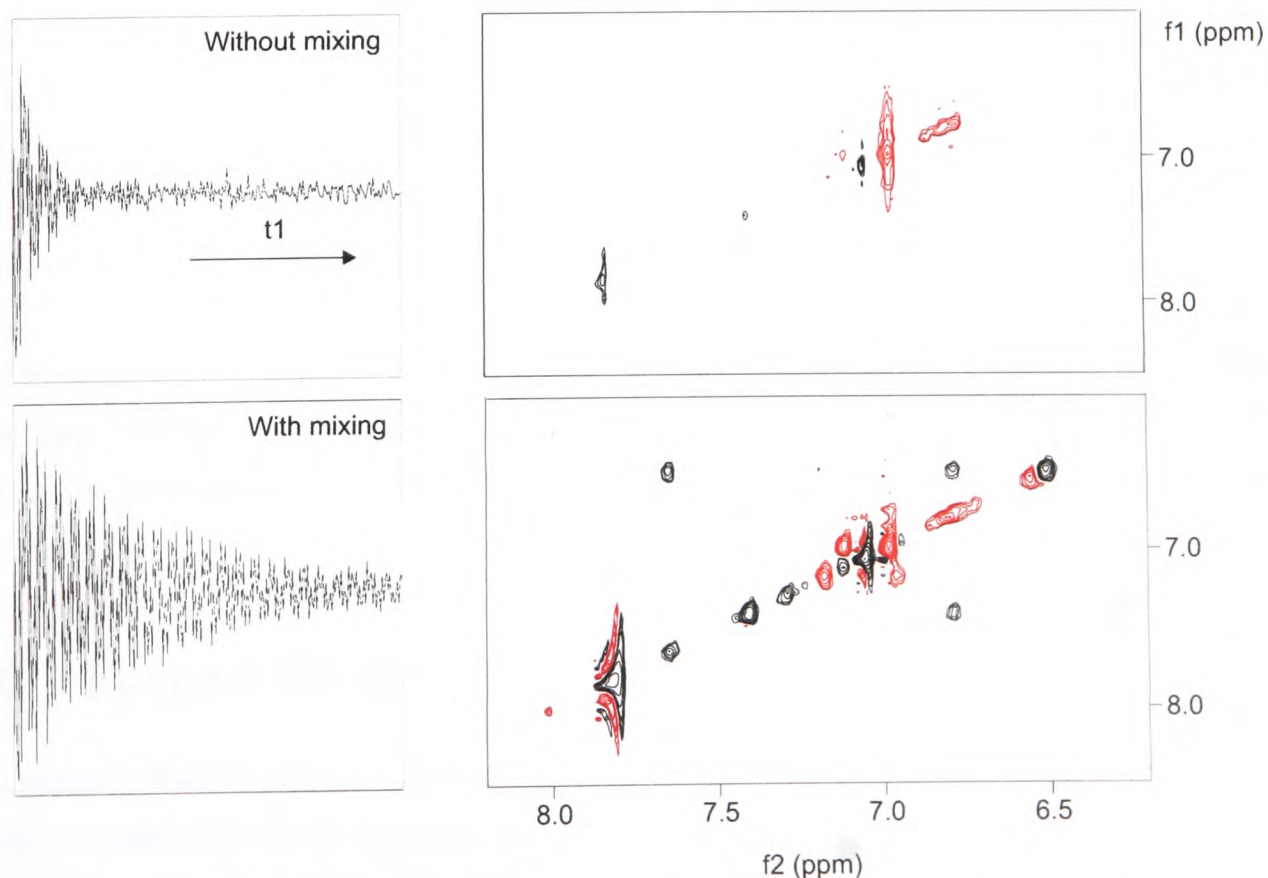


Figure 4.15: Aromatic region of 600 MHz CIDNP-NOESY spectra of bovine α -lactalbumin (1.5 mM, pH 7.4, 65 °C) obtained with and without mechanical mixing. Spectral widths are 8000 Hz in both dimensions, with 126 complex points in f_1 (i.e. two irradiations per t_1 increment). To suppress the background magnetization a “dark” spectrum was acquired and automatically subtracted from each “light” spectrum. Mixing was performed by injection of 300 μl into 500 μl after every flash. Interferograms taken from the 2D data set before Fourier transformation in the f_1 dimension are also shown for comparison.

dimension. Interferograms taken from the 2D data set before Fourier transformation in the f_1 dimension are also shown for comparison. Without mixing, the signal intensity is so weak and the linewidths in f_1 are so large that cross peaks are almost or completely indiscernible. When recorded with the mixing device on the other hand, the spectrum has clearly resolved intraresidue and interresidue cross peaks.

The potential for such a method has already been alluded to with reference to the two-dimensional ^{15}N - ^1H heteronuclear correlation experiment presented in Chapter 3 and in Chapter 7 it will be applied to two-dimensional CIDNP-EXSY/NOESY experiments on bovine α -lactalbumin.

4.5.2 Chemical oxidation - Real-time observation of protein folding

Chapter 5 will describe how photo-CIDNP in combination with a stopped-flow technique can be used to monitor the changes in side-chain accessibility that occur as a protein refolds. When refolding is fast (<1 s), as in the refolding of hen lysozyme from 10 M urea, a single CIDNP spectrum can be acquired per refolding experiment [20, 60]. The time dependence is therefore studied in a series of “one-shot” experiments in which a single CIDNP spectrum is acquired at a different time after the start of refolding. As each solution is used only once, the issue of sample degradation does not arise. However, it could be a significant problem if one were to try to record multiple spectra in the course of a somewhat slower folding reaction (>10 s), in the manner of a recent real-time NMR study of bovine α -lactalbumin in the absence of bound calcium [138].

In Chapter 5 the potential for such a method will therefore be demonstrated by real-time CIDNP observation of the slow refolding of bovine α -lactalbumin following rapid dilution from a high concentration of chemical denaturant in the absence of calcium.

4.6 Future Work

A number of alternative approaches to those presented in this chapter are currently being considered. One approach under investigation involves adding small quantities of concentrated (10 mM) flavin solution to the NMR sample between scans, in an attempt to replace that lost by reduction. This can conveniently be done using the set-up described for mixing by connecting the syringe to a small stepper motor controlled by the spectrometer. However, a number of difficulties have been encountered during preliminary experiments [139]. In particular it is difficult to know in advance how much flavin to add and at what rate; in addition reliance on diffusion to transfer flavin into the coil region causes an inhomogeneous dye concentration to

build up in the sample tube. Combining this “leakage” approach with the mixing device described earlier might alleviate such problems and is also being considered. A quite different approach to the problem would be to use a dye/laser combination that showed more favourable reversibility of the photoreaction. Various dyes have been investigated as alternatives to flavins [12]. In particular a number of xanthenes, quinones, aromatic ketones and aza-aromatics [63, 71, 140–142] have been shown to produce CIDNP with certain amino acids when excited at 308 nm. Unfortunately, most suffer from extraneous product formation and undergo non-cyclic reactions with amino acids and proteins.

Chapter 5

Kinetic studies of protein folding using photo-CIDNP

The potential of stopped-flow photo-CIDNP spectroscopy for the study of protein folding is explored. Rapid dilution of denatured protein into a buffer solution is used to initiate a refolding process which is followed using short laser pulses to generate ^1H CIDNP in the side-chains of exposed aromatic residues. The experiment is first tested on bovine α -lactalbumin (BLA) in the absence of calcium denatured in 6 M guanidinium deuteriochloride (GdmDCl) before being applied to single tryptophan mutants of histidine-containing phosphocarrier protein (HPr) also denatured in 6 M GdmDCl. In both cases, a transient intermediate is formed in the dead time of the experiment. Quantitative approaches for the analysis of the subsequent two-state conversion of this intermediate to the native state are proposed.

5.1 Introduction

The ultimate objective of any experimental description of the protein folding process is to define in molecular detail the ensembles of structure that exist at different stages of the reaction. This is most readily achieved *in vitro* by rapidly transferring a protein from denaturing conditions to an environment in which the native conformation is favoured and monitoring refolding in real-time with biophysical techniques capable of probing different aspects of the native fold [42, 44]. In this context, a whole host of complementary stopped-flow optical techniques such as intrinsic fluorescence [143], fluorescence anisotropy [144] and circular dichroism in the near- and far-UV [46] have been developed. The inherent sensitivity and fast relaxation properties of spectroscopy in the high frequency UV-visible region make such techniques ideally suited to the kinetic characterization of fast folding (>10 ms) processes. However, fast relaxation is associated with low spectral resolution and the measured signal therefore tends to be averaged both within the protein structure and over the hetero-

geneous folding population. As a consequence, whilst these techniques are crucial in describing the folding process, they reflect global changes in structure and are therefore limited to general concepts such as hydrophobic collapse and the formation of secondary and tertiary structure. NMR spectroscopy has therefore played a significant role here through its ability to provide *high resolution* spectra and hence a detailed and site specific definition of the folding process [43]. Of particular importance has been its application in the analysis of the distribution of deuterium in specific backbone amide sites resulting from quenched flow hydrogen exchange pulse labeling procedures [145]. In this chapter, however, the far simpler and more direct use of NMR spectroscopy to study folding as it takes place in *real-time* [146, 147] will be discussed. In particular, attention will be focused on the combination of real-time NMR and photo-CIDNP as recently demonstrated by Hore and Winder [20, 60].

5.2 Real-time NMR and photo-CIDNP

The simplest of real-time NMR applications involves initiating refolding within the magnet and directly monitoring the folding process by rapidly acquiring a series of 1D ^1H spectra. In sharp contrast to the stopped-flow optical techniques described earlier, this provides simultaneous information on the rates of formation of all of the tertiary interactions that produce resolved resonances. The inherent low sensitivity of NMR spectroscopy and the slow relaxation of nuclear spins can however place significant limitations on the applicability of the experiment. Conventionally, refolding is initiated by rapidly transferring a small volume of denatured protein from a lower field region of the magnet into a larger volume of refolding buffer already present within the NMR tube [138, 148]. Hence, whilst mixing may be complete within as little as 100 ms, there is an added wait necessary for spin-lattice relaxation to polarize the injected spins which can increase the experimental dead time by up to 1 s. In addition, the delay between subsequent acquisitions must be sufficiently long to allow recovery of Boltzmann population differences. For these reasons, real-time NMR has so far been limited to rather slow folding processes (>10 s) [138, 146–148]. In an attempt to reduce the dead time, an alternative mixing device in which both denatured protein and refolding buffer are initially at high field has recently been patented [149], however it requires significant probe modifications and has so far proved difficult to implement. Finally, whilst a large number of resonances can usually be resolved, protein 1D ^1H spectra remain relatively crowded. One particularly elegant approach to this problem recently pioneered by Frieden and co-workers [150–153] involves using ^{19}F NMR to study proteins in which specific residues (particularly aromatic ones) have been replaced by fluorinated analogues. The 1D ^{19}F spectra acquired contain a single resonance per labeled residue and are therefore

considerably less crowded. An alternative strategy using 2D approaches is also possible if folding is sufficiently slow. In this way, Baum and colleagues acquired a series of 2D ^{15}N - ^1H HSQC spectra (~ 4 minutes each) during the folding and assembly of ^{15}N labeled peptide fragments of collagen (~ 60 minutes) [123, 154].

In an attempt to address some of the limitations of real-time NMR, Hore and Winder recently proposed and developed a real-time photo-CIDNP experiment [20, 60]. Detection of CIDNP signals rather than equilibrium Boltzmann polarization has a number of interesting consequences. Direct information on surface exposure is provided; because polarization is induced in only a small number of residues the resulting spectra are relatively well resolved; and, there is in general a two to four fold sensitivity enhancement. In addition, the approach has a shorter experimental dead time than conventional NMR for two reasons. Nuclear polarization is produced during a 50 ms laser light flash, a somewhat faster process than waiting for spin-lattice relaxation to polarize spins that have been transferred from a lower field region of the magnet; and CIDNP signals come from the irradiated portion of the sample and hence, homogeneous mixing is only required in this volume (~ 50 - $100\ \mu\text{l}$) which is considerably smaller than the sensitive region of the NMR receiver coil ($\sim 300\ \mu\text{l}$). To illustrate the potential of the technique for the study of *fast* processes, the folding of hen lysozyme which is essentially complete within the dead time of a conventional NMR experiment was described. In this chapter a slightly modified device to that employed by Winder that requires no spectrometer modifications will be introduced and used to characterize the folding of bovine α -lactalbumin and histidine-containing phosphocarrier protein on a variety of timescales.

5.3 The rapid injection device

The rapid injection device used for all experiments described in this chapter is similar to that described by Winder [20]. However, rather than using a modified CIDNP probe with quartz rod and prism for laser irradiation a portable device using an optical fibre and coaxial insert was designed. As described in Chapter 2 this opens up the possibility of performing experiments on any available spectrometer.

As shown in Fig.5.1, to enable rapid transfer of denatured protein into the NMR tube, a thin (0.5 mm internal diameter) PTFE tube was introduced into the sample via a small hole in the coaxial insert and connected to a pneumatically driven syringe (model 500R-GT, SGE) outside the magnet. The syringe was embedded in a solid PTFE block to absorb mechanical shock and was mounted on an aluminium support along with the spectrometer controlled pneumatic triggering system [20]. The syringe and PTFE transfer line were filled and washed several times with D_2O ,

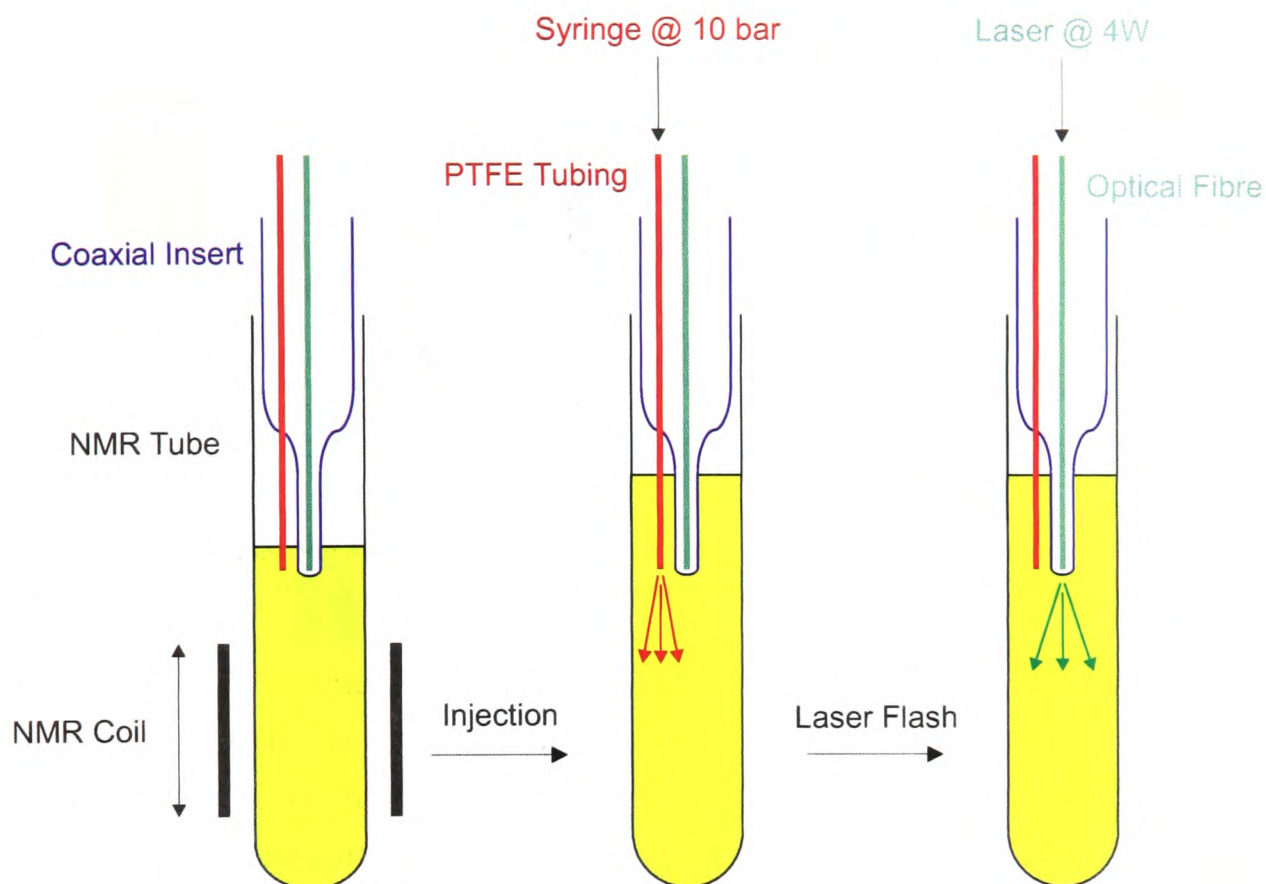


Figure 5.1: Rapid injection setup. The tip of the coaxial insert and PTFE tubing were held 4 mm above the top of the NMR coil.

and a small amount, typically $50\ \mu\text{l}$, of denatured protein was then sucked up into the end of the transfer line. The NMR tube was then filled with refolding buffer solution, typically $450\ \mu\text{l}$, and the coaxial insert with optical fibre and PTFE tubing was carefully placed into the NMR tube until both the insert and tubing dipped under the surface of the buffer solution 4 mm above the top of the NMR coil. With this arrangement, no significant loss in field homogeneity was observed and adequate linewidths were easily obtained. Shimming and tuning (particularly when using high salt concentrations as with GdmDCI) were first performed on a “dummy” sample with the denatured protein injected into the refolding buffer and then left unchanged for further experiments.

The pulse sequence used is shown in Fig.5.2 and the sequence of events in a typical experiment are shown schematically in Fig.5.1. A short injection pulse, typically 30 ms at 10 bar, transfers the denatured protein into the refolding buffer and hence initiates refolding. After a delay τ_{initial} a hard 90° r.f. pulse and short gradient pulse (G) destroy all background magnetization. A short 50 ms laser flash is then applied to generate polarization in exposed aromatic side-chains and a CIDNP spectrum is acquired. If folding is fast (<1 s) the whole process is simply repeated with a new sample and a different delay τ_{initial} . However, if folding is sufficiently slow (>5 s) several CIDNP spectra can be acquired in a single injection with the choice of delay between CIDNP spectra, τ_{recovery} , depending on the folding rate. As an example, whilst τ_{recovery} was set to 7 s for experiments on the very slow folding of bovine

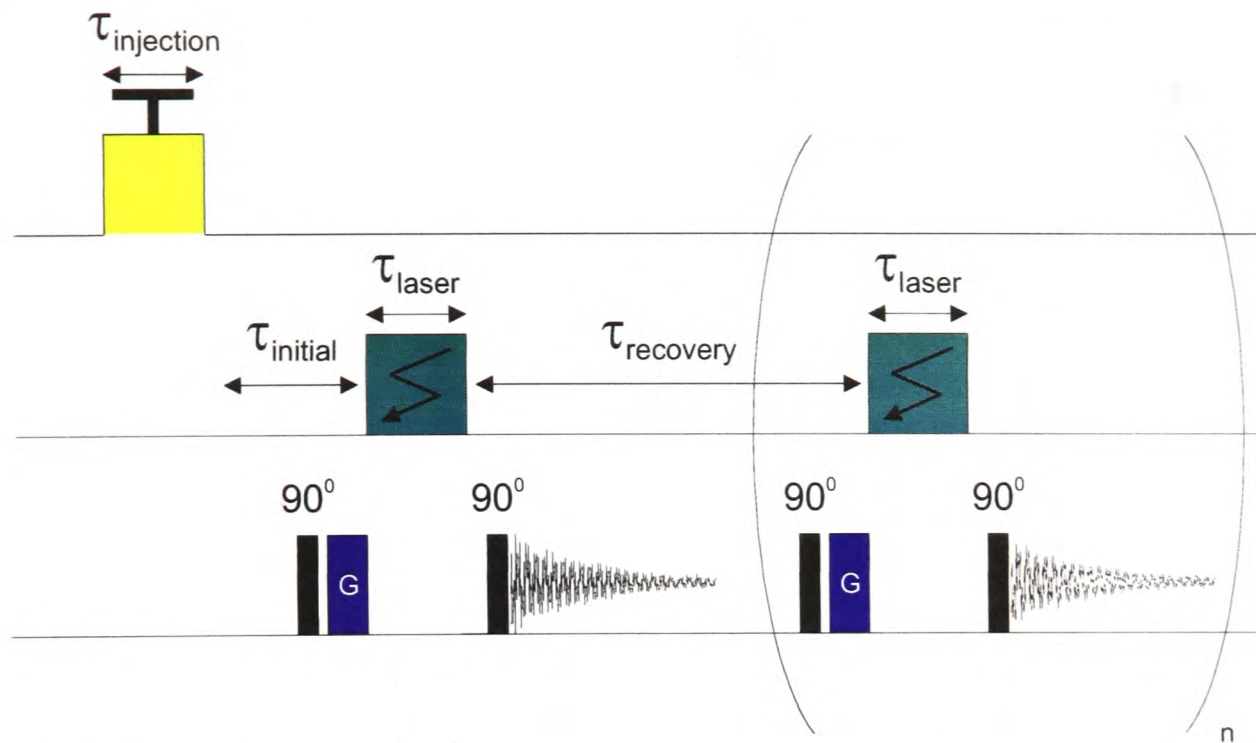


Figure 5.2: Pulse sequence for real-time observation of protein folding by photo-CIDNP. Presaturation of Boltzmann polarization prior to the laser flash was performed using a 90° r.f. pulse and short (<5 ms) gradient (G) pulse.

α -lactalbumin in the absence of bound calcium (complete in ~ 100 s) a much shorter 0.5 s delay was used for the faster folding HPr (complete in ~ 5 s).

5.4 Preliminary tests

As was highlighted by Winder [20, 60], the success of the technique depends crucially on the speed with which the protein solution can be transferred into the NMR tube, the recovery of the NMR lineshape from the shock of the stopped-flow and the efficiency of mixing. In order to assess these features, a number of preliminary experiments were therefore performed.

Injections were first examined outside the magnet by injecting a small quantity of methylene blue dye solution into water. The process was filmed at 25 Hz and the first few frames are shown in Fig. 5.3. The bulk of the mixture appears to be fairly homogeneous within 80 ms (2 frames) of injection. However, it is apparent that it takes several seconds to achieve full homogeneity in the extreme regions of the NMR tube during a far slower mixing phase. This slow phase was found to be significantly shortened by injecting greater volumes of dye and by sample spinning (using the design described by Winder [20]). In order to calibrate the system, the dependence of volume injected on the length of a 10 bar injection pulse was also measured, with a typical length of PTFE tubing (~ 2 meters) approximately 20 ms was required to inject $50 \mu\text{l}$ and 40 ms for $100 \mu\text{l}$.

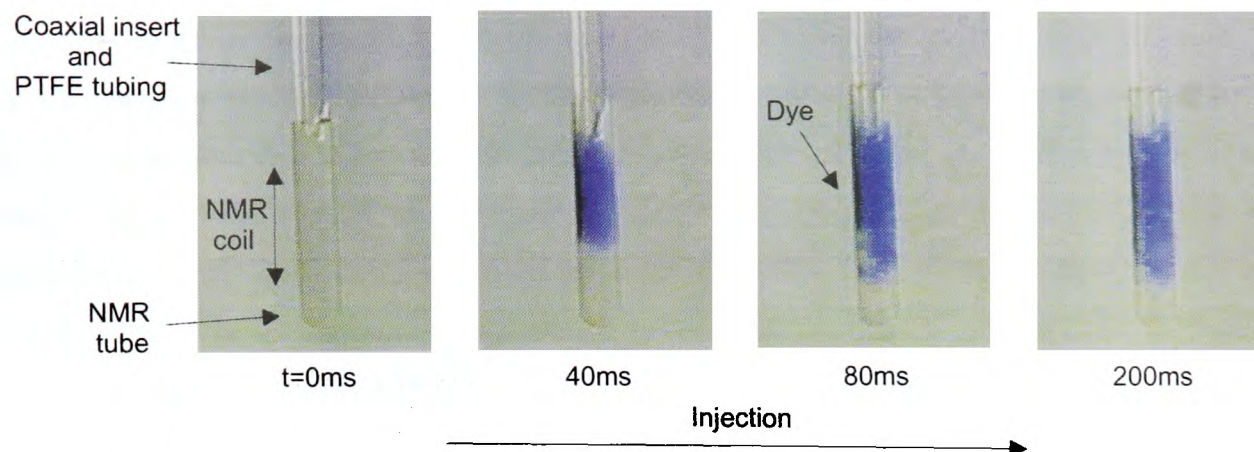


Figure 5.3: The injection of 70 μl of methylene dye at 10 bar into H_2O as filmed outside the magnet at 25 Hz. The 30 ms injection pulse occurred at some stage between the first two frames.

In an attempt to characterize the mixing and recovery timescales further, a series of photo-CIDNP controls were performed. The procedure involved injecting 70 μl of histidine solution (50 mM, pH 7) containing 10 M urea (to mimic the viscosity of denatured protein solutions) into 430 μl of flavin dye (0.25 mM, pH 7) with a 30 ms 10 bar pulse. After a variable delay (during which Boltzmann magnetization was saturated), a 50 ms 4 W laser flash was applied and the photo-CIDNP spectrum recorded. Under such conditions, no CIDNP signal can be observed until the histidine and flavin have been brought into intimate contact by mixing of the two solutions. The final concentration of histidine (7 mM) was chosen to lie in a fairly linear region of the CIDNP intensity versus histidine concentration curve [20] thereby providing a direct correlation between measured CIDNP intensity and histidine concentration within the irradiated region. Integrated CIDNP intensities as a function of the delay between injection and laser flash, obtained in a series of separate injections, are shown in Fig.5.4a. At $t=0$ (i.e. immediately after the 30 ms injection pulse) the intensity has already reached 80% of its maximum value. It

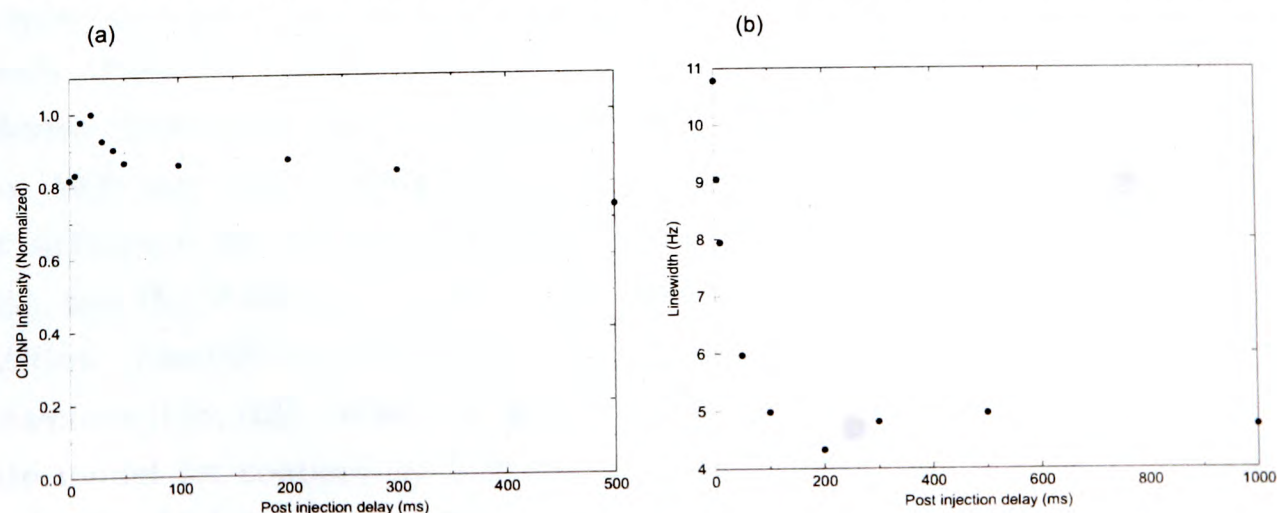


Figure 5.4: (a) CIDNP intensities and (b) linewidths of the H_2 proton of histidine as a function of the delay between the end of the injection (30 ms) and the start of the laser flash (50 ms). 70 μl of histidine solution (50 mM, pH 7) in 10 M urea was injected into 430 μl of flavin solution (0.25 mM, pH 7). Each data point represents a separate injection. The CIDNP intensities in (a) reflect the concentration of injected histidine and hence mixing in the irradiated region of the NMR tube.

then continues to increase for a short time (~ 20 ms) reflecting the time of travel from the end of the PTFE tube to the irradiated region, before gradually dropping back down to around 80% as the injected histidine diffuses out of the CIDNP sensitive region. This seems to correspond with the behaviour observed in the filmed experiments and confirms that bulk mixing ($\sim 80\%$) occurs very rapidly (< 100 ms) but is followed by a smaller amplitude ($\sim 20\%$) slow phase (> 1 s). To examine the rate of recovery from mechanical shock, linewidths of the histidine CIDNP signals from this experiment were also measured and are shown in Fig.5.4b. Whilst early spectra (0-25 ms) are severely broadened by sample inhomogeneity, the linewidth recovers remarkably quickly and after 50 ms it has almost narrowed to its limiting value.

These results suggest that strong well resolved CIDNP signals may be acquired within 100 ms of injection confirming that photo-CIDNP has the potential to dramatically reduce the dead time of real-time NMR experiments.

5.5 Bovine α -lactalbumin

5.5.1 Structure and folding properties of BLA

Bovine α -lactalbumin is a small monomeric protein of 123 residues (14.2 kDa), found in bovine milk whose biological function is the regulation of lactose biosynthesis [155, 156]. The structure of bovine α -lactalbumin itself has not yet been solved by X-ray crystallography or NMR, but a model structure was derived from the X-ray structures of its baboon [157] and human [158] analogues and was illustrated in Chapter 1 (Fig.1.10). α -lactalbumins and c-type lysozymes (e.g. hen lysozyme in Chapter 3) show close sequence homology [159] and in the native state they are closely similar in conformation [160, 161]. The α -lactalbumins are however quite different from the c-type lysozymes in two respects: their tight binding of Ca^{2+} ions [162] and their folding properties. The structure and biological activity are not influenced by the presence (*holo* form) or the absence (*apo* form) of calcium [163], but the stability is considerably reduced if the calcium is removed [164]. In addition, α -lactalbumins fold up to three orders of magnitude slower in the absence of calcium [138, 165]. While lysozymes conform rather closely to the classical two-state model for cooperative folding, it is well established that α -lactalbumins do not [102]. At low concentrations of denaturant, or on thermal or acid unfolding, a so-called molten globule state is generated and is characterized by pronounced secondary structure, compact shape, a hydrophobic core, and the absence of rigid sidechain packing [166–168]. This equilibrium molten globule state has been shown to be similar to a transient folding intermediate formed in the refolding from the fully unfolded state by use of stopped-flow CD [169], hydrogen exchange pulse la-

beling combined with 2D NMR [145] and other methods [170, 171]. These results have recently been confirmed by both real-time NMR on the slow folding (>100 s) of apo-BLA from 6 M GdmDCI [138] and photo-CIDNP on the fast folding (<1 s) of holo-BLA from 10 M urea [20]. In both cases spectra very similar to that of the molten globule state were obtained at intermediate times along the refolding pathway from the fully unfolded state.

The slow refolding of apo-BLA from 6 M GdmDCI was chosen as a test system for the set up described in Section 5.3. As was mentioned in Chapter 4, slow refolding processes allow multiple CIDNP spectra to be acquired in the course of a single refolding reaction and are therefore likely to encounter problems with flavin degradation. A small amount of H_2O_2 was therefore added to the refolding mixture to illustrate the use of hydrogen peroxide in protecting flavin-protein solutions against photo-reduction.

5.5.2 Comparison of equilibrium and kinetic spectra

Figure 5.5a shows the aromatic region of selected CIDNP spectra taken from a series recorded after the injection of 40 μl of 7 mM apo-BLA in 6 M GdmDCI into 510 μl of 10 mM H_2O_2 in D_2O at 20 $^\circ\text{C}$; both solutions contained 0.2 mM FMN and 200 mM cacodylic acid buffer at pH 7.2. Apo-bovine α -lactalbumin was used as supplied by Sigma-Aldrich-Fluka and may therefore have contained up to 0.3 moles of Ca^{2+} per mole of bovine α -lactalbumin. The pulse sequence and experimental set up were as described in Section 5.3. Laser flashes were of 100 ms each at a power of 4 W, the first spectrum was recorded 1 s after injection and subsequent spectra were then obtained at 7 s intervals over a period of ~ 3 minutes. In Fig.5.5b, these kinetic spectra are compared to *equilibrium* spectra of the pre-injection mixture (i.e. denatured state), the equilibrium molten globule state recorded at pH 2 (also known as the acid state or A-state) and the native state recorded under similar final conditions. As was observed by real-time NMR under similar conditions [138], the first spectrum recorded bears a strong resemblance to the CIDNP spectrum of the A-state. This suggests a rapid collapse from the denatured state that is stable in 6 M GdmDCI (which has a rather different CIDNP spectrum) to the A-state in a time much less than the time resolution of the measurement. Later spectra show the growth of sharp lines from the native state and the concomitant disappearance of the A-state as the protein refolds.

In an attempt to characterize the kinetics of the slow step monitored in the experiment, the complete set of spectra from which those in Fig.5.5a were taken were simulated as a linear combination of the first (A-state, $t=1$ s) and the last (native state, $t=166$ s) spectrum using a program developed by Dr.K.Maeda. The time

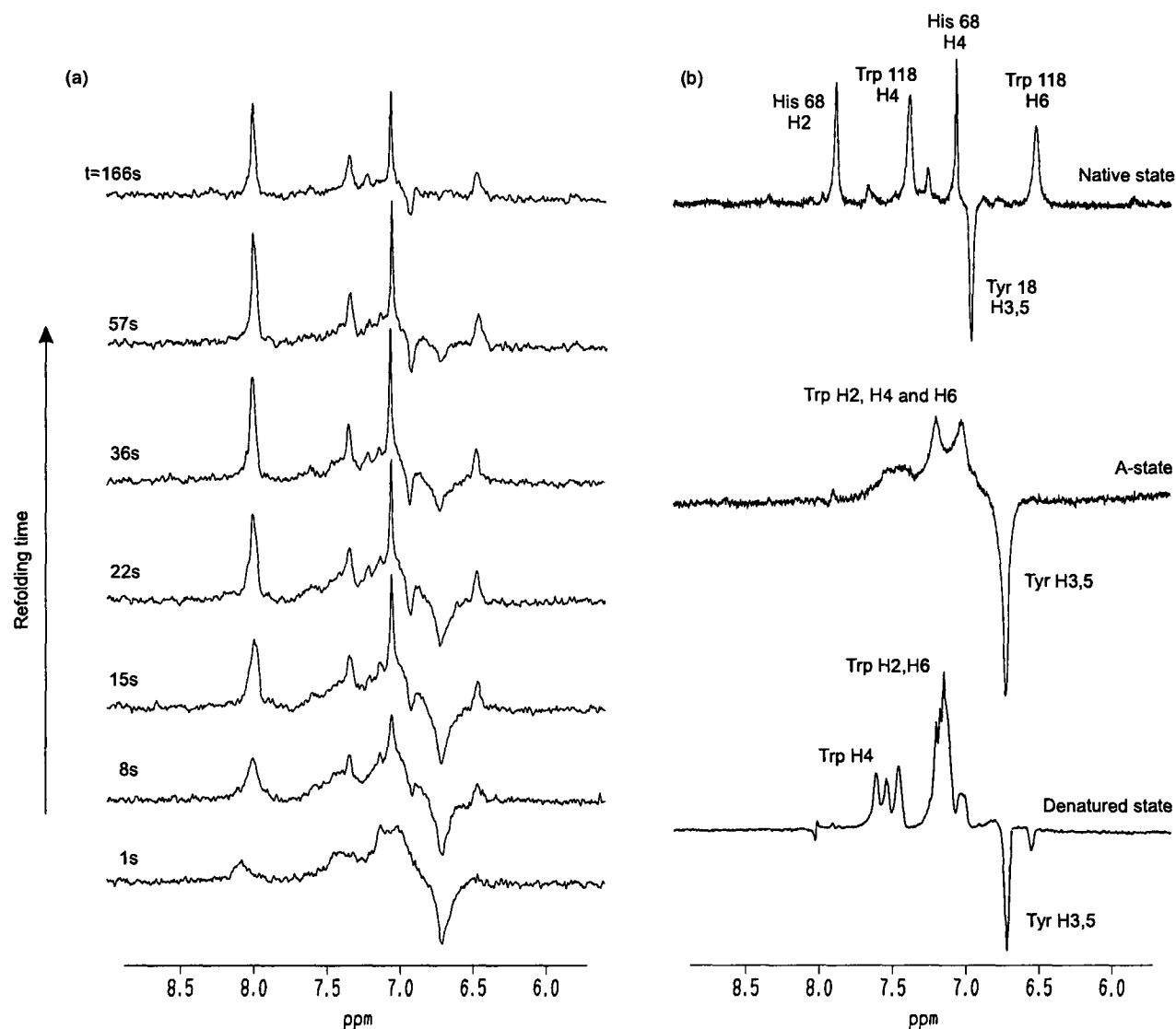


Figure 5.5: (a) Real-time 600 MHz ^1H photo-CIDNP spectra of the refolding of bovine α -lactalbumin following dilution from 6 M GdmDCI at 20 °C in the absence of calcium. Spectra were obtained at 7 s intervals in the course of a single experiment. (b) Equilibrium photo-CIDNP spectra of the pre-injection mixture (i.e. denatured state), the molten globule state recorded at pH 2 (also known as the acid state or A-state) and the native state recorded under similar final conditions. Assignments for the native state were taken from Ref.[31].

dependence of the contributions of the two states were fitted using single exponentials and are shown in Fig.5.6. Whilst all spectra could be adequately fitted, the time constant for the native state contribution ($\tau=12$ s) appears significantly shorter than that of the A-state ($\tau=28$ s), in addition, the native state contribution shows a $\sim 15\%$ “overshoot” at intermediate times. These observations suggest that the intensity of the final spectrum is weaker than it should be thereby inflating early contributions, and would therefore indicate that even in the presence of hydrogen peroxide, CIDNP decay can become confused with refolding kinetics. To confirm this, the CIDNP decay of the equilibrium native state of apo-BLA was measured with and without hydrogen peroxide using the same conditions as in the refolding experiment (i.e. 100 ms 4 W laser flash, 7 s delay). Whilst the presence of hydrogen peroxide did reduce sample degradation, there was still a significant decay in intensity which was later shown to be reduced by dialysis of the supplied bovine α -lactalbumin [136]. One somewhat crude approach to this decay problem would involve multiplying the series of spectra by a “correction” function determined by

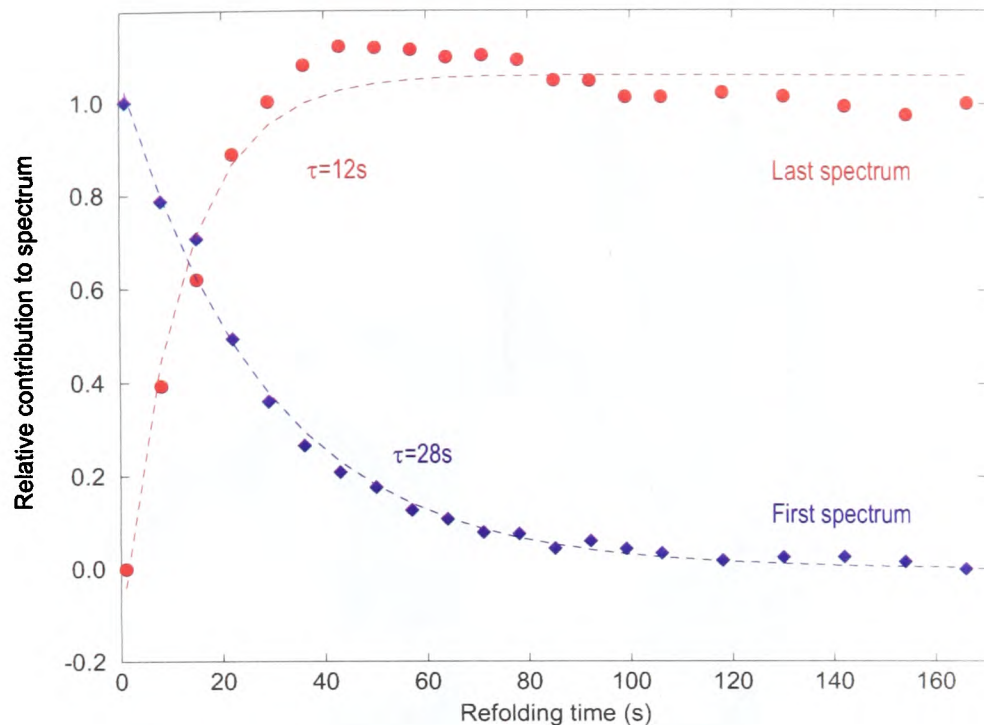


Figure 5.6: Time dependence of the contribution to the spectra in Fig.5.5a from the first ($t=1$ s) and last ($t=166$ s) spectra. Each data set was fitted using a single exponential function as shown.

the decay rate at equilibrium. A more elegant approach will be described in the following section and applied to the refolding of histidine-containing phosphocarrier protein.

5.6 *E.coli* histidine-containing phosphocarrier protein

5.6.1 Structure and folding properties of Wild-type HPr

Histidine-containing phosphocarrier protein (HPr) is the central component of the phosphotransfer system (PTS), which regulates sugar accumulation in bacterial cells [172,173]. It is a small monomeric protein of 85 residues (9.6 kDa). The structure has been determined by X-ray crystallography [174–176] and by multi-dimensional NMR experiments in solution [177–181] and is shown in Fig.5.7. It contains three α -helices (16-27, 47-52, 70-83) and a small sheet region consisting of four strands (1-8, 32-37, 40-43, 60-66). Unlike other proteins studied in this thesis (i.e. lysozyme and α -lactalbumin) HPr lacks cysteines and therefore has no covalent disulphide bridges. The active residue, where the phosphate is bound, is His 15. In the wild-type protein (WT HPr) only one other photo-CIDNP active residue is present, His 76 which lies partially buried in a hydrophobic region [181].

At pH 7.6, 20 °C HPr undergoes reversible unfolding above 2.2 M GdmDCl [182]. The 600 MHz ^1H NMR and CIDNP spectra of the native (0.55 M GdmDCl) and denatured (6 M GdmDCl) states of WT HPr are shown in Fig.5.8a and b respectively. Assignments were taken from Ref.[178]. The spectra are in good agreement with those previously obtained by Winder [20]. Whilst in the native state the relative

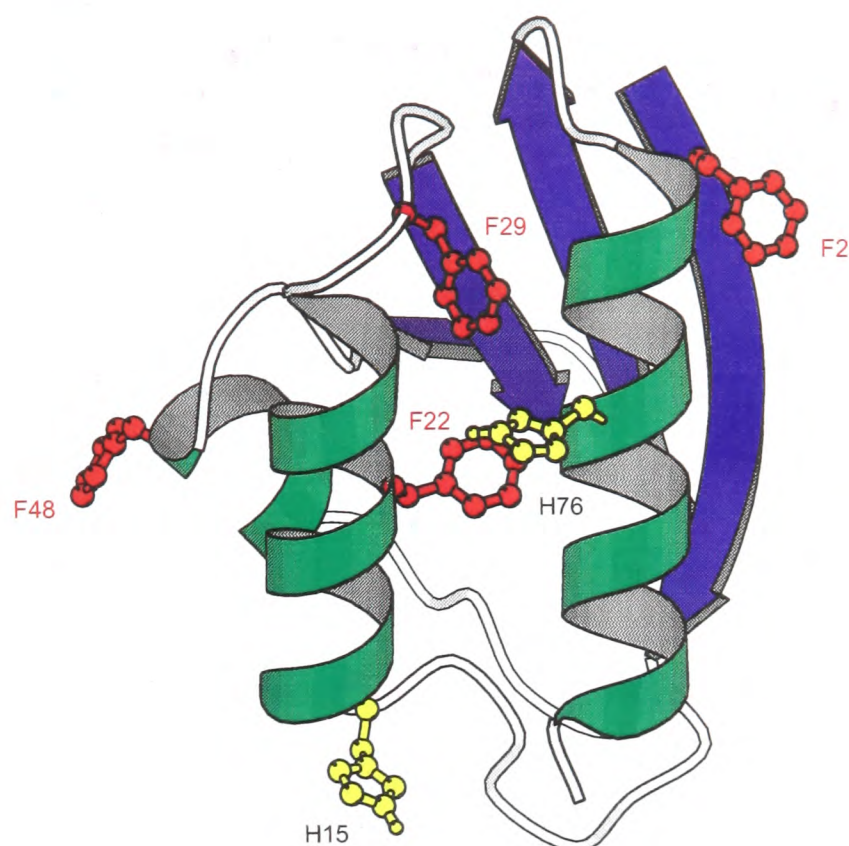


Figure 5.7: Molscript representation of native histidine-containing phosphocarrier protein (HPr). α -helices have been coloured in green and the β -sheet in blue. In the wild-type, His 15 and His 76 are the only CIDNP active residues and are shown in yellow. The four phenylalanine residues are also shown and are drawn in red.

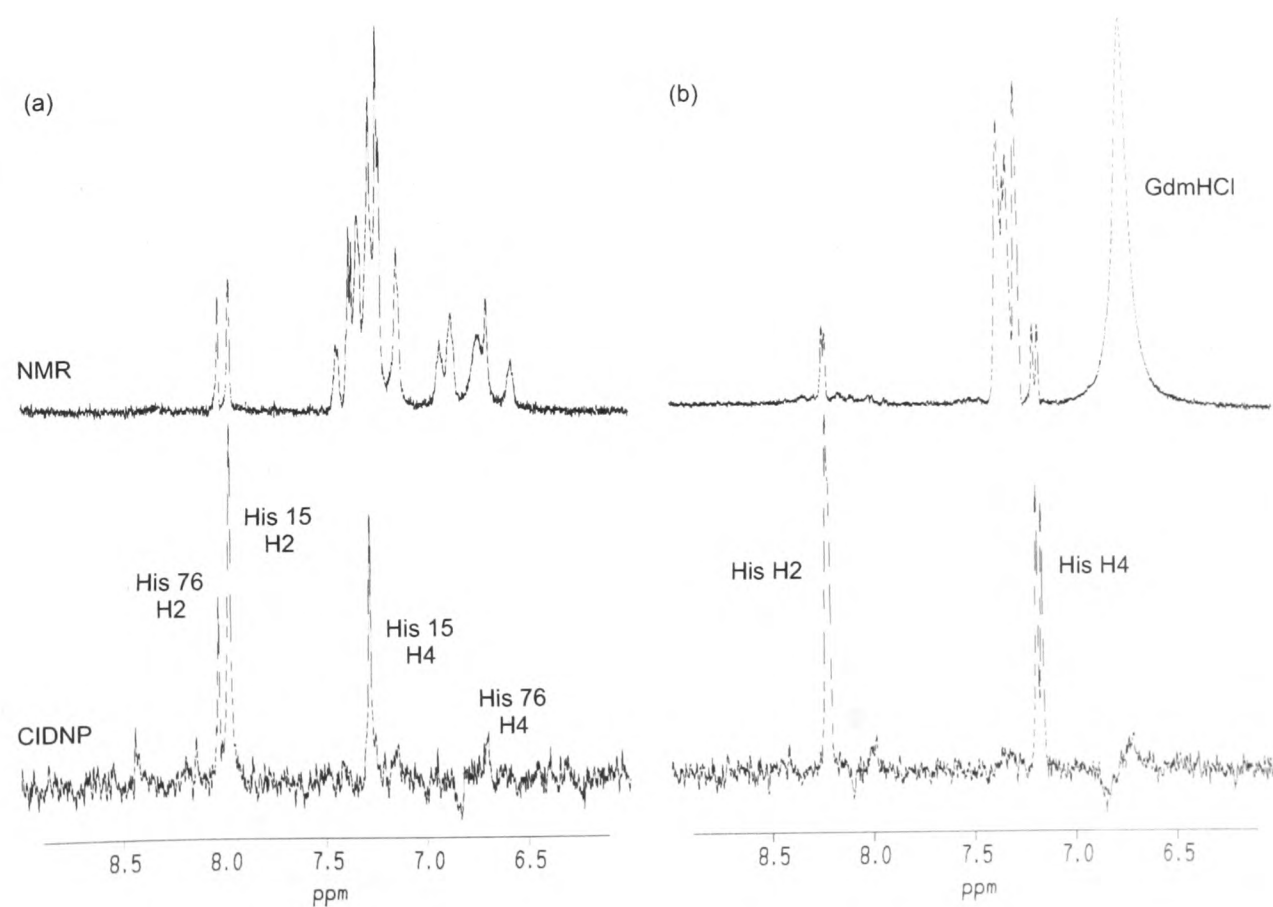


Figure 5.8: 600 MHz ^1H NMR and CIDNP spectra of (a) native (0.55 M GdmDCI, pH 7.6, 20 °C) and (b) denatured (6 M GdmDCI, pH 7.6, 20 °C) states of wild-type HPr.

enhancements for His 15 (exposed) and His 76 (partially buried) match their relative solvent accessibilities, the denatured state shows a loss in chemical dispersion and similar CIDNP intensities for all peaks indicating similar environments for the two histidine residues.

As a result of the lack of intrinsic fluorescent residues (Trp and Tyr), the spectroscopic probes available for conventional folding studies on wild-type HPr are limited. Nevertheless, CD in the far- and near-UV, and fluorescence associated with the binding of 8-anilino-1-naphthalenesulphonic acid (ANS) (which monitors the formation and disruption of hydrophobic regions [183]) were recently combined with NMR spectroscopy and differential scanning calorimetry (DSC) to study equilibrium and kinetic features of the folding and unfolding of HPr [182]. From both kinetic and equilibrium studies, it was found that the unfolding of HPr could be described as a two-state process without the accumulation of intermediates.

5.6.2 Single tryptophan mutants of HPr

As was mentioned in the introduction to this chapter, when *several* spectroscopic probes (e.g. fluorescent tryptophans) are present within a protein the measured signal in low resolution stopped-flow optical experiments tends to be averaged over the different sites within the folding protein. In direct contrast, when the protein contains a *single* probe, site specific information can be obtained. By introducing single tryptophans at different sites within wild-type HPr and following the folding process by intrinsic fluorescence (which monitors core packing [143]) and fluorescence anisotropy (which monitors side-chain mobility [144]) one could therefore in theory get complementary site specific information on the folding process. Expression systems have therefore been developed [184] that allow each of the four phenylalanine residues shown in Fig.5.7 (Phe 2, Phe 22, Phe 29 and Phe 48) to be replaced in turn by a tryptophan to produce four single tryptophan mutants of HPr (F2W, F22W, F29W and F48W). As will be shown in the following sections, whilst these mutants were originally expressed for stopped-flow fluorescence experiments, they also present an intriguing folding system for a kinetic study by real-time photo-CIDNP.

When using mutants to describe the folding of any protein, care must first be taken to ensure that the mutated residue has no adverse effect on either the structure or stability of the protein. The four mutants have therefore been characterized by activity assay, by ultra-violet and fluorescence spectroscopy and by NMR [184]. The equilibrium folding of the four mutants has also been studied by both circular dichroism and fluorescence, and compared to that of the wild-type protein [184]. The results obtained indicate that whilst F2W and F48W show roughly the same stability and folding behaviour as WT HPr, both F22W and F29W are slightly

destabilized (with a midpoint of thermal denaturation of 60 °C as compared to 65 °C in the wild-type). As the crystal structure (Fig.5.7) shows, whilst Phe 2 and Phe 48 lie on the surface of the protein, Phe 22 and Phe 29 are buried. The loss of stability for the latter mutants has therefore been attributed to disruption of the hydrophobic core due to the introduction of a tryptophan, and hence slight care should be taken when interpreting data from these two mutants.

Small amounts of the four mutants were made available for photo-CIDNP studies. Equilibrium CIDNP spectra of the denatured and native states of three of the mutants (F2W, F22W and F48W) were first obtained and are described in Section 5.6.3. Real-time CIDNP studies were then performed under fast (<1 s) and slow (5-10 s) folding conditions and are described in Section 5.6.4 and 5.6.5 respectively.

5.6.3 Photo-CIDNP equilibrium studies

Figure 5.9 shows the aromatic and aliphatic regions of the 600 MHz ^1H CIDNP spectra of three of the mutants (F2W, F22W and F48W) *denatured* in 6 M GdmDCl taken at pH 7.6, 20 °C. In such a high concentration of denaturant and in the ab-

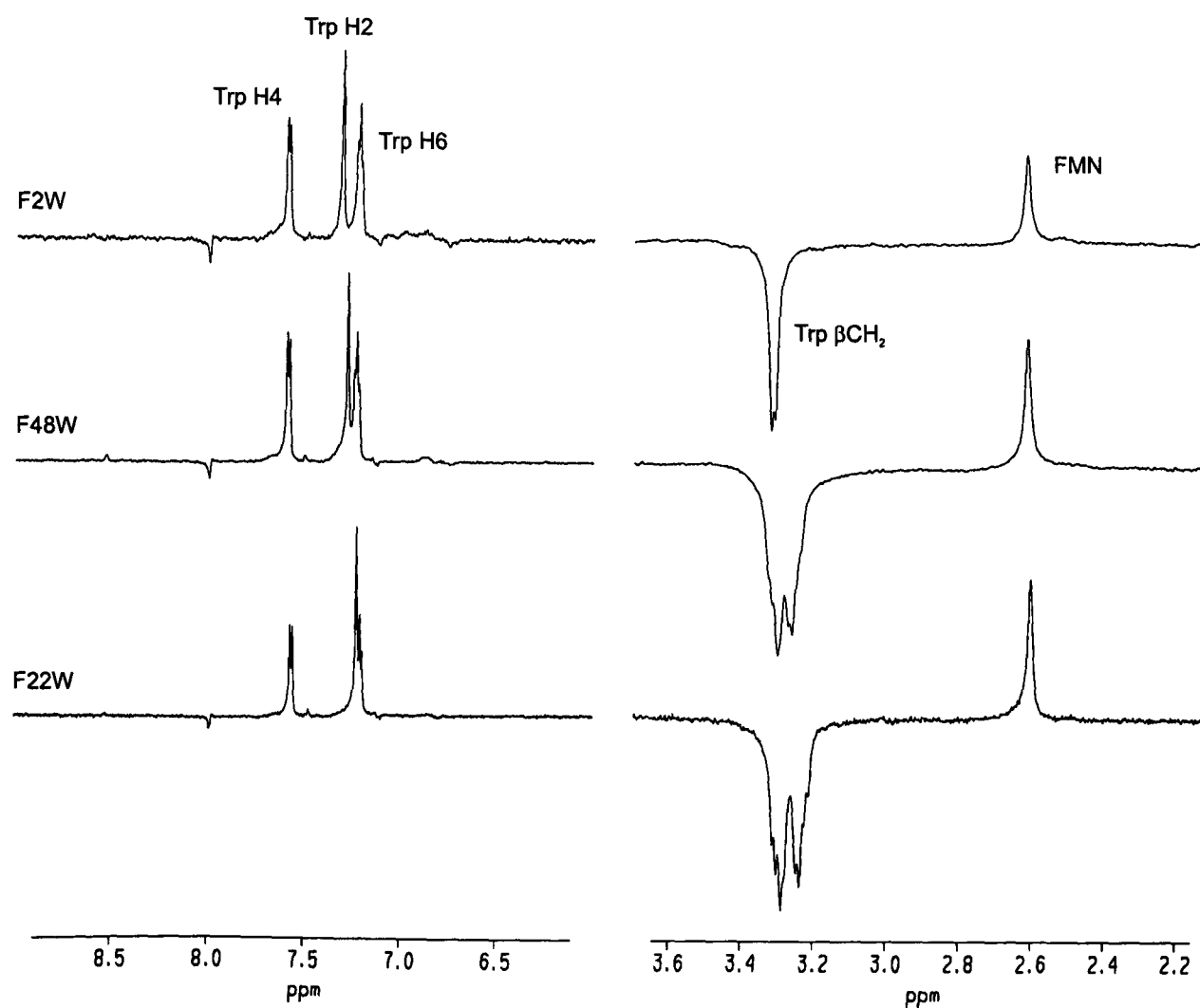


Figure 5.9: Aromatic and aliphatic regions of the 600 MHz ^1H CIDNP spectra of the *denatured* states (6 M GdmDCl, pH 7.6, 20 °C) of three of the single tryptophan mutants of HPr. Assignments are based on random coil chemical shifts (Ref.[185]) and multiplicities (H2 singlet, H4 doublet and H6 triplet).

sence of disulphide bridges, the protein should be highly unfolded. The spectra of the three mutants are remarkably similar and differ dramatically from that of the wild-type (Fig.5.8b). Whilst both histidines were visible in the wild-type spectrum, they are absent in all the mutant spectra and have been replaced by strong tryptophan H2, H4, H6 and β -CH₂ signals at roughly random coil chemical shifts [185]. The small deviations observed for the H2 and β -CH₂ protons are probably caused by local sequence effects. As was mentioned in the introduction to this thesis (Section 1.2.5), the relative rate constants for quenching of triplet flavin derivatives by the free amino acids tryptophan and histidine have been investigated at pH 7 by both photo-CIDNP competition experiments [30] and transient absorption [29], and were found to be in the approximate ratio of 40:1 and 8:1 respectively. The absence of histidine signals is therefore likely to be due to poor competition for excited flavin in the presence of an equally exposed tryptophan. The lack of histidine peaks in all three spectra suggests that the three mutated sites are equally exposed and therefore unlikely to be involved in any form of hydrophobic clustering as was observed for denatured hen lysozyme in Chapter 3.

Figure 5.10 shows the aromatic and aliphatic regions of the 600 MHz ¹H CIDNP spectra of the *native* states of these three mutants (F2W, F22W and F48W) in 0.55 M GdmDCl taken at pH 7.6, 20 °C. Unlike under denatured conditions, the histidine

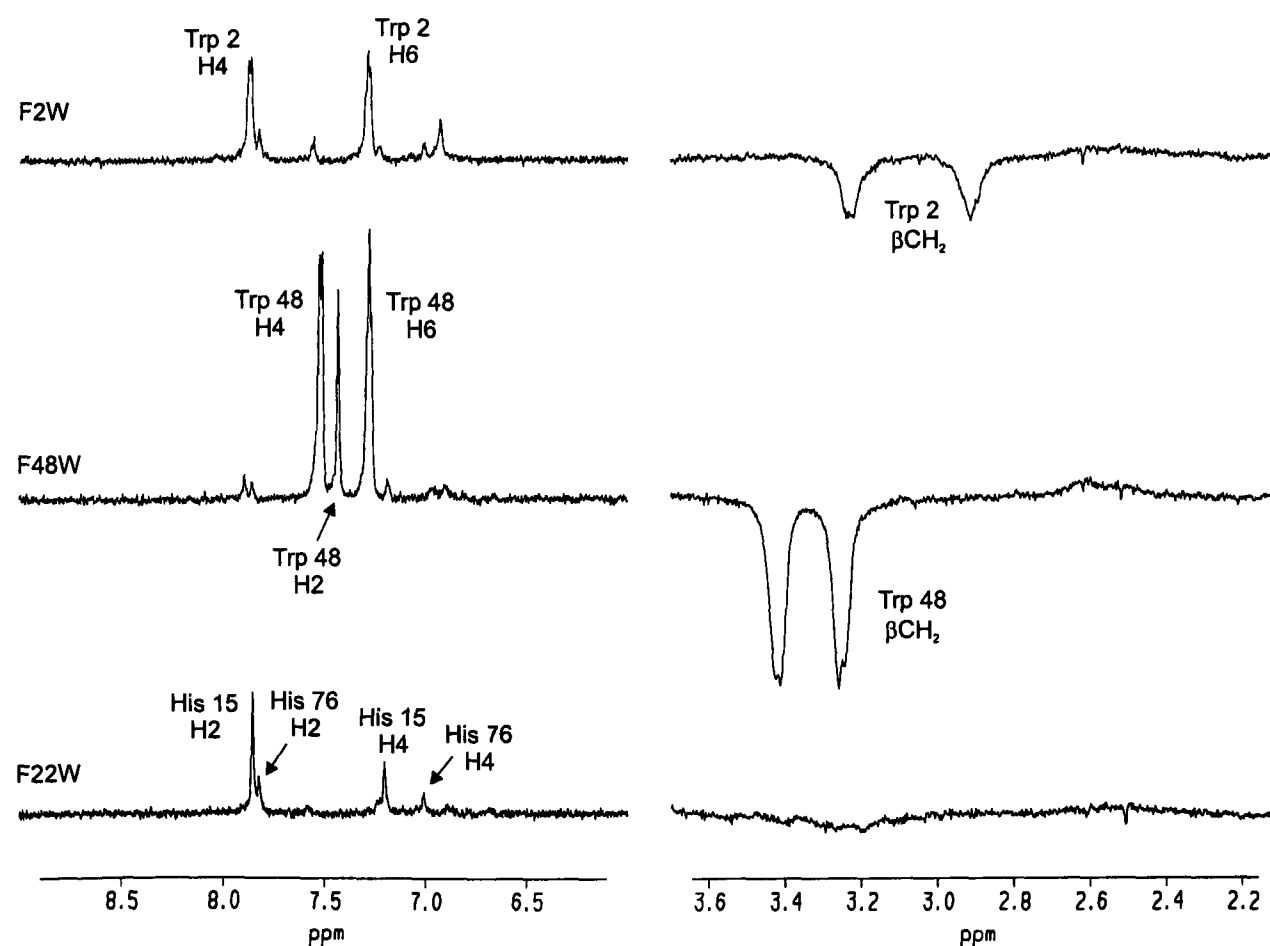


Figure 5.10: Aromatic and aliphatic regions of the 600 MHz ¹H CIDNP spectra of the *native* states (0.55 M GdmDCl, pH 7.6, 20 °C) of three of the single tryptophan mutants of HPr. Assignments are described in the text.

and tryptophans are no longer equally exposed. For F2W and F48W in which the tryptophan is on the surface of the protein strong tryptophan and weak histidine signals are observed. The tryptophan in F22W on the other hand is buried and not surprisingly, the native CIDNP spectrum resembles that of the wild-type protein (Fig.5.8a) with signals from both His 15 and His 76. Resonance assignments for the tryptophans and histidines of the native states of F48W and F29W (F29W is shown in Fig.5.11) are known and were kindly provided by Dr.N.A.J.van Nuland. Resonance assignments for the native states of F2W and F22W on the other hand have not yet been performed, and hence assignments for these two mutants were only made when they seemed unambiguous and were based on both the crystal structure predictions discussed above and multiplet multiplicities (e.g. whilst Trp H4 is a doublet Trp H6 is a triplet).

These results illustrate one of the inherent problems associated with competition effects in a photo-CIDNP experiment. Namely that in a protein, the CIDNP intensity for a given residue is determined by both its accessibility *and* its intrinsic reactivity. In the presence of several potentially CIDNP active residues, the *absence* of a CIDNP signal for a given residue cannot therefore be taken as an indication of weak exposure, but instead should be interpreted as an indication of poor overall reactivity as compared to the other residues present. Notwithstanding some of the other factors determining CIDNP intensities, knowledge of the relative intrinsic reactivities can however be used to *suggest* relative accessibilities.

5.6.4 Photo-CIDNP kinetic studies - fast folding

The folding of HPr initiated by 11-fold dilution from 6 M GdmDCI at pH 7.6 and 20 °C is fast and essentially complete in about 1 s [182]. The majority of stopped-flow optical studies on HPr have been performed at this temperature, but a recent real-time NMR experiment was carried out at the lower temperature of 2.8 °C where folding takes over 20 s [182]. To illustrate the potential of photo-CIDNP at following *fast* processes the refolding of F29W and F48W was first monitored at 20 °C.

Figure 5.11 shows CIDNP spectra obtained in separate injections and taken 50 and 500 ms after the injection of 50 μ l of 5 mM (a) F48W and (b) F29W HPr in 6 M GdmDCI into 500 μ l of D₂O at 20 °C. Both solutions contained 0.2 mM FMN and 100 mM sodium phosphate buffer at pH 7.6. The pulse sequence and experimental set up were as described in Section 5.3. Laser flashes were of 50 ms at a power of 4 W. Also shown for comparison are CIDNP spectra of the pre-injection mixture (i.e. denatured state) and the native state. For both F29W and F48W, the spectrum obtained 500 ms after injection appears identical to that of the native state confirming that folding is fast under these conditions. In contrast, the spectrum obtained 50 ms

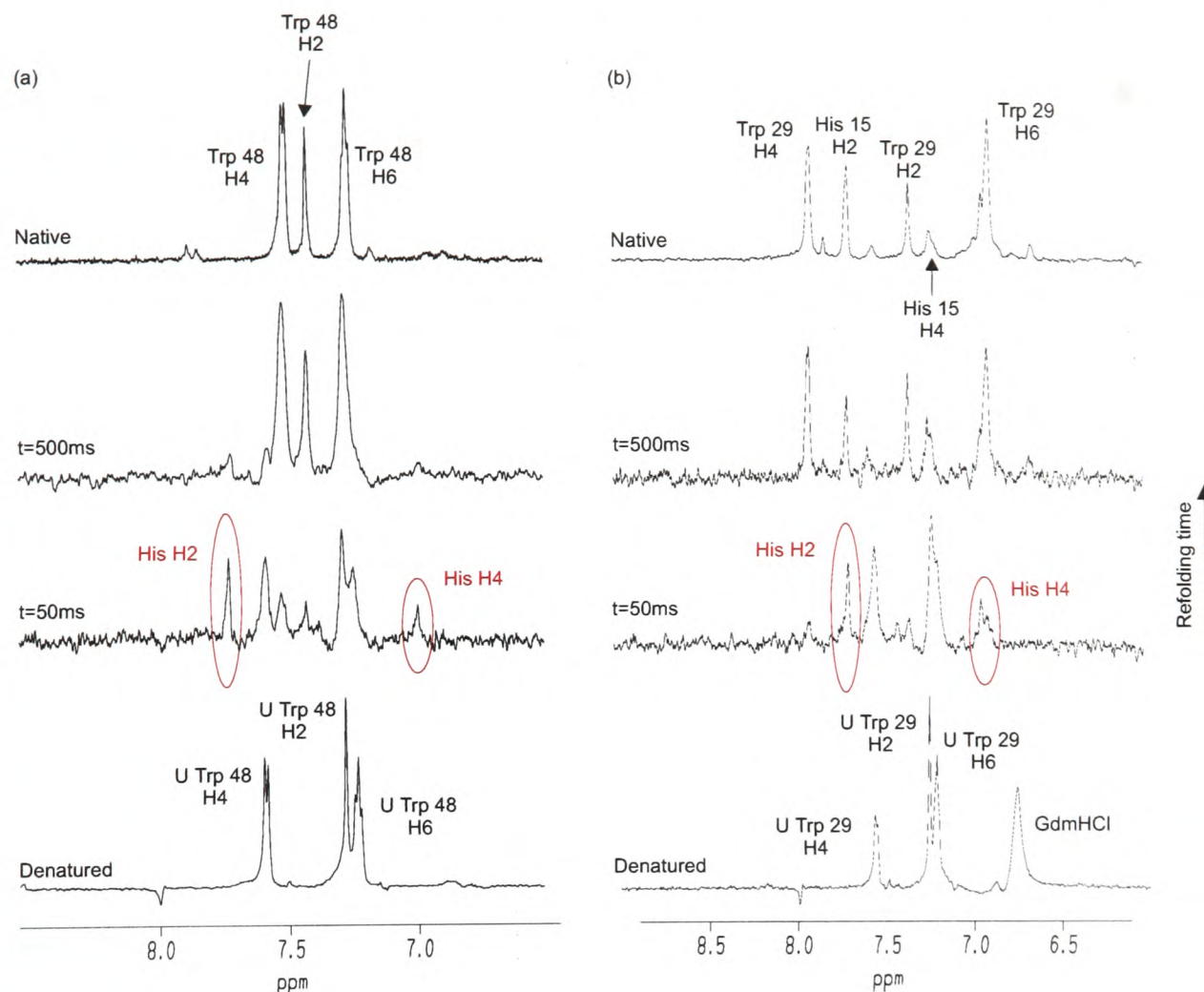


Figure 5.11: (a) Comparison of real-time CIDNP spectra taken 50 and 500 ms after rapid dilution of F48W HPr from 6 M GdmDCI at 20 °C with equilibrium CIDNP spectra of the pre-injection mixture (i.e. denatured state), and the native state recorded under the same final conditions. Strong histidine peaks are observed at 50 ms that are absent in both the pre-injection and native spectra and have been circled in red. (b) The same as in (a) but for F29W. Assignments are described in the text.

after injection for F29W and F48W differs from that of the pre-injection mixture; in both cases, strong peaks at denatured tryptophan chemical shifts suggest that the protein is still largely unfolded, however several new peaks (circled in red) are also present. Based on the known chemical shifts of the tryptophan residues in both the native and denatured states of the two mutants, these new peaks can be assigned to one *or* both of the histidines (specific assignment makes no difference to the argument). The fact that these two peaks (labeled His H2 and H4) occur at 7.68 and 6.93 ppm in both the spectrum obtained with F48W (Fig.5.11a) *and* F29W (Fig.5.11b) suggests that the same state is formed with both mutants. In addition, the lack of native tryptophan peaks after 50 ms suggests that these histidine peaks belong to the same state as the strong denatured tryptophan peaks. However, as was shown in Section 5.6.3, no histidine peaks were observed in any of the mutants under denatured conditions because of competition effects. The appearance of these peaks must therefore be as a result of some dramatic change in the *relative* exposure of histidine and tryptophan residues in the denatured state. The observation of strong histidine resonances in the denatured wild-type protein (Fig.5.8b) suggests that any change in

the relative exposure is unlikely to be as a result of an increase in exposure of the histidines but rather a decrease in the exposure of the tryptophans and would indicate the rapid formation within the dead time of the experiment of a compact collapsed state with a hydrophobic core involving residues 29 and 48. A burst phase involving collapse and burial of the tryptophans has also been suggested by stopped-flow fluorescence anisotropy under identical conditions [186]. This type of hydrophobic collapse has been shown to play an important role in a large number of other refolding studies including the α -lactalbumins and lysozymes [117, 166, 187, 188] and appears to be a key stage in the formation of persistent native-like structure during refolding.

5.6.5 Photo-CIDNP kinetic studies - slow folding

Whilst the spectra obtained at 20 °C with each mutant paint an elegant qualitative picture of the folding process, a greater range of refolding times are necessary for any quantitative discussion. With the small amounts of protein available (<20 mg) the number of injections that could be performed at 20 °C were limited. The temperature was therefore dropped to 5 °C for F48W and 10 °C for F2W and F22W where folding is ~ 10 and 5 times slower respectively [182]. At these temperatures, a full set of CIDNP spectra can then be obtained with a single shot.

F48W at 5 °C

The aromatic and aliphatic region of selected spectra taken from a single series at 5 °C with F48W are shown in Fig.5.12. Apart from the refolding temperature conditions were as described in Section 5.6.4. The first spectrum was taken 100 ms after injection and in order to obtain a reasonable time resolution the delay between subsequent spectra was set at 500 ms. As was observed at 20 °C, early spectra are dominated by denatured tryptophan peaks. These gradually disappear with time and are replaced by native tryptophan peaks. The final spectrum obtained after 7.6 s is essentially identical to that of the native state (Fig.5.11a and Fig.5.10). Histidine peaks are again observed at early times and seem to disappear gradually along with the denatured tryptophan peaks, confirming that they originate from the same collapsed intermediate state.

The photo-CIDNP results presented so far suggest that within the dead time of the experiment (at both 20 and 5 °C) a transient intermediate is formed, and that the process actually monitored in the experiment is the slower two-state conversion of this intermediate to the native state (i.e. $U \xrightarrow{fast} I \xrightarrow{slow} N$). This is very similar to the results described earlier for apo-BLA. The quality and time resolution of these results suggest that one might be able to extract some quantitative information from

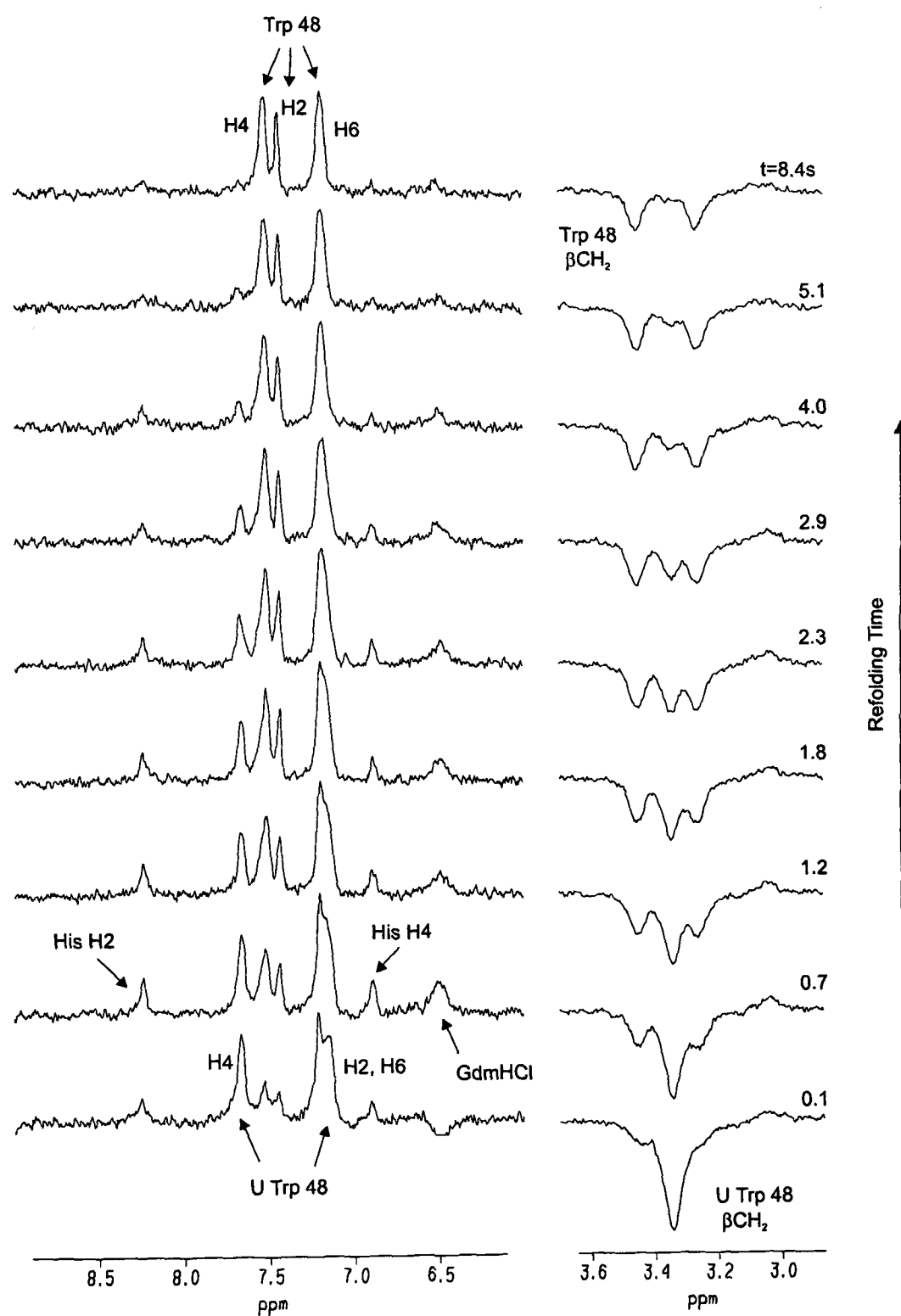


Figure 5.12: Aromatic and aliphatic regions of the real-time 600 MHz ^1H photo-CIDNP spectra of the refolding of F48W HPr following dilution from 6 M GdmDCI at 5 °C. The first spectrum was obtained 100 ms after injection with a 50 ms laser flash and an acquisition time of 500 ms.

the data. However, rather than attempting to reconstruct all spectra as a linear combination of the first and last in the series as for apo-BLA, an alternative strategy was adopted.

If the folding process as monitored by the time resolution of the CIDNP experiment is indeed the second cooperative two-state step ($I \xrightarrow{\text{slow}} N$), then the folding system can be treated as a binary mixture in which the concentrations of intermediate and native states as a function of refolding time t are given respectively by:

$$[Int] \propto e^{-t/\tau} \text{ and } [Nat] \propto 1 - e^{-t/\tau} \quad (5.1)$$

where τ is the folding time constant. The competition for flavin at any time will therefore be between exposed residues in both the intermediate and native states and any other existing quenching mechanism (e.g. fluorescence). If an arbitrary number n potentially CIDNP active residues are present in the protein, the CIDNP intensity for a given residue l in the *intermediate* state can be represented by:

$$I_l^{Int} \propto \frac{k_l^{Int}[Int]}{k_q + \sum_{i=1}^n k_i^{Int}[Int] + \sum_{i=1}^n k_i^{Nat}[Nat]} \quad (5.2)$$

where k_q is the (pseudo-) first order rate constant for the decay of triplet flavin by fluorescence and/or quenching by molecular oxygen and k_i^{Nat} and k_i^{Int} are the second order rate constants for quenching of triplet flavin by residue i in the native and intermediate state respectively. Similarly, the CIDNP intensity for residue j in the *native* state will be given by:

$$I_j^{Nat} \propto \frac{k_j^{Nat}[Nat]}{k_q + \sum_{i=1}^n k_i^{Int}[Int] + \sum_{i=1}^n k_i^{Nat}[Nat]} \quad (5.3)$$

These two equations illustrate the complexity introduced into the interpretation of CIDNP intensities by competition for excited flavin. The situation can be simplified somewhat by taking the ratio of Eqn. 5.3 and Eqn. 5.2 to give:

$$\frac{I_j^{Nat}}{I_l^{Int}} \propto \frac{[Nat]}{[Int]} \quad (5.4)$$

and finally, by inserting expressions from Eqn. 5.1 and simplifying, one obtains:

$$\frac{I_j^{Nat}}{I_l^{Int}} \propto e^{t/\tau} - 1 \quad (5.5)$$

By plotting the CIDNP intensity ratio of any peak in the native state and any peak in the intermediate state against the refolding time t and fitting the data with Eqn.5.5 one should then in theory be able to extract a value for the folding time

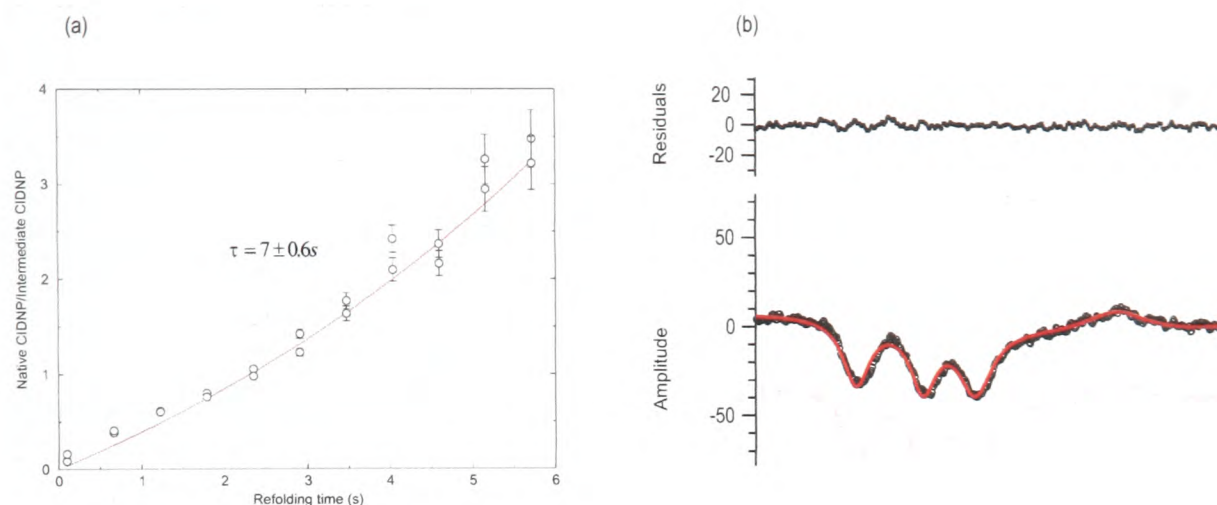


Figure 5.13: (a) Plot of the ratio of native to unfolded CIDNP intensity as a function of refolding time. Tryptophan β -CH₂ signals were taken from Fig.5.12. The data was fitted as described in Eqn.5.5. (b) Illustration of the lorentzian fitting procedure for the $t=2.3$ s spectrum in Fig.5.12.

constant τ . Fig.5.13a illustrates this for the β -CH₂ signals of the native and intermediate states taken from Fig.5.12. Intensities were estimated by multiplying the widths and heights obtained for each well resolved peak using a lorentzian fitting procedure developed by Dr.K.Maeda (Fig.5.13b). As illustrated in Fig.5.13a, the error increases dramatically with refolding time, and time points beyond $t=6$ s were therefore not included in the fitting procedure. The agreement between experiment and theory is good and yields a value for τ of 7 ± 0.6 s in good agreement with values obtained by real-time NMR ($\tau=8.3 \pm 0.7$ s) and ANS fluorescence ($\tau=8.4$ s) at the slightly lower temperature of 2.8°C [182].

It is interesting to note that by using short 50 ms flashes and limiting their number to ~ 16 the decay of CIDNP due to progressive flavin degradation as described in Chapter 4 does not seem to be a problem in these experiments. In addition, whilst decay poses problems to the approach described for apo-BLA in which all spectra are reconstructed as a linear combinations of the first and last in the series, by taking the *ratio* of native and intermediate CIDNP intensities as just described this problem is in theory avoided.

F2W and F22W at 10°C

The refolding experiment was also performed with both F2W and F22W mutants at the slightly higher temperature of 10°C where folding is complete within ~ 5 s. Apart from the refolding temperature, conditions were as described in Section 5.6.4. In both cases, the first spectrum was taken 100 ms after injection and the delay between subsequent spectra was set at 1 s. The aromatic and aliphatic region of a selection of spectra are shown in Fig.5.14 and Fig.5.15 for F2W and F22W respectively. In both cases, early spectra are again dominated by denatured tryptophan peaks, and the last spectrum is essentially identical to the native state spectra shown in

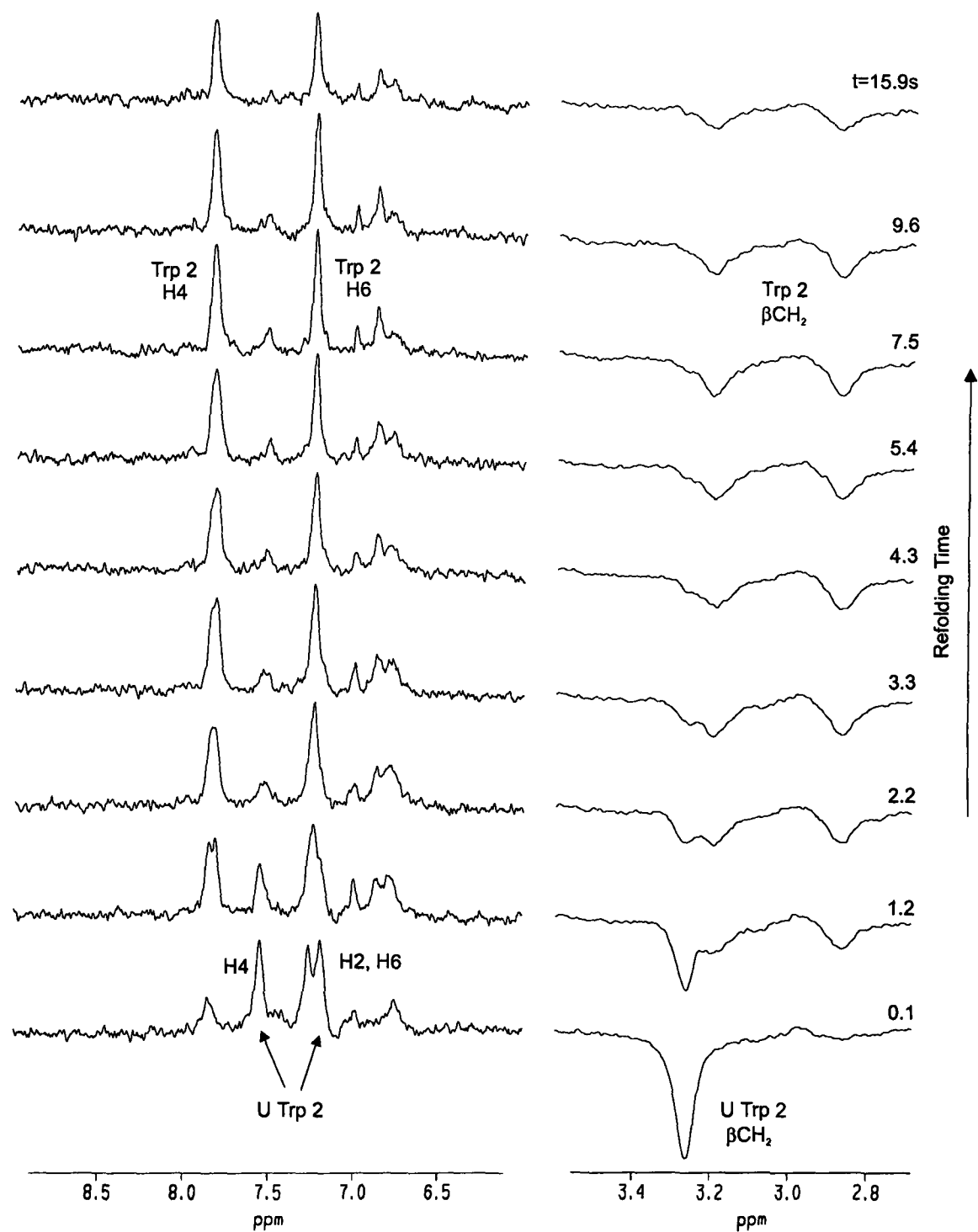


Figure 5.14: Aromatic and aliphatic regions of the real-time 600 MHz ^1H photo-CIDNP spectra of the refolding of F2W HPr following dilution from 6 M GdmDCl at 10 °C. The first spectrum was obtained 100 ms after injection with a 50 ms laser flash and an acquisition time of 1 s.

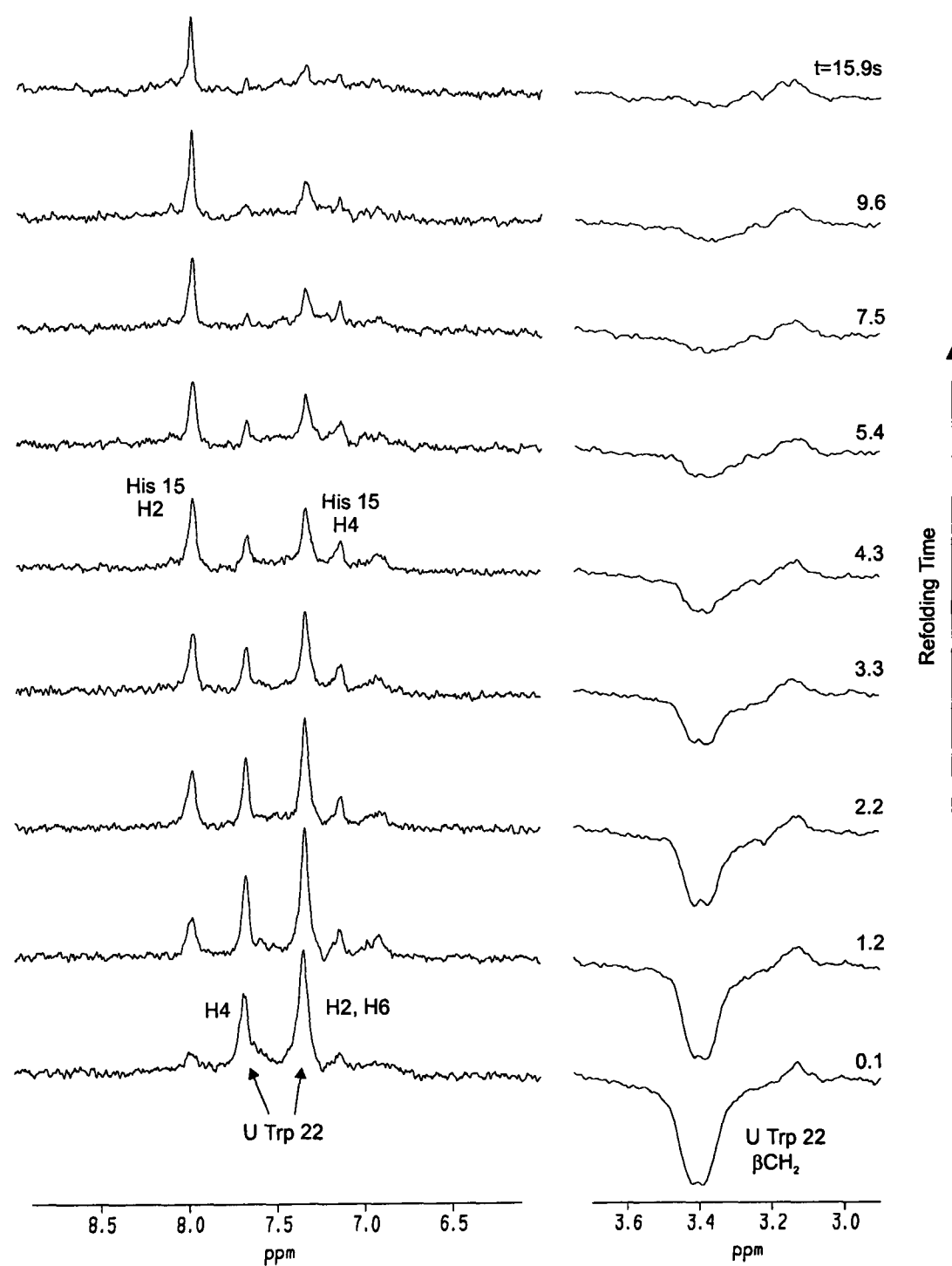


Figure 5.15: Aromatic and aliphatic regions of the real-time 600 MHz ^1H photo-CIDNP spectra of the refolding of F22W HPr following dilution from 6 M GdmDCI at 10 °C. The first spectrum was obtained 100 ms after injection with a 50 ms laser flash and an acquisition time of 1 s.

Fig.5.10 with native tryptophan peaks for F2W and native histidine peaks for F22W. Unlike with F29W and F48W, no strong histidine peaks are apparent at early times which might suggest that residues 2 and 22 are *not* involved in the hydrophobic collapse suggested for the other two mutants. However as was described earlier any interpretation based on the *absence* of a CIDNP signal needs to be confirmed by other methods before being taken seriously. Note that the faster folding at 10 °C and the poorer time resolution (spectra every 1 s rather than every 0.5 s) in both data sets prevented the refolding kinetics from being analyzed in the same way as for F48W at 5 °C (Fig.5.13).

5.7 Future Work

The results presented in this chapter highlight the simplicity and great potential of the real-time photo-CIDNP technique. By generating polarization through short laser pulses rather than spin-lattice relaxation it offers considerable improvements in dead time and time resolution over real-time NMR. In addition, photo-CIDNP provides direct information on side-chain accessibility, and whilst real-time ^1H NMR spectra are crowded and usually require signal averaging, the inherent signal enhancement and selectivity of photo-CIDNP produces strong well resolved signals. However, a number of improvements can still be envisaged.

In order to avoid dynamic range problems associated with a large solvent signal [74] all experiments described in this chapter were carried out in D_2O . However, most modern spectrometers are now equipped with pulse field gradients and can readily cope with samples prepared in high concentrations of H_2O [90]. As was described in Chapter 3, samples prepared in this way allow detection of CIDNP signals from the exchanging indole NH protons of exposed tryptophans. These singlet peaks resonate around 10 ppm [185] in a particularly uncrowded region of the protein 1D ^1H spectrum. To illustrate the potential of such an approach, the *unfolding* of hen lysozyme in 9.1 M urea was followed by photo-CIDNP in 95% H_2O /5% D_2O . Unfolding was initiated by injecting 50 μl of 7 mM hen lysozyme into 500 μl of 10 M urea at 55 °C; both solutions contained 0.2 mM FMN, 100 mM NaCl (to prevent aggregation) and were adjusted to pH 3.8. The first spectrum was obtained 1 s after injection and subsequent spectra were obtained at 6 s intervals. The indole region of a selection of spectra are shown in Fig.5.16. The first spectrum is essentially native with a strong signal from Trp 62 and a weaker signal from Trp 123 (Section 3.4). Over a relatively slow timescale, these native peaks disappear with a concomitant growth in the poorly resolved unfolded tryptophan peaks (U Trp) that were also described in Chapter 3 (Section 3.5). The simplicity and clarity of the spectra in this region is striking and provides a dramatic improvement in spectral resolution

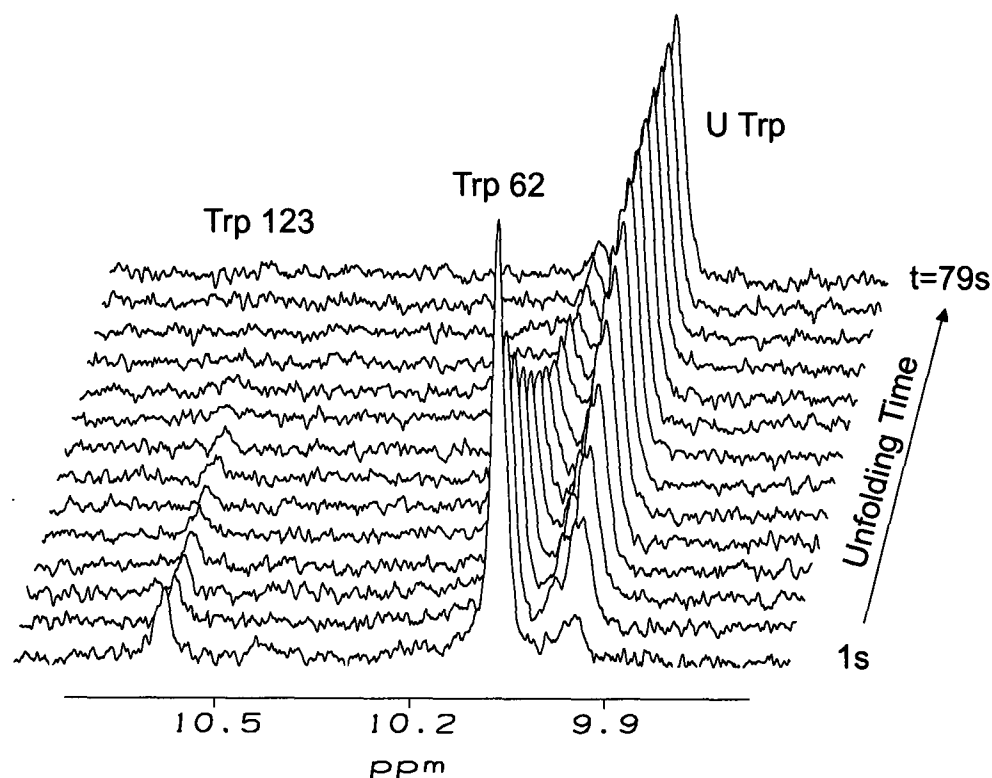


Figure 5.16: Indole region of the real-time 600 MHz ^1H photo-CIDNP spectra of the *unfolding* of hen lysozyme in 9.1 M urea at 55 °C. The first spectrum was obtained 1 s after injection with a 100 ms laser flash and an acquisition time of 1 s.

over the crowded aromatic region.

Photo-CIDNP spectra obtained during the course of a refolding experiment such as those described in this chapter provide time-dependent information on the multi-component nature of the refolding mixture, enabling a relatively simplistic description of changes in the relative concentrations of the native, intermediate and unfolded states. Unfortunately the poorly dispersed and often broad nature of intermediate and unfolded spectra make a detailed and site specific description difficult. In order to extract such information, a simple variation of the experiment described in this chapter can be envisaged. In the current experiment, the radio frequency (r.f.) pulse is placed 5 ms after the laser pulse so that the CIDNP spectrum obtained corresponds to the refolding mixture at the time of irradiation. If folding is fast, the whole process is repeated for each time point and the delay separating injection and laser flash is varied. If folding is faster than spin-lattice relaxation (<200 ms) one could consider placing a *fixed* delay, sufficient to allow complete refolding to the native state, in between the injection and r.f. pulses. Then by irradiating the sample at different times after the onset of folding, one should in theory be able to label the folding mixture at different stages whilst monitoring the CIDNP signals at native chemical shifts. This approach is a transient analogue of the “pulse labeling” experiment that will be described in Chapter 7, in which polarization generated in an equilibrium denatured state is transferred to the native state by rapid refolding for observation. The advantage of such approaches, as highlighted by the great success of the conceptually similar pulse labeling hydrogen/deuterium exchange ex-

periments [145], lies in their ability to use the well dispersed native chemical shifts to assign exposed residues in partially folded states. As will be discussed in Chapter 7, folding needs to be fast to avoid loss of CIDNP through spin-lattice relaxation, the recovery of Boltzmann polarization and the build up of cross-polarized peaks during the delay separating laser and r.f. pulses. In order to maintain reasonable time resolution with such fast folding processes (<200 ms), lasers with higher output powers would also be particularly attractive.

Finally, as an alternative to the ^1H CIDNP approach presented in this chapter, preliminary studies were performed on ^{19}F labeled tyrosine and tryptophan. The fluorine shielding parameter is extremely sensitive to molecular environment [189–191]. As an example, whilst the random coil chemical shifts of H6 in tryptophan and H3 in tyrosine are only 0.4 ppm apart, F6 and F3 in the fluorine labeled analogues are separated by 15 ppm. Therefore unlike for ^1H , individual residues in *denatured* proteins can often be resolved and therefore assigned on the basis of the ^{19}F chemical shift dispersion alone [151–153, 192]. The feasibility of ^{19}F photo-CIDNP with labeled tyrosine [19, 189] and tryptophan [189] has been demonstrated previously, to illustrate the sensitivity of the fluorine shielding parameter, the ^{19}F NMR and photo-CIDNP spectra of an equimolar mixture of 3-fluoro-tyrosine, 4-fluoro-tryptophan and 6-fluoro-tryptophan taken at pH 4.5 are shown in Fig.5.17. Note

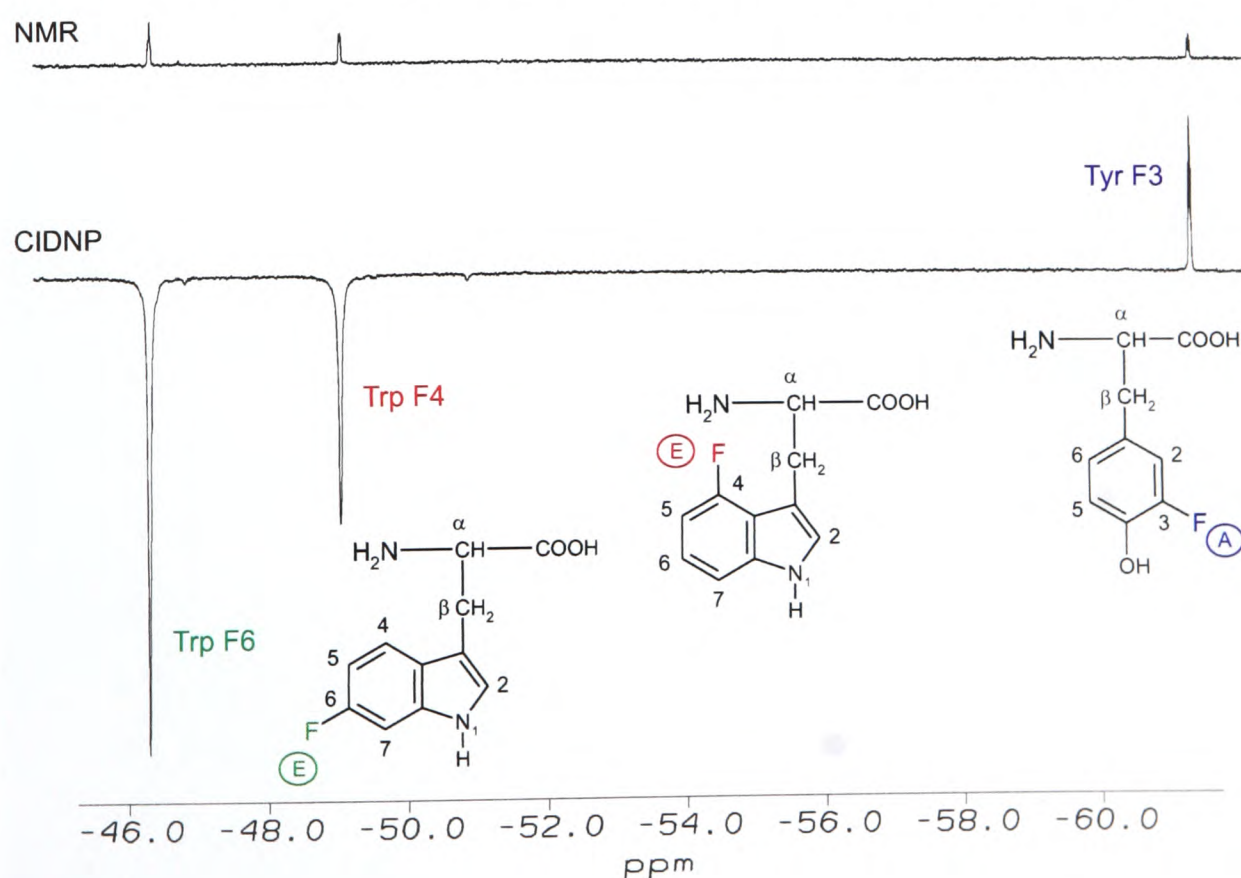


Figure 5.17: 564 MHz ^{19}F NMR and photo-CIDNP spectra of an equimolar mixture of 3-fluoro-tyrosine, 4-fluoro-tryptophan and 6-fluoro-tryptophan (0.7 mM, pH 4.5, 20 °C). Both spectra were averaged over 16 scans and the photo-CIDNP spectrum was obtained with 100 ms 4 W laser flashes. Chemical shifts are shown relative to that of trifluoroacetic acid.

that the phases of the enhancements are opposite to those obtained with unlabeled amino acids as expected from the opposite signs of ^{19}F and ^1H hyperfine coupling constants [193]. The high resolution of one-dimensional ^{19}F CIDNP protein spectra should lend itself well to stopped-flow studies of protein folding as recently demonstrated for dihydrofolate reductase using ^{19}F NMR [151–153].

Chapter 6

Photo-CIDNP field dependence

The field dependence of amino acid photo-CIDNP intensities is investigated. A stopped-flow CIDNP device that allows sample irradiation over a range of magnetic fields (0.1-7 T) within the bore of a 9.4 T NMR magnet and rapid transfer (<200 ms) into the NMR tube for detection is described. A comparison of the experimental field dependence and simulations based on Adrian's diffusion model is presented, and the possibility of improved signal-to-noise ratios in CIDNP experiments with particular emphasis on methionine and protein applications is assessed.

6.1 Introduction

In a conventional photo-CIDNP experiment, polarization is both generated and detected within the strong magnetic fields found at the centre of typical NMR magnets (e.g. 9.4 T for a 400 MHz spectrometer) [11, 12]. Whilst detection of CIDNP signals at such high fields has a number of advantages, in particular high spectral resolution, a crude “back of the envelope” calculation suggests that maximum CIDNP intensities for a flavin/amino acid mixture should be obtained by generating polarization at fields around 0.5-3 T. Therefore, unlike in conventional NMR where larger magnets provide both greater sensitivity and resolution [194], in photo-CIDNP experiments the gain in resolution is often associated with a loss in signal intensity. In this chapter, the possibility of enhancing sensitivity in protein experiments by generating CIDNP in a “low” field whilst maintaining high resolution by transferring polarization to high field for detection will therefore be explored.

The nuclear spin sorting process that lies at the heart of the radical pair mechanism and is responsible for the CIDNP phenomenon, originates from the nuclear spin dependence of singlet-triplet mixing rates (Chapter 1). At its simplest level, the origin of the field dependence of CIDNP can be understood somewhat qualitatively in terms of the EPR spectra of the free radicals that make up each radical pair. As

shown in Fig.6.1, for the simplest case of a radical pair with a single proton on one of the radicals, at very weak (in red) and very strong (in green) magnetic fields, the difference in Larmor frequency between the two component radicals shows little dependence on the nuclear spin state of the proton (i.e. $|\Delta\omega^\alpha| \simeq |\Delta\omega^\beta|$) and as a consequence, CIDNP generated at such extreme fields will be weak. For a given

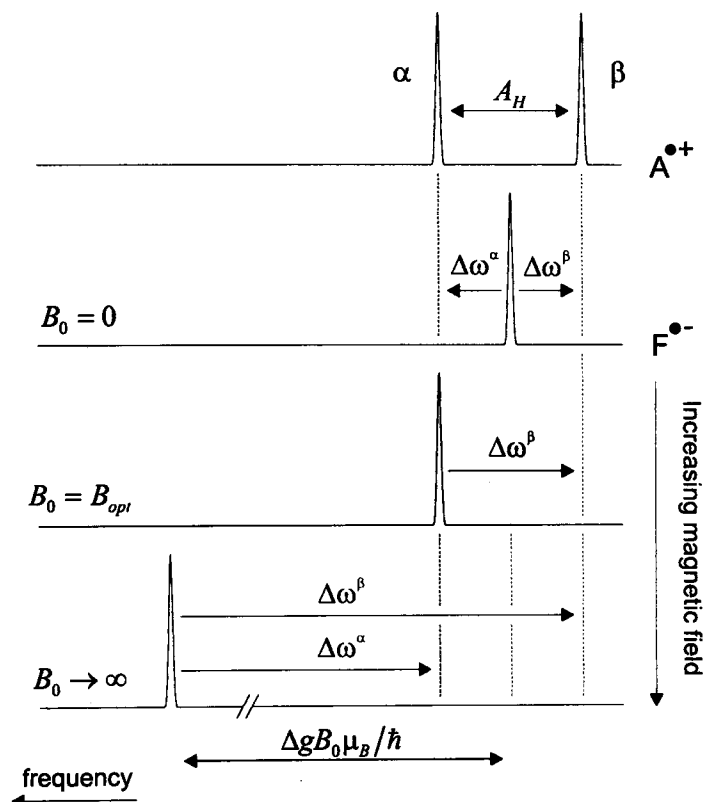


Figure 6.1: EPR spectra of the free radicals $A^{\bullet+}$ and $F^{\bullet-}$ as a function of the external magnetic field. α and β denote respectively the $m_I = +\frac{1}{2}$ and $-\frac{1}{2}$ spin states of the proton on $A^{\bullet+}$ and $\Delta\omega^{\alpha,\beta} = \omega_A^{\alpha,\beta} - \omega_F$.

hyperfine coupling A_H , the maximum CIDNP effect would be expected to arise at an intermediate field $B_0 = A_H \hbar / 2\mu_B \Delta g$ as shown in blue. Hence radical pairs with large Δg values will exhibit a maximum at lower fields than radical pairs with small Δg values and nuclei with large hyperfine couplings should favour high magnetic fields.

In the following section, a quantitative approach, the so called “diffusion model” proposed by Adrian [14, 15] and Kaptein [16] that takes into account the relative timescales of the spin dynamics just described and the motional behaviour of the component radicals will be outlined. As will become apparent in the subsequent discussion, this model provides a sound theoretical basis for the quantitative description of the field dependence of amino acid photo-CIDNP intensities.

6.2 The diffusion model

As was described in Chapter 1, geminate radical pairs formed by the chance encounter of an excited triplet flavin and an amino acid molecule are short lived

(10^{-11} s), in addition, at such short radical separations the exchange interaction is large and hence, S- T_0 mixing (10^{-9} - 10^{-8} s) in these primary pairs is limited. As a consequence, reaction within this “cage” will not lead to a polarized product and for polarization to develop, the radicals of the geminate pair must diffuse apart thereby initiating singlet-triplet mixing (the exchange coupling drops off rapidly with increasing radical separation) before re-encountering at a later time to form a secondary radical pair. Typical re-encounter times (10^{-10} - 10^{-7} s) are such that singlet-triplet mixing can take place while the radicals are separated. The extent of secondary recombination will then be determined by the singlet character of the radical pair at the time of recombination. As shown in Eqn.6.1, the overall probability of secondary recombination P_n for a given nuclear state n is therefore obtained by integrating over time the product of the singlet character $S_n(t)$ and the re-encounter probability $f(t)$:

$$P_n = \lambda \int_0^{\infty} S_n(t) f(t) dt \quad (6.1)$$

where λ is a steric factor that determines the probability of reaction upon encounter in the singlet state. The singlet character of the radical pair oscillates according to the rate of S- T_0 mixing and for a triplet precursor is given by [195]:

$$S_n(t) = \frac{1}{3} \sin^2 \omega_n t \quad (6.2)$$

where ω_n describes the rate of S- T_0 mixing (in angular frequency units) for a given nuclear state n and account has been taken of the fact that only 1/3 of the triplets will be in the T_0 state. In early communications [7, 196], an exponential function was adopted to describe the diffusive behaviour of the components of the radical pair with limited success. An alternative function based on the diffusion theory developed by Noyes [197] that provides a better description of the long term behaviour was therefore later suggested by Adrian [14, 15] and Kaptein [16]. The probability of re-encounter of two molecules which have separated from an initial encounter is then given by:

$$f(t) = mt^{-3/2} e^{-m^2/p^2 t} \quad (6.3)$$

where m (typically $10^{-6} \text{ s}^{1/2}$ [8]) is determined mainly by the frequency of diffusive steps and p is the total probability of at least one re-encounter (in most solvents ~ 0.5 [8]). At times sufficiently long to be of importance to S- T_0 mixing the exponential part of Eqn.6.3 goes to unity. By inserting expressions for the singlet probability (Eqn.6.2) and the simplified re-encounter probability (Eqn.6.3) into Eqn.6.1 and integrating by parts [195], one finally obtains for the overall probability of secondary recombination [8]:

$$P_n = \frac{\lambda}{3(1-p)} m (\pi |\omega_n|)^{1/2} \propto |\omega_n|^{1/2} \quad (6.4)$$

where the factor $(1 - p)^{-1}$ accounts for the effect of multiple re-encounters [8]. The rate of S-T₀ mixing ω_n depends on the difference in Larmor frequencies of the two radicals (Chapter 1), giving for the simple case described in Fig.6.1 of a radical pair with a single proton with magnetic quantum number m_I and hyperfine coupling A_H ,

$$\omega_{m_I} = \frac{\Delta g \mu_B B_0}{\hbar} + m_I A_H \quad (6.5)$$

and hence the polarization generated per radical pair,

$$P_{+1/2} - P_{-1/2} \propto \left| \frac{\Delta g \mu_B B_0}{\hbar} + \frac{1}{2} A_H \right|^{1/2} - \left| \frac{\Delta g \mu_B B_0}{\hbar} - \frac{1}{2} A_H \right|^{1/2} \quad (6.6)$$

In a typical radical pair, several nuclei with hyperfine couplings are likely to be present on one or both radicals. For a radical pair with N nuclei, Eqn.6.6 will need to be evaluated and summed over the 2^{N-1} permutations the other nuclei may assume. A program developed for this purpose by Mr.J.J.Lopez was used for all simulations.

To illustrate the effect of adding increasing numbers of hyperfine couplings on one or both radicals, Figure 6.2 compares the theoretical field dependence for tyrosine H3 (or H5) simulated using different subsets of the known hyperfine couplings for both neutral tyrosine [21] and flavin [198] radicals shown in Table 6.1. Whilst simulation

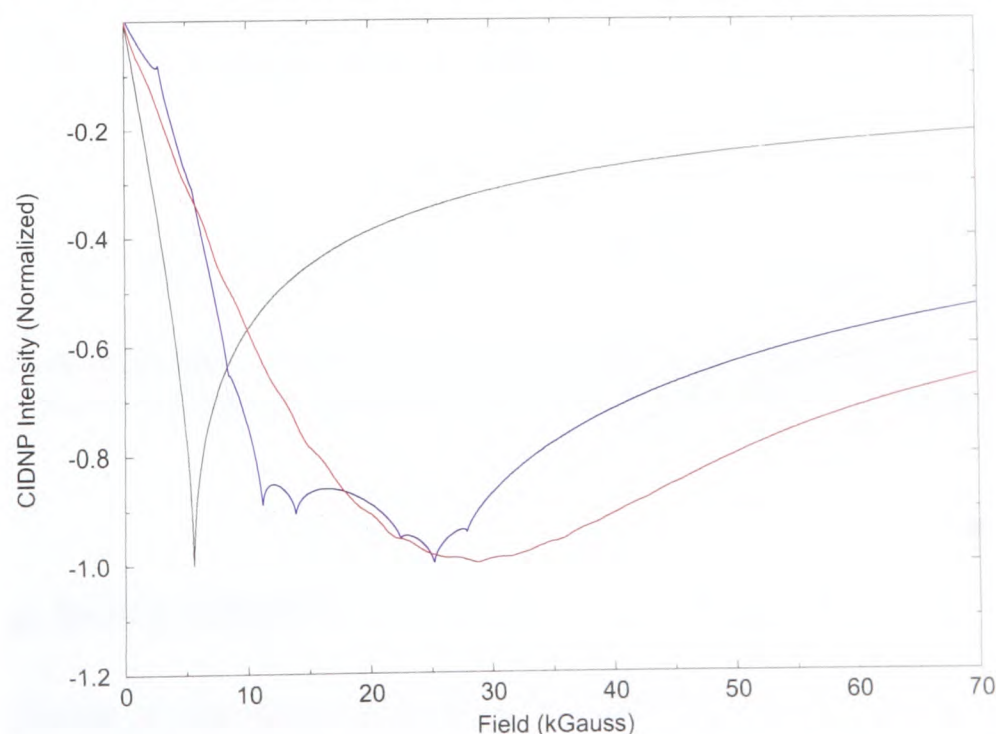


Figure 6.2: Field dependence simulations for tyrosine H3 (or H5) using a single hyperfine coupling for H3 (in black), including all the other tyrosine hyperfine couplings (in blue) and including all tyrosine *and* flavin hyperfine interactions (in red).

based on a single hyperfine coupling H3 (or H5) on tyrosine is rather sharp and predicts an optimum field of ~ 0.6 T, adding further couplings on tyrosine and including the interactions on the flavin counter radical shifts this optimum to higher field and broadens out the overall field dependence curve. In addition, as shown in Fig.6.3a

Tyr• $g=2.0041$		FMN• $g=2.0030$	
H2	1.5	N5 (x2)	7.8
H3	-6.5	H6	-1.7
H5	-6.5	8-CH ₃ (x3)	2.4
H6	1.5	N10 (x2)	3.7
β -CH ₂	7.7		

Table 6.1: Hyperfine couplings (in Gauss) and g -values used for the neutral tyrosine [21] and flavin [22, 198] radicals. Note that ^{14}N are spin-1 nuclei, however, the simulation program was designed for spin-1/2 nuclei only and all ^{14}N nuclei were therefore included as two spin-1/2 nuclei as shown. Such an approach doubles the degeneracy of the $m_I=0$ line but was found to be the closest possible approximation.

whilst increasing the magnitude of the hyperfine coupling on H3 (or H5) when all other hyperfine interactions are included, increases the absolute CIDNP enhancement, it has very little effect on the overall position of the curve, however, as shown in Fig.6.3b small changes in the Δg value can shift the maximum considerably, in particular around small Δg values.

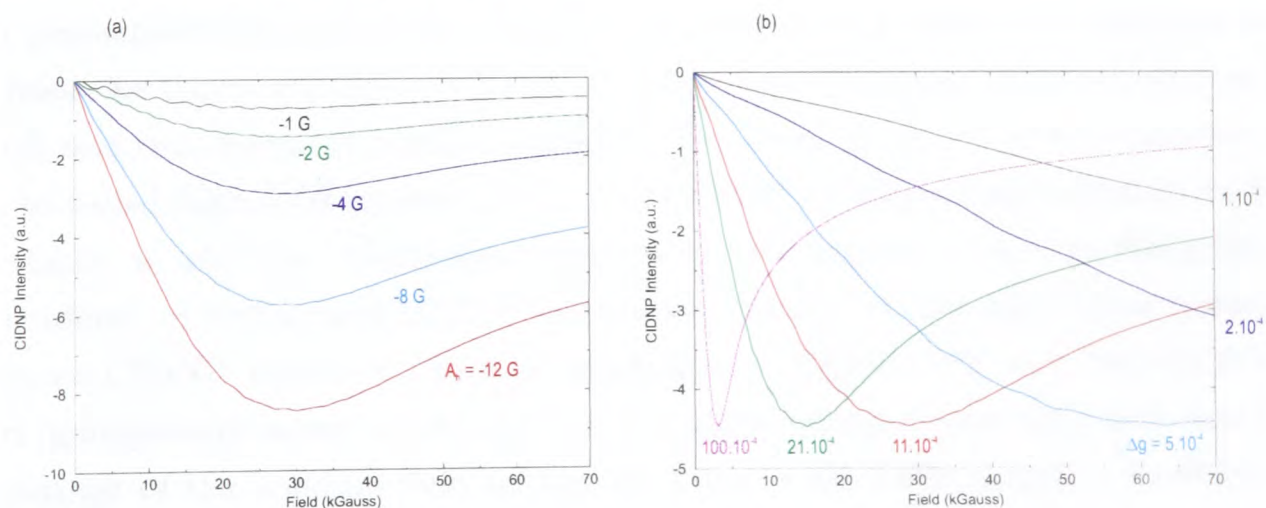


Figure 6.3: Field dependence simulations for tyrosine H3 (or H5) including all tyrosine and flavin hyperfine interactions. (a) Changes caused by varying the H3 hyperfine coupling and (b) the radical pair Δg -value.

6.3 The bore insert

A growing interest in the generation of photo-CIDNP over a range of fields (in particular very low fields <0.1 T) has led to the development of several field dependent CIDNP setups. These generally involve the use of an NMR magnet in conjunction with a smaller magnet of variable field strength (e.g. an adjacent EPR magnet). During the course of an experiment, the radicals are generated in the auxiliary magnet and then transferred into the centre of the NMR probe for measurement [199]. During the transfer period, polarization will decay by relaxation and whilst this may not be a problem for small molecules (e.g. amino acids) that have relaxation times

on the order of seconds, it becomes a limiting factor for proteins where relaxation occurs over an order of magnitude faster. The key element of any field dependent experimental design therefore lies in minimizing the transfer time between magnets [199]. Whilst some of the earliest experiments were performed by manual transfer or by slow continuous flow (e.g. using a peristaltic pump) on compounds with long relaxation times (~ 10 s), shorter transfer times have recently been obtained (0.5-1 s) using pneumatically driven flowing systems and by mechanical transfer. In this context, Stob *et al.* [200] developed a “falling tube” system in which the NMR tube is transferred by gravity from an auxiliary magnet placed concentrically above the NMR magnet and Grosse *et al.* [201] have recently proposed an alternative approach that involves driving the whole probe head from a smaller magnet located beneath the super-conducting solenoid.

While the methods described above offer the possibility of very weak homogeneous fields and thus the possibility of studying CIDNP generated by mixing of the S and $T_{\pm}$ states [199], they involve considerable technical efforts, and are more or less permanent once installed. Transfer of these setups onto other magnets would therefore be time consuming, a factor of importance if a range of spectrometers from which one can choose is readily available. However, the focus of this chapter is on the so-called high field region (>0.1 T) of CIDNP where mixing is limited to S and T_0 states, in addition, whilst the emphasis in *this* chapter is on describing the field dependence of amino acid CIDNP intensities, the underlying motivation remains to enhance CIDNP signals for protein applications (Chapter 7), and therefore, strict field homogeneity is not necessary. An alternative stopped-flow approach that takes advantage of the residual field within the bore of all NMR magnets was therefore developed and is shown in Fig.6.4.

The key components of the bore insert have been highlighted and consist of an optical fibre, a right angle prism (model 10-RB-10, Comar) and a thin glass tube (2 mm internal diameter) connected at both ends with PTFE tubing (0.6 mm internal diameter) using two-way liquid chromatography connectors (model 1001, Omnifit) and O-ring seals. A pneumatically driven gas-tight syringe that sits outside the bore was connected to the upper PTFE tubing enabling rapid transfer of irradiated solution into the NMR tube. The 5 mm NMR tube was fitted with a coaxial insert (model WGS-5BL, Wilmad) with its stem cut down to allow the end of the lower PTFE tubing to dip into the solution 4 mm above the top of the NMR coil. The pneumatic triggering system and syringe support were as described in Chapter 5 for real-time refolding, except that a larger syringe (model 2.5MDR-GT, SGE) was used to allow injection of larger volumes. After each injection and acquisition, the syringe was automatically retracted thereby sucking up the transferred sample. Typically,

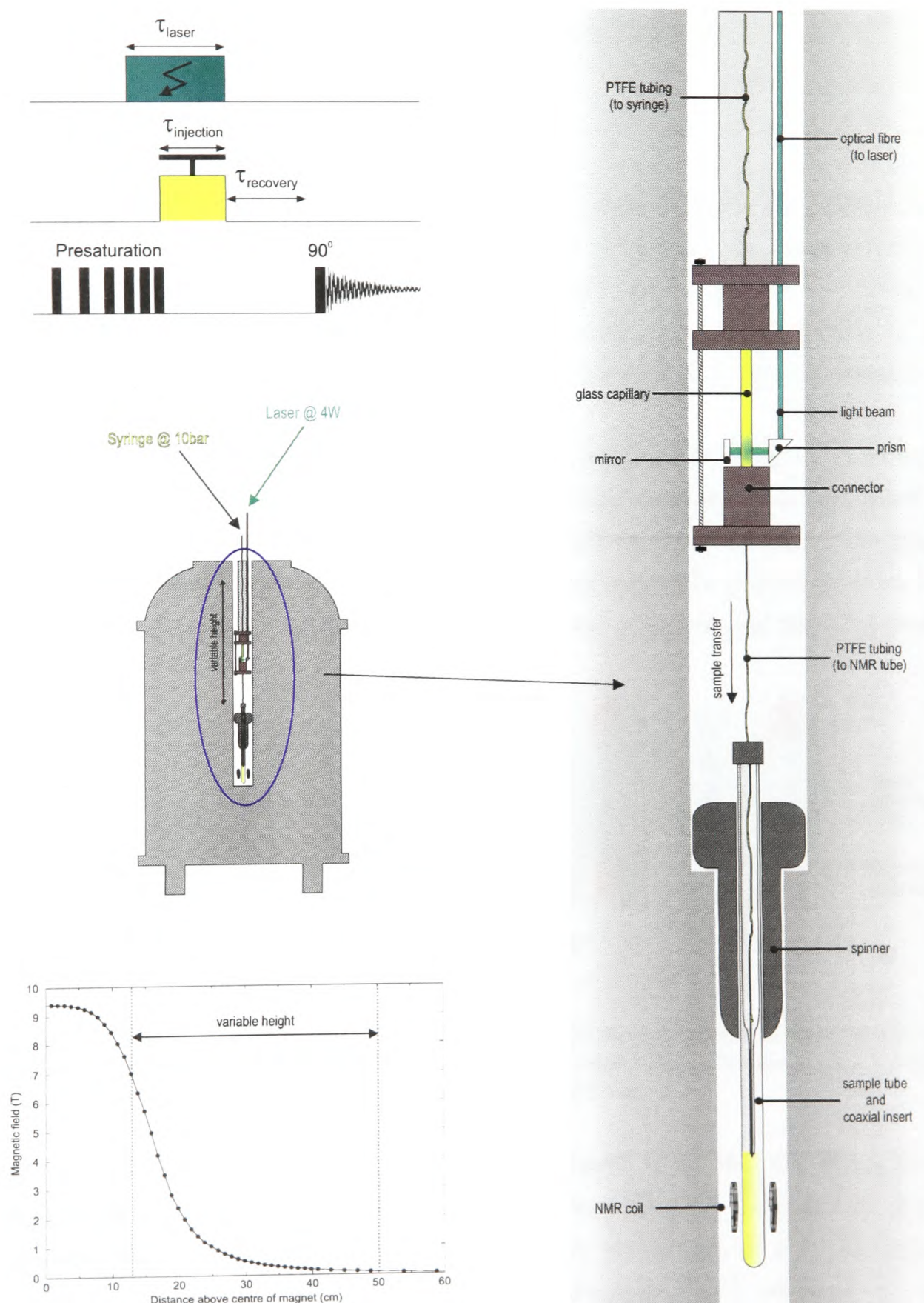


Figure 6.4: The stopped-flow CIDNP device and pulse sequence. Note that laser and injection pulses overlap. During irradiation and before the onset of transfer to high field, the “dead” sample within the NMR tube was presaturated by a random series of hard 90° pulses. Also shown is the residual field within the bore measured with an axial hall probe (model MMA-1808-WL, Lakeshore) as a function of distance above the centre of the 9.4 T magnet. Polarization can be generated at any field within the range 0.1–7 T (~ 50 –13 cm above the magnet centre respectively).

300 μl of solution was transferred into 400 μl of the same solution within the NMR tube. The dead volume between the irradiated region and NMR tube was estimated to be ~ 100 μl with a lower PTFE tubing of ~ 30 cm in length.

6.4 Preliminary tests

As was mentioned earlier, the success of the technique depends crucially on minimizing the delay between generation and detection of CIDNP and therefore on both the speed with which the polarized solution can be transferred into the NMR tube and the recovery of the NMR lineshape from the shock of the stopped-flow. In order to assess these features, a number of preliminary experiments were performed.

Injectons were first examined outside the magnet as shown in Fig.6.5. The stopped-flow device (i.e. syringe, PTFE transfer line and glass capillary) was first filled with water, 300 μl of methylene blue dye was then sucked up into the end of the transfer line and the coaxial insert with PTFE tubing was carefully placed into an NMR tube containing 400 μl of water. The methylene dye solution was then injected via

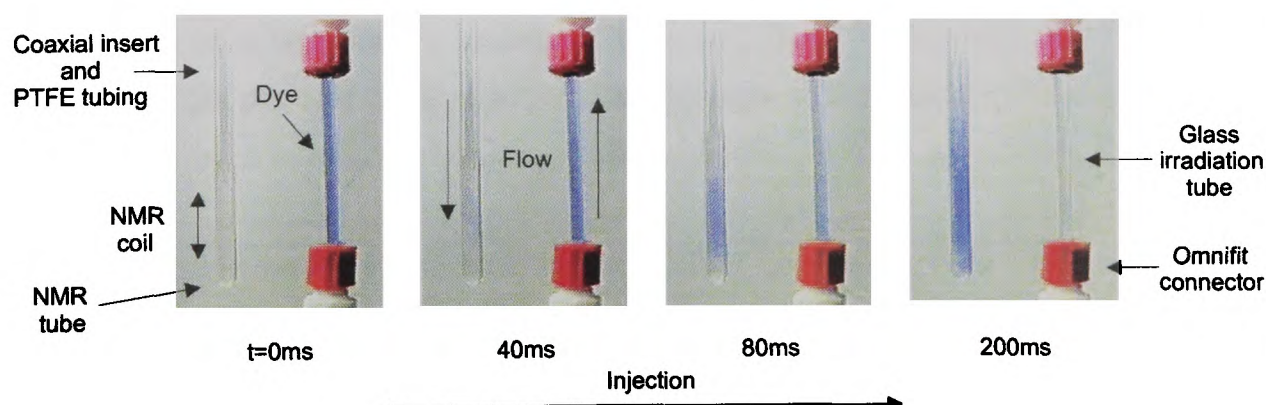


Figure 6.5: The injection of 300 μl of methylene dye at 10 bar into 400 μl H_2O as filmed outside the magnet at 25 Hz. The 200 ms injection pulse began at some stage between the first two frames. Transfer of dye into the NMR tube by injection appears efficient.

a 200 ms pulse at 10 bar and the process was filmed at 25 Hz. Bulk transfer and mixing of the dye within the injection time appears efficient. In order to calibrate the system, the dependence of volume injected on the length of a 10 bar injection pulse was also measured. With a typical length of upper PTFE tubing (~ 2 meters) approximately 100 ms was required to inject 150 μl and 200 ms for 300 μl .

To examine the rate of recovery from mechanical shock, linewidths of the histidine H_2 CIDNP signals after injection were also measured and are shown in Fig.6.6a. Whilst early spectra (0-50 ms) are severely broadened by sample inhomogeneity, the linewidth recovers remarkably quickly and after 100 ms it has almost narrowed to its limiting value. The efficiency of mixing within the NMR sensitive region was

also estimated by injecting 300 μl of D_2O into 400 μl of H_2O and measuring the “diluted” H_2O NMR intensity as a function of the post-injection delay (Fig.6.6b). At $t=0$ (i.e. immediately after the 200 ms injection pulse) the final dilution factor

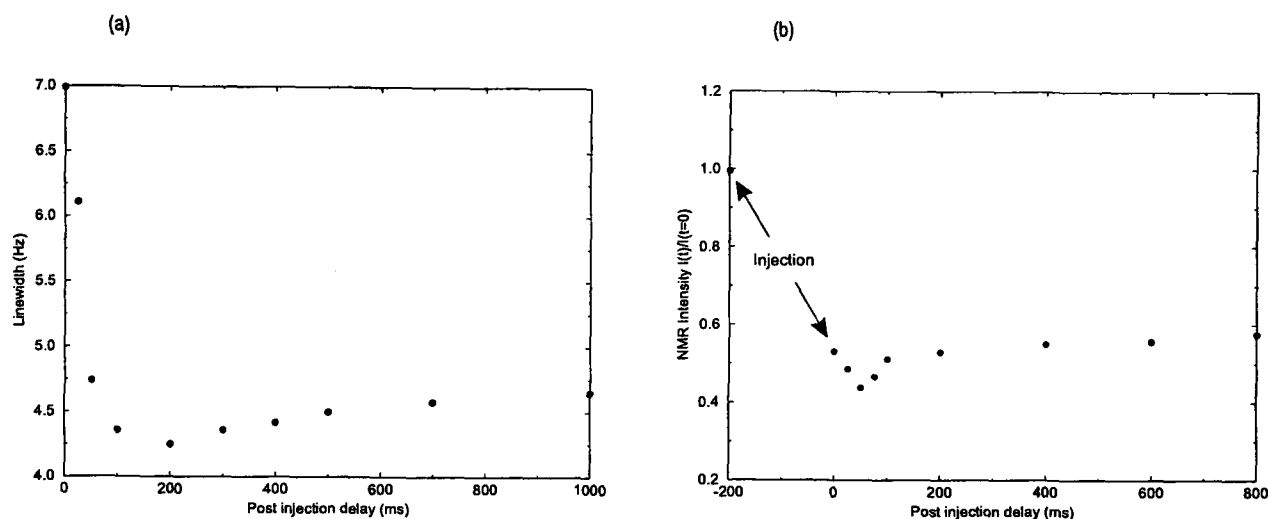


Figure 6.6: (a) Recovery from mechanical shock after injection as monitored by the linewidth of the histidine H2 CIDNP signal and (b) mixing as monitored by the H_2O NMR signal (integrated intensity *not* peak height) after injection of 300 μl of D_2O into 400 μl of H_2O .

of ~ 0.6 (i.e. 400 $\mu\text{l}/700 \mu\text{l}$) has already been reached, however as with the real-time device described in Chapter 5 (Fig.5.4a) a small “overshoot” occurs reflecting the time of travel of a temporary excess of injected D_2O within the NMR sensitive region. Within 50 ms the final dilution factor is re-established although as with the real-time device, a small slow mixing phase ($<5\%$) is also apparent.

In order to maximize the available polarization, the dependence of tyrosine (4 mM, pH 4.5) H3,5 CIDNP intensity on flavin concentration (Fig.6.7a) and on the volume of irradiated sample transferred to high field (Fig.6.7b) was also measured. The

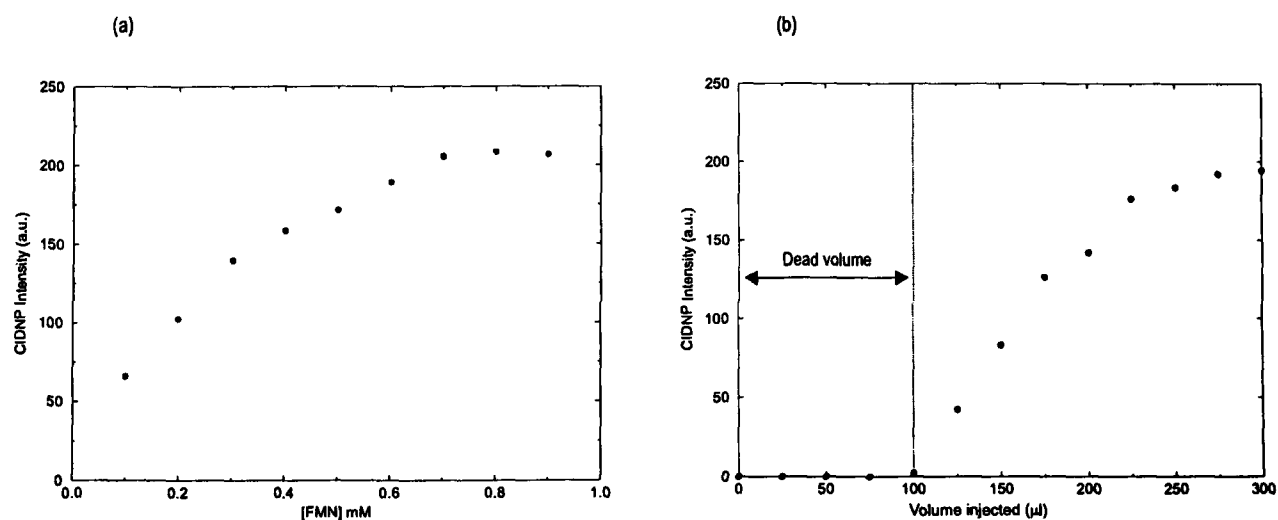


Figure 6.7: Dependence of tyrosine H3,5 CIDNP intensity on (a) flavin concentration and (b) volume of irradiated sample transferred to high field. Note the $\sim 100 \mu\text{l}$ dead volume in (b).

optimum flavin concentration (0.8 mM) is significantly greater than for the optical fibre and coaxial insert used at high field (0.2 mM) as described in previous chapters. This is most likely to be a simple consequence of the different path lengths in the

two different experimental setups (~ 2 mm here vs. ~ 10 mm at high field). The dependence on injected volume confirms that the dead volume below the irradiated region is $\sim 100 \mu\text{l}$ and that no gain in signal is obtained by injecting more than $300 \mu\text{l}$.

These results suggest that strong well resolved CIDNP signals may be acquired using a combination of a 100 ms initial laser flash, 200 ms transfer pulse (laser remains on during this period) in which $300 \mu\text{l}$ is injected into $400 \mu\text{l}$ and a 100-200 ms post-injection delay for sample recovery before acquisition. The optimal FMN concentration (0.8 mM) was used throughout.

As an aside and to illustrate the quality of spectra obtained using such a combination of parameters, the dependence of net and multiplet effect contributions to the overall CIDNP spectrum on the r.f. rotation angle (α) was measured at ~ 3 T for the histidine $\beta\text{-CH}_2$ protons and is shown in Fig.6.8. Whilst net polarization

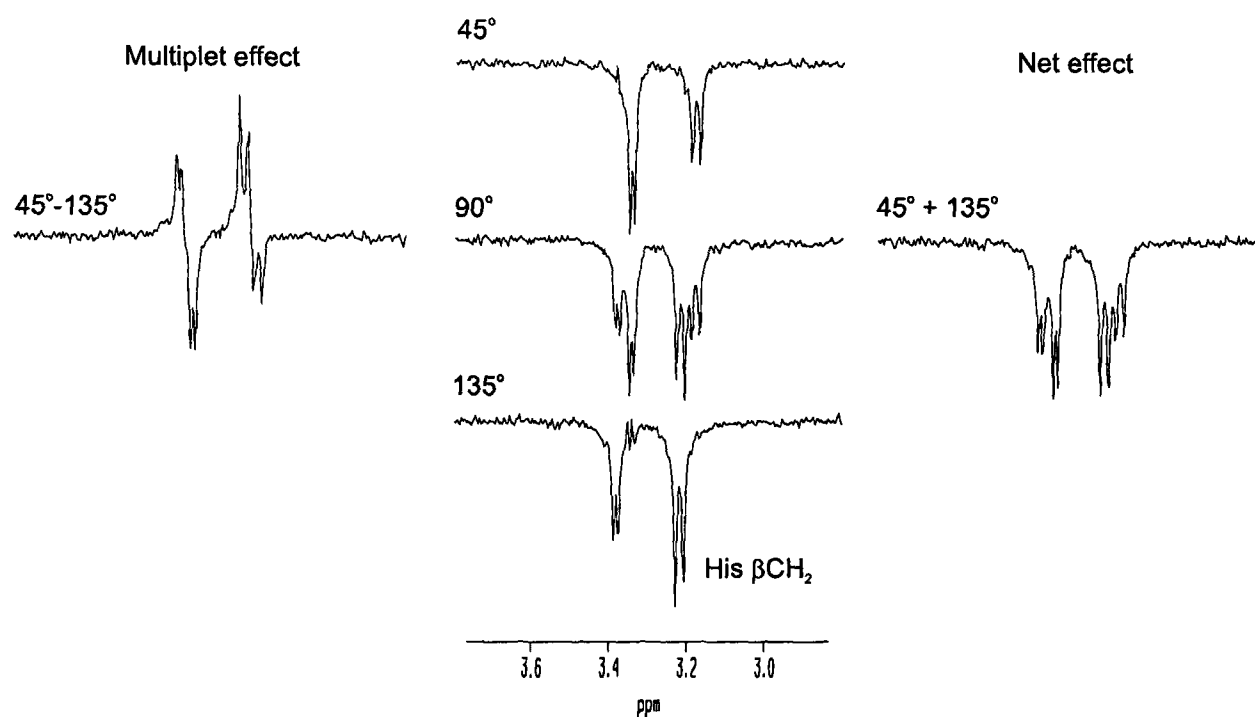


Figure 6.8: A comparison of the dependence of net and multiplet effect contributions to the histidine (20 mM, pH 7) βCH_2 CIDNP spectrum on the final r.f. pulse angle.

depends on $\sin \alpha$, the multiplet effect depends on $\sin 2\alpha$, therefore addition of free induction decays taken after 45° and 135° pulses, yields pure net polarization and subtraction yields the pure multiplet effect [202]. All experiments described in this chapter were performed using a 90° flip angle and therefore monitored the field dependence of the net effect.

Finally, the effect of photo-degradation on the signal due to dye exhaustion over successive laser flashes, was examined for tyrosine (4 mM, pH 4.5) and is compared in Fig.6.9 to the CIDNP decay obtained under identical conditions in Chapter 4 for a static sample at high field. As was observed using the mixing device in Chapter

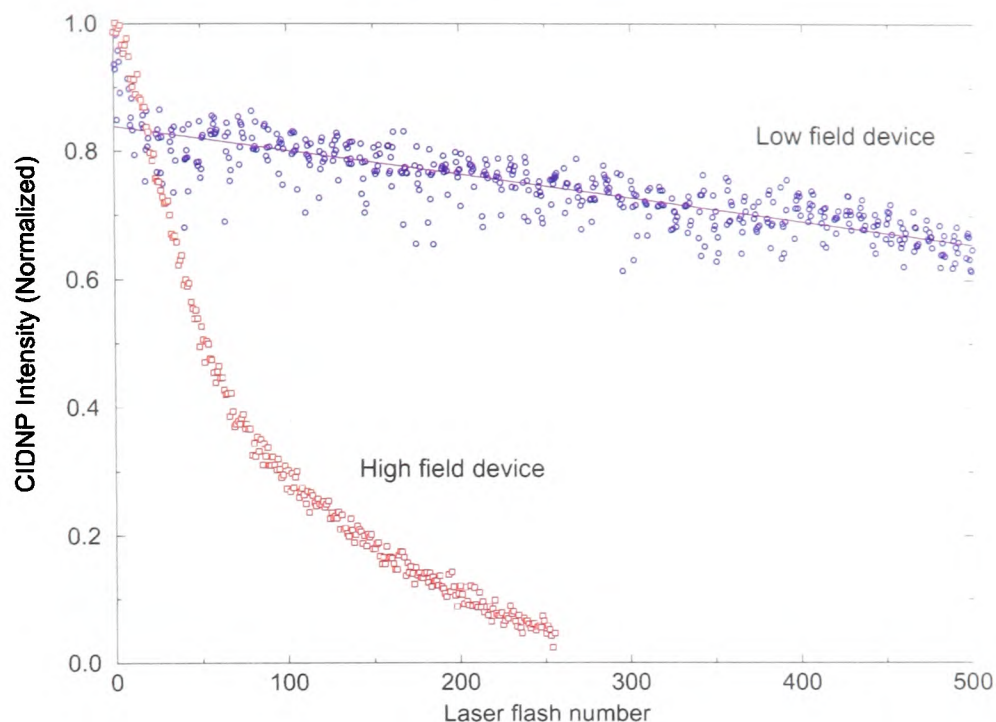


Figure 6.9: Comparison of the decay in CIDNP over successive laser flashes for tyrosine H3,5 using the variable field stopped-flow device and the conventional high field static device.

4, the lifetime of the sample is dramatically prolonged by the regular sample mixing that occurs with each injection. After an initial drop over the first ~ 8 flashes, a steady state linear decay is observed and experimental data in the following sections was therefore corrected accordingly.

6.5 Tyrosine

It is well established that tyrosine reacts with flavin via hydrogen abstraction to form a radical pair composed of neutral tyrosine and flavin radicals (Chapter 1). The g -factors and hyperfine couplings for both components are known and were shown in Table 6.1. Comparison of the tyrosine CIDNP field dependence measured experimentally to that simulated with these parameters should therefore provide a rigorous test of the experiment. Figure 6.10 shows the field dependence for tyrosine (4 mM, pH 4.5) measured over the range 0.1-7 T. At each field strength, 4 “light” and 4 “dark” free induction decays were recorded, subtracted and Fourier transformed to give photo-CIDNP difference spectra. Note that whilst dark spectra were often very weak, their intensity is field dependent and hence could not be ignored. Two separate sets of measurements were obtained with fresh samples and, in order to check for errors introduced by dye exhaustion, the direction in which the field was “sampled” was reversed for the two sets. Individual multiplets were integrated and are shown normalized after correction for photo-degradation (each data set and nucleus was normalized separately). Simulations were calculated using hyperfine couplings on tyrosine only (dashed line) and on both tyrosine and flavin (solid line). Vertical scaling was the *only* adjustable parameter. Agreement between experiment and theory for (a) H3,5 and (c,d) β -CH₂ is extremely good, particularly considering

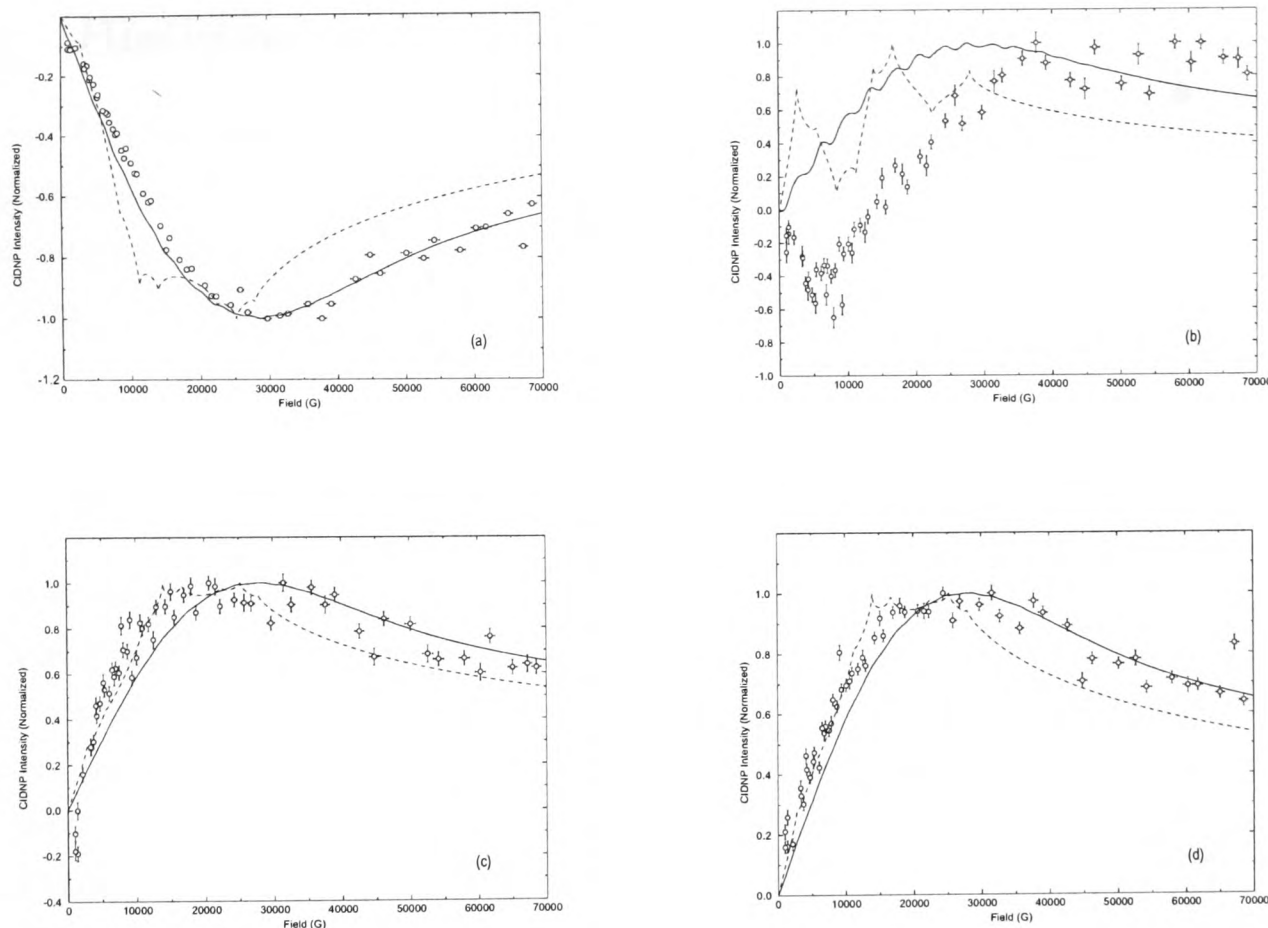


Figure 6.10: Comparison of experimental and simulated field dependence for tyrosine (a) H3,5 (b) H2,6 (c) H β_1 and (d) H β_2 . The simulations were calculated using hyperfine couplings on tyrosine only (dashed line) and on both tyrosine *and* flavin (solid line).

the simple nature of the experiment. Small deviations from the simulated curve and a sign reversal at low fields are however observed for the ~ 10 fold weaker (b) H2,6 suggesting that nuclei with smaller hyperfine couplings may deviate from this somewhat simple approach. The deviation appears to be field dependent and could potentially be caused by weak electron-nuclear relaxation at “low” (~ 1 T) fields that may go unnoticed for the strongly polarized H3,5 and β -CH₂ protons. The phase of the electron polarization (i.e. chemically induced *electron* polarization or CIDEP) on the neutral tyrosine radical is given by the sign rule $\Gamma_{net} = \mu \cdot \Delta g \cdot J = + \cdot + \cdot - = -$ (E) where J is the sign of the exchange interaction and all other parameters are as defined in Section 1.1.2 [203]. A crude “back of the envelope” calculation assuming a rotational correlation time τ_c for the free tyrosine amino acid of $\sim 5 \times 10^{-11}$ s and an electron Larmor frequency ω_0 (in angular frequency units) of $\sim 2 \times 10^{10}$ - 10^{12} s⁻¹ in the range 0.1-10 T suggests that $\omega_0 \tau_c > 1$, and hence that the emissive electron polarization should be transferred to neighbouring protons via double quantum relaxation processes and thus with retention of phase in agreement with the observed deviation. At higher fields, electron spin relaxation is likely to be dominated by the g -anisotropy and hence as observed, the deviation should decrease with increasing field. Note that the details of such a mechanism would depend on a number of factors, in particular the lifetime of radicals and the anisotropy of both the g -value and the hyperfine coupling on H2,6.

6.6 Histidine and Tryptophan

Figure 6.11 shows the field dependence for (a) histidine (20 mM, pH 7) and (b) tryptophan (2 mM, pH 8) over the range 0.1-7 T. Conditions and procedure were as described for tyrosine in Section 6.5. However, unlike for tyrosine, the hyperfine couplings and g -values for the neutral histidine and tryptophan cation radicals have not been accurately measured and calculated hyperfine interactions are inconsistent [93, 94], therefore, rather than simulating the field dependence, an alternative fitting procedure was adopted.

As was described in Section 6.2, the overall form of the field dependence curve is most sensitive to the Δg value as long as all hyperfine couplings are included and are roughly of the right magnitude. The g -value and hyperfine couplings for both the neutral flavin [22, 198] and flavin anion [23, 133] radicals are known (Table 6.2), an educated guess of the magnitude and sign of histidine and tryptophan hyperfine couplings based on photo-CIDNP data was therefore made and is shown in Table 6.2. Note that recent experiments [204] found that the phase of photo-CIDNP tryptophan signals were inverted when using eosin Y ($\text{EYH}_2^{\bullet-}$, $g=2.0025$ [205]) rather than flavin ($\text{FMN}^{\bullet-}$, $g=2.0034$) as sensitizer, hence assuming that reaction with eosin Y occurs via electron transfer, the tryptophan radical cation g -value should occur in the range $2.0025 < g < 2.0034$. The data sets were then fitted by varying the g -value of the amino acid in question as shown in Fig.6.11a and b. Reasonable agreement between theory and experiment is obtained for both histidine (H2 and H4) and tryptophan (H2, H4 and H6) with g -values of 2.0023 ± 0.0001 and 2.0027 ± 0.0002 respectively. It is interesting to note that these values are similar to those recently measured for a histidine *cation* radical [206] ($g=2.0023$) and a *neutral* tryptophan radical [94] ($g=2.0029 \pm 0.0002$) respectively.

His [•] $g < 2.0030$		FMN [•] $g=2.0030$		Trp ^{•+} $2.0025 < g < 2.0034$		FMN ^{•-} $g=2.0034$	
H2	-5	N5 (x2)	7.8	N1 (x2)	7	N5 (x2)	7.3
H4	-5	H6	-1.7	H2	-5	H6	-3.5
β -CH ₂ (x2)	5	8-CH ₃ (x3)	2.4	H4	-5	8-CH ₃ (x3)	4.0
		N10 (x2)	3.7	H6	-5	N10 (x2)	3.1
				β -CH ₂ (x2)	5		

Table 6.2: Hyperfine couplings (in Gauss) and g -values used for the histidine and flavin neutral [22, 198] radicals (Fig.6.11a), and the tryptophan cation and flavin anion [23, 133] radicals (Fig.6.11b).

tophan signals were inverted when using eosin Y ($\text{EYH}_2^{\bullet-}$, $g=2.0025$ [205]) rather than flavin ($\text{FMN}^{\bullet-}$, $g=2.0034$) as sensitizer, hence assuming that reaction with eosin Y occurs via electron transfer, the tryptophan radical cation g -value should occur in the range $2.0025 < g < 2.0034$. The data sets were then fitted by varying the g -value of the amino acid in question as shown in Fig.6.11a and b. Reasonable agreement between theory and experiment is obtained for both histidine (H2 and H4) and tryptophan (H2, H4 and H6) with g -values of 2.0023 ± 0.0001 and 2.0027 ± 0.0002 respectively. It is interesting to note that these values are similar to those recently measured for a histidine *cation* radical [206] ($g=2.0023$) and a *neutral* tryptophan radical [94] ($g=2.0029 \pm 0.0002$) respectively.

The results presented so far for tyrosine ($\Delta g=0.0011$), tryptophan and histidine (both $\Delta g \sim 0.0007$) indicate that the maximum net effect for all three amino acids arises over a somewhat broad range of magnetic fields centered around 4 T (i.e. ~ 20

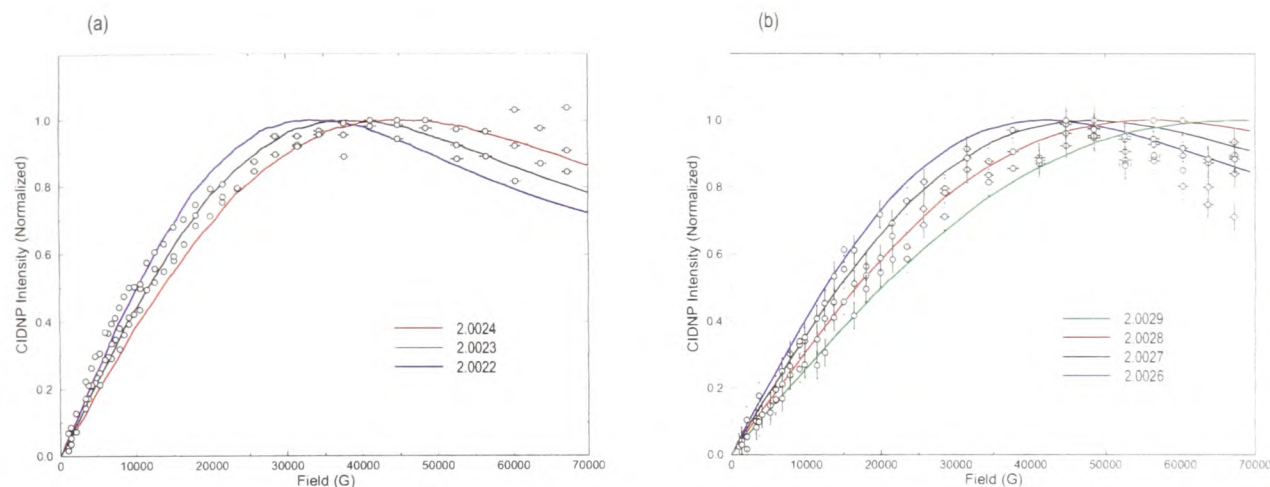


Figure 6.11: Comparison of experimental and simulated field dependence for (a) histidine (H2 and H4) and (b) tryptophan (H2, H4 and H6). Simulations were calculated for a range of amino acid g -values using the hyperfine couplings shown in Table 6.2.

cm above the centre of the 400 MHz spectrometer). Optimized CIDNP intensities using both the high field device described in previous chapters at 9.4 T and the “low” field device at 4 T were therefore compared for all three amino acids and the native states of hen lysozyme and bovine α -lactalbumin. Somewhat disappointingly, the absolute intensities for all systems were found to be roughly identical within experimental error for both setups suggesting that the high field approach is more efficient in maximizing the theoretical polarization. Considering the differences between the two approaches namely the injection and delay between generation and detection of polarization in the “low” field setup, this is perhaps not so surprising. This comparison suggests that the “low” field device as described in this thesis is of little use for the study of proteins under *equilibrium* conditions at least as far as tyrosine, tryptophan and histidine signals are concerned. However, as will be described in the following section this does not apply to methionine where an order of magnitude enhancement has been obtained. In addition, as will be described in Chapter 7, for *kinetic* protein experiments that require an injection to initiate refolding, the “low” field approach provides a five fold signal enhancement over a high field analogue. On another note, improvements to the current “prototype” design in particular with regards to the efficiency of sample transfer and laser illumination are surely possible and should provide greater enhancements. It might also be possible to perform these experiments with the sample enclosed in a μ -metal box so as to get fields much smaller than currently possible (i.e. $B_0 \simeq A_H$) at which point S-T $_{\pm}$ mixing [199] (Chapter 1) becomes important and could potentially give much larger enhancements with the same transfer times and efficiency of irradiation. A final alternative that was not explored would simply involve monitoring the magnitude of *multiplet* effects rather than *net* effects as shown in Fig.6.8. Whilst the net effect disappears at low fields, multiplet effects do not and could therefore potentially provide a greater polarization.

6.7 Methionine

Aside from histidine, tryptophan and tyrosine, methionine is the only commonly occurring amino acid susceptible to the photo-CIDNP technique [12, 19]. Figure 6.12 compares the 600 MHz ^1H NMR and photo-CIDNP spectra obtained for methionine (3 mM, pH 7) with the same number of scans. The CIDNP enhancement for the

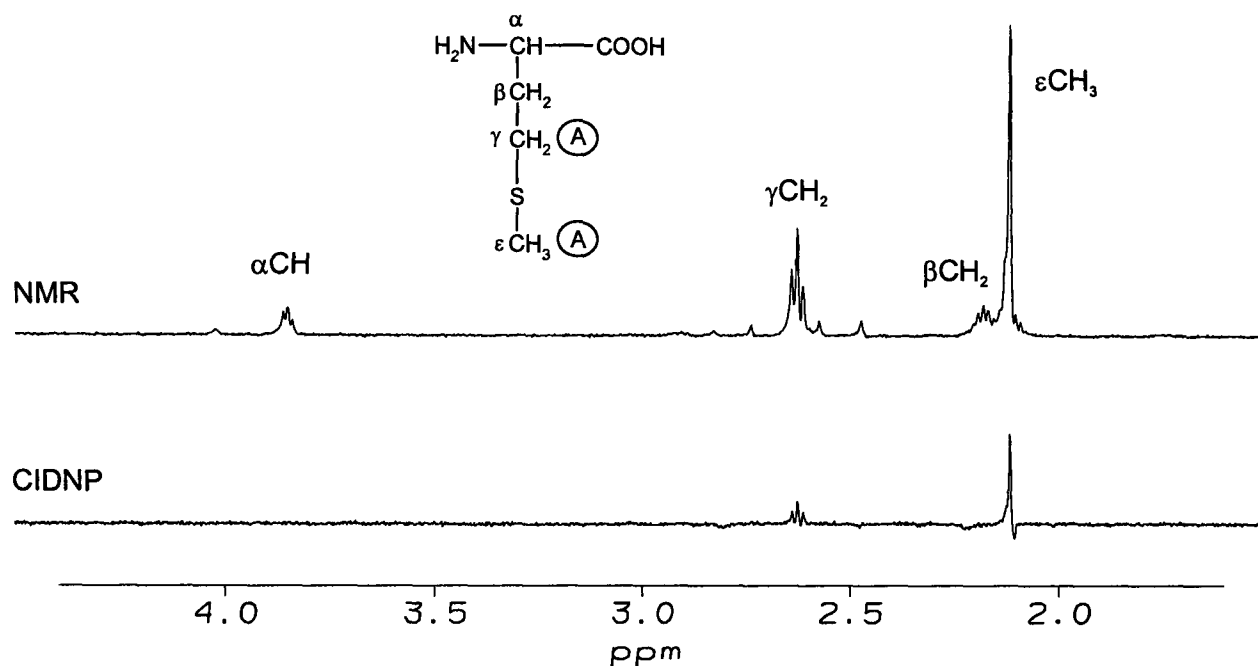


Figure 6.12: 600 MHz ^1H NMR and photo-CIDNP spectra of methionine (3 mM, pH 7). Both spectra were obtained with four scans and the photo-CIDNP spectrum was obtained with 100 ms 4 W laser flashes. At such a high field (14.1 T), the CIDNP enhancement for methionine is much weaker than for tyrosine, tryptophan and histidine suggesting a large Δg -value.

γ -CH₂ and ϵ -CH₃ protons at such a high field (14.1 T) is over an order of magnitude smaller than that obtained for tyrosine, tryptophan and histidine under the same conditions (Chapter 1) and as a consequence, of the four CIDNP active amino acids, methionine has largely been ignored in photo-CIDNP studies of proteins. It has been suggested [19] that the reaction between methionine and the excited triplet state of the flavin proceeds via an electron abstraction mechanism to give a radical ion pair comprising a methionine cation radical and a flavosemiquinone anion ($g=2.0034$). The weakness of the effect has been attributed to a large Δg value due to strong spin-orbit coupling at the sulphur atom in the methionine cation radical [19]. The only report of directly polarized methionine in a protein has been in the *lac*-repressor and its headpiece [207]. In the following section, the relative contributions of the field dependence, the competition for excited flavin and the surface accessibility to methionine photo-CIDNP intensities in proteins will be assessed.

It is worth noting at this stage that the photoreactions of sulphur-containing amino acids have recently received particular attention because of the biological importance they are thought to play in long-range electron transfer across cell membranes [208] and in oxidative damage of cell components [209]. Photo-CIDNP experiments

using 4-carboxybenzophenone as sensitizer and 308 nm excitation have been described [210, 211], however the emphasis appears to have been placed on describing in great detail the radical intermediates and non cyclic reaction pathways rather than developing the technique for the study of protein structures.

6.7.1 Field dependence

Figure 6.13 shows the field dependence for methionine (5 mM, pH 7) over the range 0.1-7 T. Conditions and procedure were as described for tyrosine in Section 6.5. As

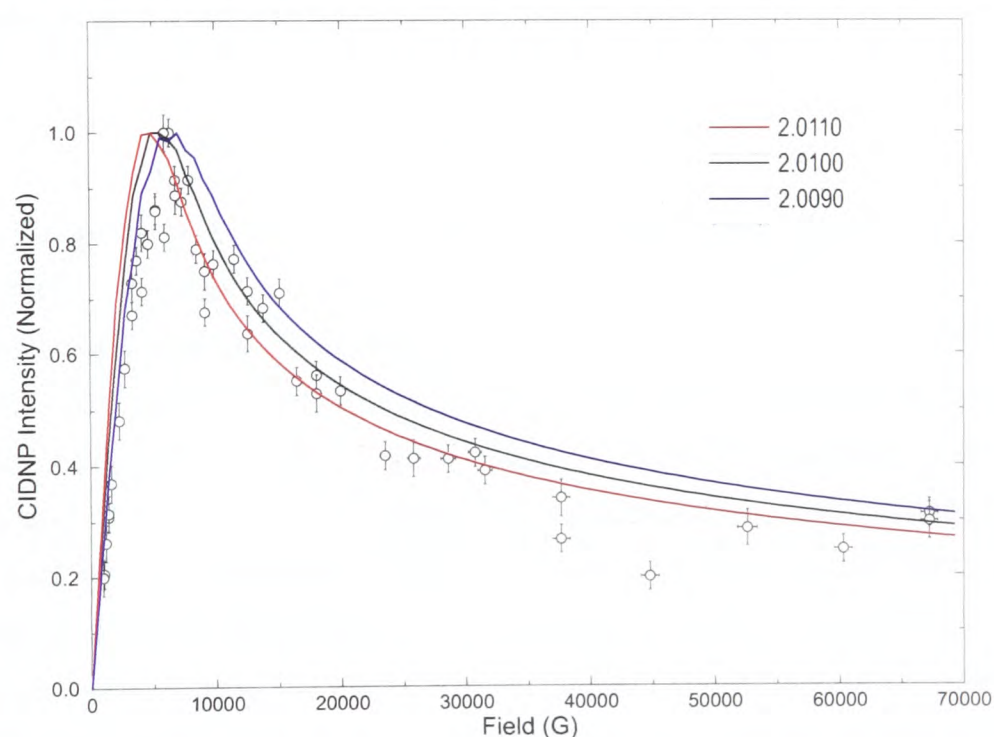


Figure 6.13: Comparison of experimental and simulated field dependence for methionine (ϵ -CH₃). Simulations were calculated for a range of amino acid g -values using the hyperfine couplings shown in Table 6.3.

compared to tyrosine, tryptophan and histidine, the optimum field is shifted down towards ~ 0.7 T suggesting a large Δg -value. Just as for histidine and tryptophan, the g -value and hyperfine couplings for the methionine radical are unknown, the same strategy as described in Section 6.6 was therefore adopted and the hyperfine couplings used are shown in Table 6.3. Note that a number of $R_2S^{\bullet+}$ radical species

Met ^{•+}		FMN ^{•-}	
$g \gg 2.0034$		$g=2.0034$	
γ -CH ₂ (x2)	8	N5 (x2)	7.3
ϵ -CH ₃ (x3)	8	H6	-3.5
		8-CH ₃ (x3)	4.0
		N10 (x2)	3.1

Table 6.3: Hyperfine couplings (in Gauss) and g -values used for the methionine cation and flavin anion [23, 133] radicals.

(usually described as radical dimers $R_2S^{\bullet}SR_2^+$) have been studied by solid state EPR with anisotropic g -values in the regions of 2.02, 2.01, 2.00 (i.e. isotropic 2.01)

and hyperfine interactions to the protons on either side of the sulphur centre of 5-10 G [212, 213]. In accordance with these values, reasonable agreement between theory and experiment is obtained with a g -value of 2.0100 ± 0.0010 confirming that the weak CIDNP observed for methionine at high fields is due in part to the large Δg value for the methionine cation/flavin anion radical pair.

6.7.2 Competition effects

As was described in Chapter 1, the CIDNP intensity of a proton in an amino acid A in a binary mixture of amino acids A and B can be considered as a product of three terms:

$$I_A = p_A \frac{k_A[A]}{k_q + k_A[A] + k_B[B]} \frac{T_{1A}^{-1}}{k_A^{ex}[A] + T_{1A}^{-1}} \quad (6.7)$$

The first is the polarization p_A generated per radical pair, which depends inter alia on the hyperfine coupling of the proton in question, the difference in g -values of the radicals that constitute the pair and the strength of the magnetic field as described in Section 6.2. Second is the fraction of triplet flavin molecules (3F) that react with A to give radical pairs (Section 1.2.5). This quantity is determined by the concentration of A and B, by the second order rate constants (k_A and k_B) for their reaction with (3F), and by the (pseudo-) first order rate constant k_q for decay of (3F), e.g. by fluorescence and/or quenching by molecular oxygen. Thirdly at higher amino acid concentrations, it may be necessary to include the effects of degenerate electron exchange (Section 1.2.2) between a polarized radical and its parent amino acid (T_{1A} is the nuclear spin-lattice relaxation time in the radical of amino acid A). However at low amino acid concentrations, the final term in Eqn.6.7 may be neglected and under these conditions, a Stern-Volmer plot ($1/I_A$ against $1/[A]$) should have a gradient $(k_B[B] + k_q)/k_A p_A$ and intercept $1/p_A$. A second plot, of the ratio of these two quantities against $[B]$ should give k_B/k_A and k_q/k_A as the gradient and intercept respectively [20, 30].

Previous experiments investigating the relative values of the second order rate constants of tryptophan, tyrosine and histidine with 3F at pH 7 have shown that they are in an approximate ratio of 40:15:1 respectively [20, 30]. Preliminary experiments performed with binary mixtures of methionine with these three amino acids indicated that methionine had a relatively low second order constant and histidine was therefore chosen as a partner for this investigation. Histidine CIDNP intensities were recorded at high field for mixtures of methionine and histidine in various concentrations. Some typical spectra (Fig.6.14) show the profound effect addition of increasing amounts of methionine can have on the histidine polarization. The Stern-Volmer plot and the graph of $(k_{Met}[Met] + k_q)/k_{His}$ against $[Met]$ are shown in Fig.6.15a and b respectively. The ratio of the rate constants k_{Met}/k_{His} obtained

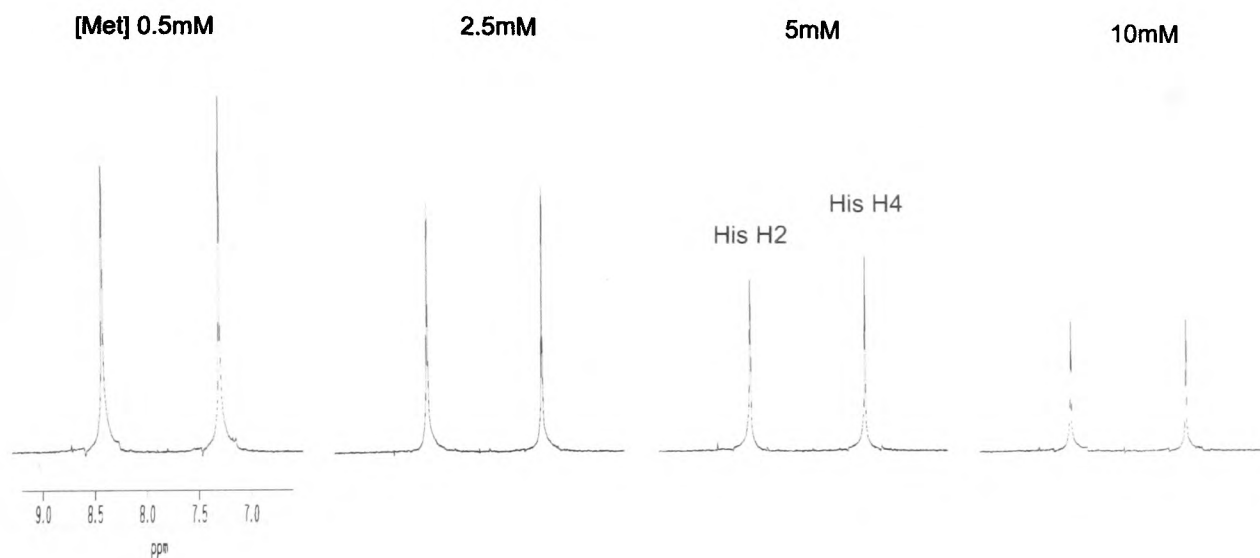


Figure 6.14: Aromatic region of 400 MHz ^1H CIDNP spectra of histidine (5 mM, pH 7) in the presence of increasing concentrations of methionine.

from this data was 4.0 ± 0.5 , and the value for k_q/k_{His} , $13 \pm 2 \times 10^{-3} \text{ mol dm}^{-3}$, was found to be in good agreement with data obtained in other studies (i.e. $9 \pm 3 \times 10^{-3} \text{ mol dm}^{-3}$ in Ref.[20]). This result suggests that methionine competes relatively well

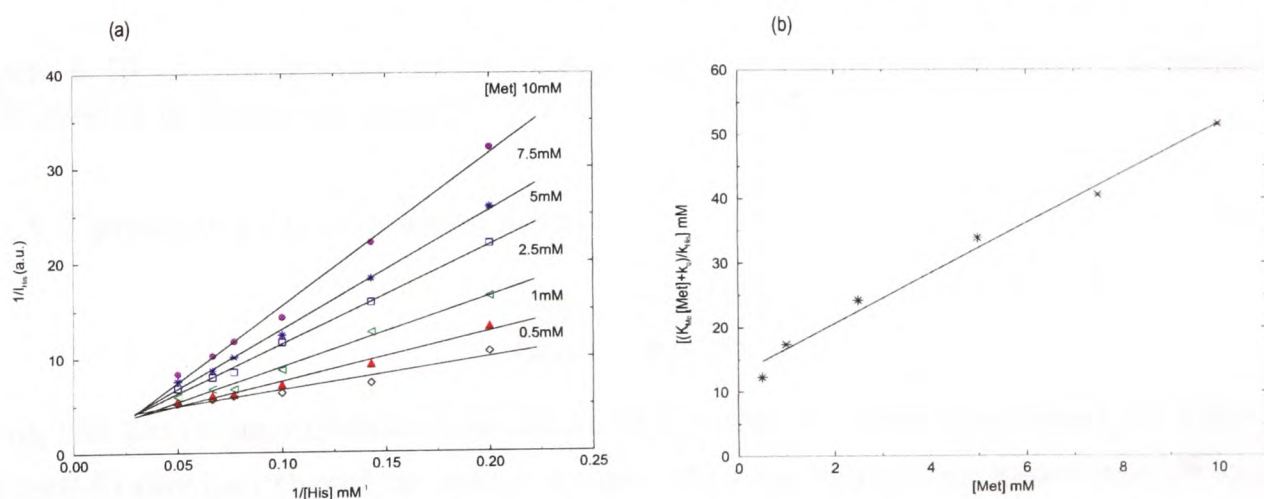


Figure 6.15: Stern-Volmer plot. (a) $1/I_{\text{His}}$ vs. $1/[\text{His}]$ for different values of $[\text{Met}]$ as shown. (b) Slope/Intercept from (a) vs. $[\text{Met}]$.

with the three other amino acids for ^3F , however as with histidine, care should be taken when interpreting CIDNP intensities under conditions where competition for ^3F is strong (e.g. high concentration of accessible tryptophan residues).

6.7.3 Equimolar studies

To illustrate the combined effects of the competition for ^3F and the field dependence, CIDNP spectra obtained at both high (9.4 T) and “low” (0.7 T) field for an equimolar mixture of methionine and histidine are shown in Fig.6.16. At high field the signals for both histidine and methionine are comparable, reflecting the fact that methionine is able to compete favourably for ^3F . As expected, there is a dramatic increase in the methionine enhancement when going to low field. Under *low* equimolar conditions,

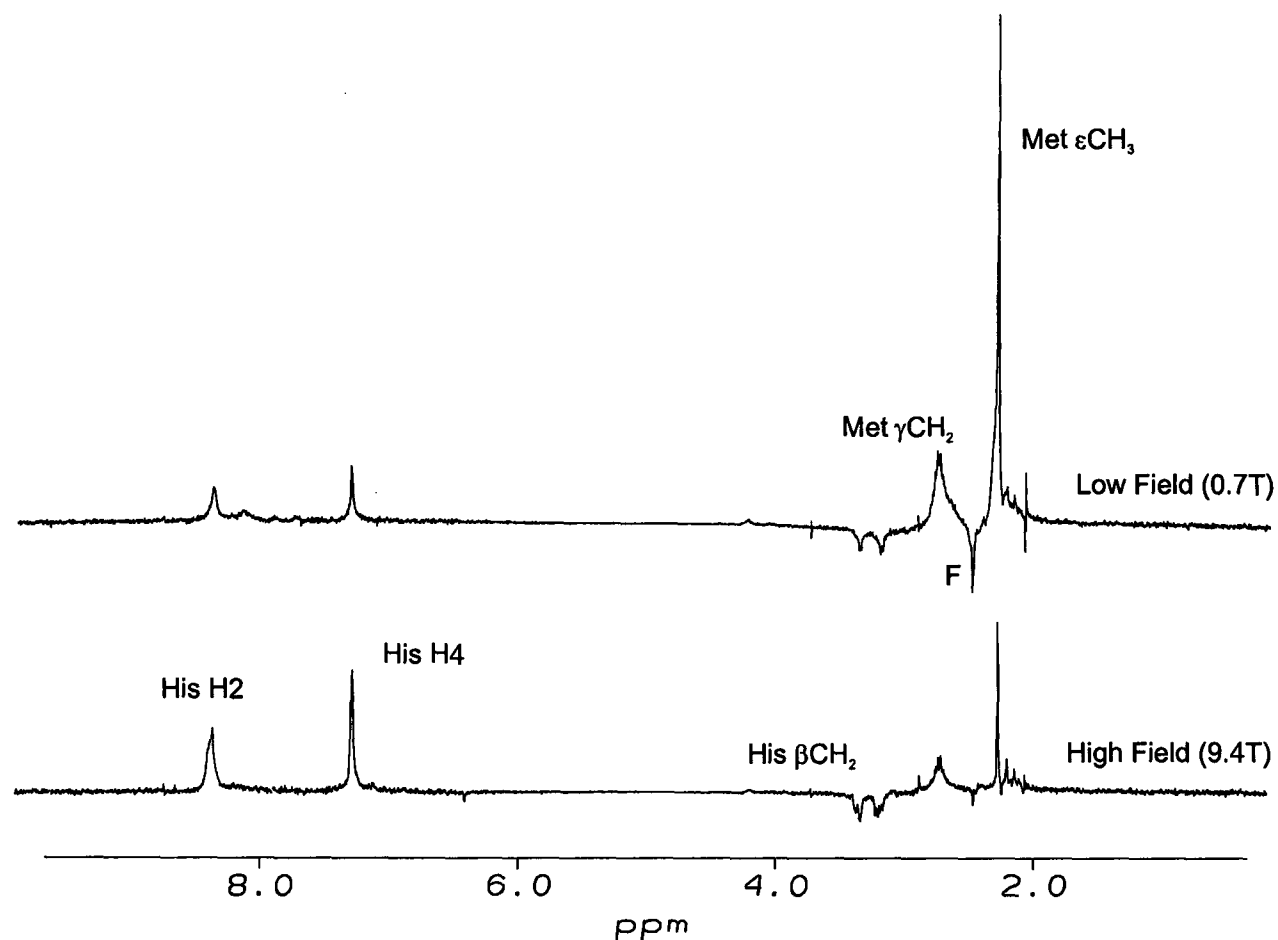


Figure 6.16: A comparison of CIDNP spectra taken with an equimolar mixture of histidine and methionine (1 mM, pH 7) at high field (9.4 T) and “low” field (0.7 T).

Eqn.6.7 predicts that at a given field:

$$\frac{I_{Met}}{I_{His}} = \frac{p_{Met}k_{Met}}{p_{His}k_{His}} \quad (6.8)$$

Using the previously obtained estimate of the ratio of rate constants (i.e. $k_{Met}/k_{His} = 4.0 \pm 0.5$) one can therefore obtain a ratio of the polarizations generated for methionine and histidine at both high ($p_{Met}/p_{His} = 4.1 \times 10^{-2}$) and low ($p_{Met}/p_{His} = 5.5 \times 10^{-1}$) fields. These results therefore suggest that an order of magnitude increase in this ratio occurs upon moving from 9.4 T to 0.7 T and hence that the technique is potentially a very powerful tool for observing methionine signals under conditions where the magnetic field and not competition is the limiting factor.

6.7.4 Methionine in proteins

For a methionine residue to show CIDNP in a protein it has to be able to react with the bulky 3F and therefore has to be on the surface of the protein molecule. In addition due to its relatively low rate constant for reaction with 3F , the presence of exposed tyrosine or tryptophan residues would need to be avoided. As was mentioned earlier, the only reported observation of methionine CIDNP in a protein is that of the *lac*-repressor head piece and intact *lac*-repressor [207]. This is surprising when one considers that under equimolar conditions even at high field, methionine and histidine signals are of comparable magnitude (Fig.6.16) and that

histidine residues are frequently observed in proteins, even under conditions where they have to compete with exposed tryptophan and tyrosine residues as in bovine α -lactalbumin [31].

It is therefore interesting to note that methionine is a hydrophobic residue with a water to vapour partition coefficient $K_D = [R]_{water}/[R]_{vapour}$ of ~ 10 , whilst histidine is a hydrophilic residue with K_D of $\sim 10^7$ [214]. This will affect the propensity for either residue to be found in contact with the solvent on the surface of the protein or buried in the hydrophobic core found at the centre of all globular proteins. A study based on the known structures of 23 globular proteins has confirmed this tendency [215] and would therefore suggest that one might be more likely to observe methionine CIDNP in unfolded rather than folded states of proteins.

To test this hypothesis the 600 MHz photo-CIDNP spectra of *denatured* hen lysozyme and histidine-containing phosphocarrier (HPr) protein were obtained and are shown in Fig.6.17 and 6.18 respectively. Hen lysozyme contains six tryptophans, three

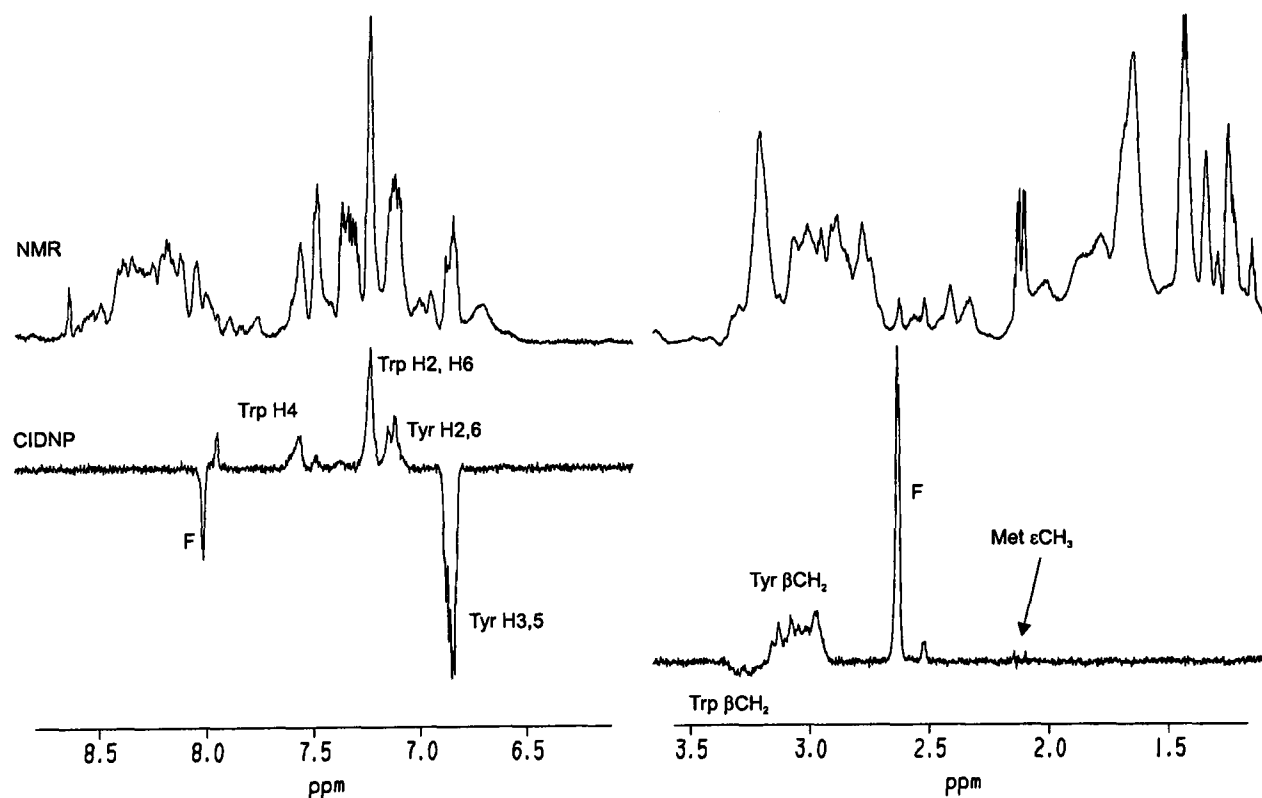


Figure 6.17: 600 MHz ^1H NMR and photo-CIDNP spectra of the aromatic and aliphatic region of denatured hen lysozyme (1 mM, pH 3.6, 10 M urea, 45 °C). In the presence of six tryptophans, three tyrosines and one histidine, the two methionines are unable to compete for excited flavin.

tyrosines and one histidine as well as two methionines (Met 12 and Met 105). Under such conditions neither histidine nor methionine residues are capable of competing for excited flavin and no methionine CIDNP is observed. HPr on the other hand contains just two histidines and two methionines (Met 1 and Met 81) and in accordance with the equimolar experiments with free histidine and methionine amino acids, comparable signals are observed for both histidine and methionine around

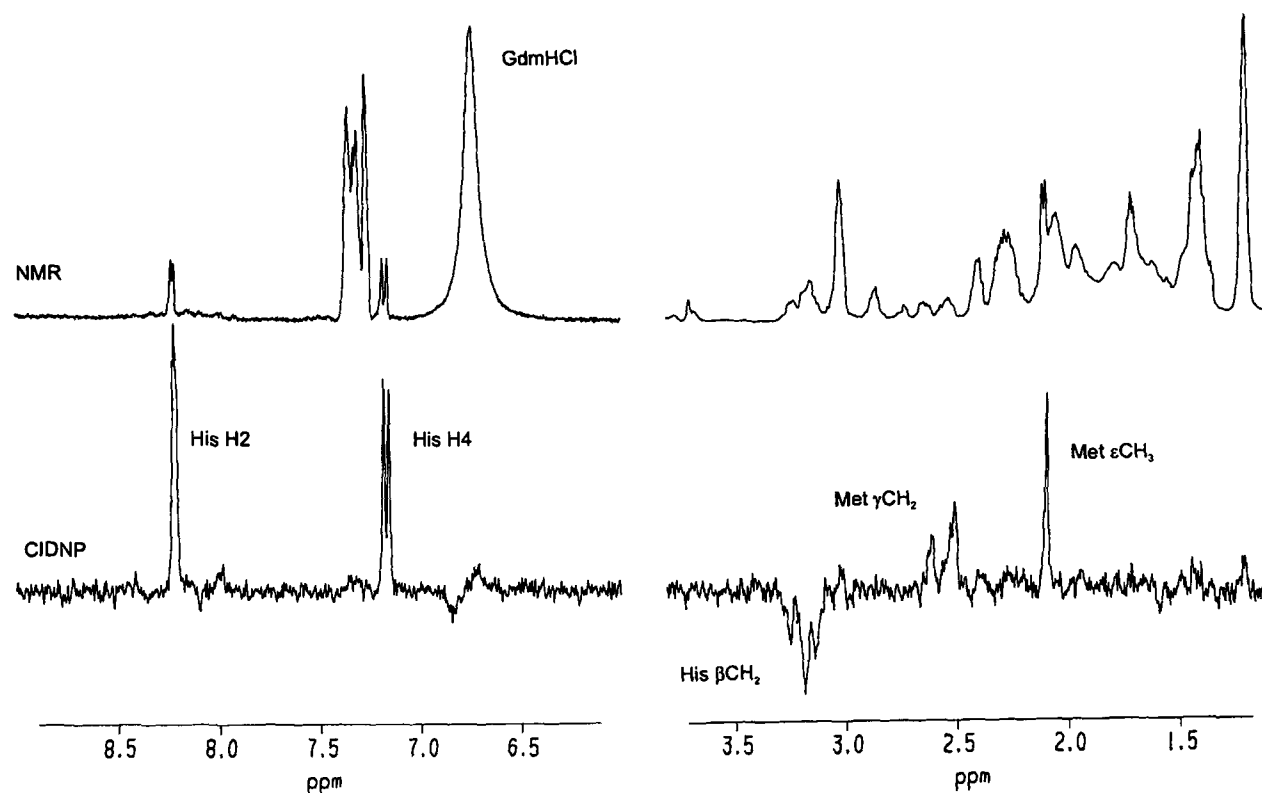


Figure 6.18: 600 MHz ^1H NMR and photo-CIDNP spectra of the aromatic and aliphatic region of denatured HPr (1.5 mM, pH 7, 4 M GdmDCI, 35 °C). In the presence of just two histidines, the two methionines are able to compete for excited flavin.

random coil chemical shifts.

It would therefore seem that the weakness of methionine CIDNP enhancements is a compound effect of the large Δg value for the methionine cation/flavin anion radical pair, the relatively low rate constant for reaction of methionine with ^3F and in the case of native proteins, to the lack of surface exposure that is inherent for such a hydrophobic residue. It has been shown that the first of these factors can be circumvented somewhat by initiating polarization at lower fields than those found at the centre of NMR magnets, alternatively one could consider searching for an alternative dye with a heavy atom centre that would give a larger g -value and hence might make methionine CIDNP more attractive at high fields.

Chapter 7

Photo-CIDNP magnetization transfer techniques

Two photo-CIDNP techniques that probe the exposure of aromatic residues in partially folded states are described. Both involve transfer of polarization to the native state for detection. One approach achieves this kinetically by rapid refolding, and the other involves monitoring exchange cross peaks in a two-dimensional CIDNP spectrum under conditions where the two states are interconverting. The former makes use of the “low” field device described in Chapter 6 and the latter illustrates the potential of the mixing device described in Chapter 4.

7.1 Introduction

The transient nature of kinetic folding intermediates restricts their detailed structural characterization by most direct methods and the search for stable analogues of these short lived states has therefore played an important role [46, 47]. Of particular interest in this regard is the so-called molten globule state that has been shown to occur under mildly denaturing conditions for a number of proteins [80]. As was described in Chapter 5, unlike the largely unfolded and somewhat structureless states observed at high concentrations of denaturant, the molten globule state is characterized by pronounced secondary structure, compact shape, a hydrophobic core and the absence of rigid side chain packing [166–168]. Most importantly however, the equilibrium molten globule state has been shown to be similar to a kinetic intermediate in refolding reactions of globular proteins [216, 217].

In this context, particular interest has centered on the α -lactalbumins that unfold to a molten globule state under a variety of denaturing conditions (e.g. extremes of pH, removal of bound Ca^{2+} ion, reduction of disulphide bonds and high temperature) [218]. The most extensively characterized of these is the one obtained

by acid-unfolding at pH 2 (the A-state). Figure 7.1 compares the ^1H NMR and CIDNP spectra of (a) the native state and (b) the A-state of bovine α -lactalbumin. The wide dispersion of chemical shifts in the native spectrum is characteristic of

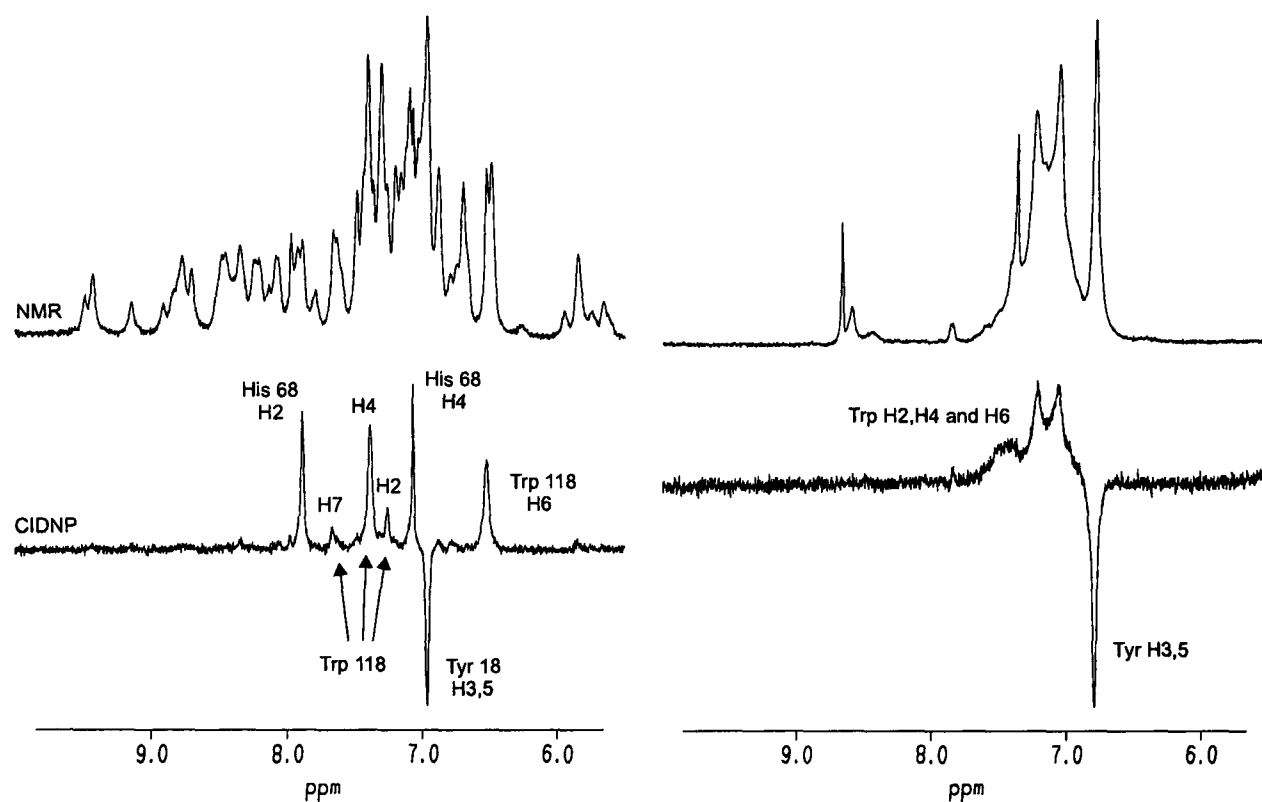


Figure 7.1: Comparison of the ^1H NMR and CIDNP spectra of (a) the native state (1.5 mM, pH 7) and (b) the A-state (1.5 mM, pH 2) of bovine α -lactalbumin.

globular proteins and reflects the highly specific interresidue interactions within the compact folded structure. In contrast, the A-state spectrum shows a dramatic loss of chemical shift dispersion typical of unfolded proteins. In addition, many peaks are much broader in the A-state than in either the native or fully unfolded (not shown) states, a fact that can be attributed to conformational fluctuations throughout the protein on a millisecond time scale [219]. The combination of poor chemical shift dispersion and broad lineshapes makes assignment by conventional multidimensional homonuclear or heteronuclear methods such as those described in Chapter 3 near impossible. For example in a recent study of human α -lactalbumin [122] only 3 of the 122 backbone amides were observed in the ^{15}N - ^1H HSQC spectrum at pH 2, whilst under more extreme denaturing conditions (8 M GdmHCl, 50 °C) where conformational fluctuations occur on a faster timescale and hence lines are sharper, all 122 resonances were clearly resolved.

In this chapter, the possibility of assigning CIDNP peaks in the A-state indirectly by methods that correlate resonances in the broad and poorly resolved spectrum of the molten globule state with those of the sharp well resolved peaks of the native state will therefore be assessed. In Section 7.2 a somewhat novel *kinetic* method (CIDNP pulse labeling) in which polarization generated in the partly folded state is detected in the spectrum of the native state following refolding will be described

and in Section 7.3, the more conventional approach to such magnetization transfer experiments which involves correlating resonances of different states where they coexist at *equilibrium* and interconvert at rates comparable to, or faster than the nuclear spin-lattice relaxation rates will be presented.

7.2 CIDNP pulse labeling

The idea of a kinetic “CIDNP pulse labeling” experiment originally came from a recently described “radio frequency (r.f.) pulse labeling” experiment [220] in which nuclear Overhauser effects (NOEs) rather than CIDNP enhancements generated in the A-state of bovine α -lactalbumin were transferred by refolding to the native state for detection. The NOEs were generated somewhat unselectively by applying a 1 s r.f. pulse to the aromatic envelope of the A-state and detected in the aliphatic region 800 ms after refolding was initiated. Whilst the results from this approach provide a general idea of the contacts between residues within the partly folded state, the inherent lack of selectivity limits the technique. Photo-CIDNP should enable a far more selective generation of polarization and hence a site specific assignment of the exposed tyrosine and tryptophan residues that contribute to the broad CIDNP spectrum in Fig.7.1b.

7.2.1 Preliminary tests

The success of the technique depends crucially on maximizing the final signal detected in the native state and hence on both maximizing the initial signal generated in the partially folded state and minimizing any losses between generation and detection of CIDNP. In order to assess these features, a number of preliminary experiments were therefore performed.

The initial experimental strategy adopted was similar to that described in the “r.f. pulse labeling” experiment and involved using the high field rapid mixing setup described in Chapter 5 for real-time refolding. The procedure involved irradiating 400 μ l of the A-state of bovine α -lactalbumin at pH 2 present within the NMR tube, and then initiating refolding by rapidly injecting 300 μ l of pH 7 refolding buffer. Preliminary tests based on this high field approach showed that by the end of the 200 ms injection period very little signal remained. To determine the origin of this dramatic loss of signal, the CIDNP intensity of the equilibrium A-state (i.e. *without* refolding) was measured at high field after a 500 ms laser flash *with* and *without* a subsequent 200 ms injection of unpolarized A-state protein into the polarized sample. The CIDNP intensities as a function of the post laser flash delay are compared in Fig.7.2 and confirm that by the end of the injection, the intensity has already dropped to 20% of its initial value. During the 200 ms injection period 50% of the

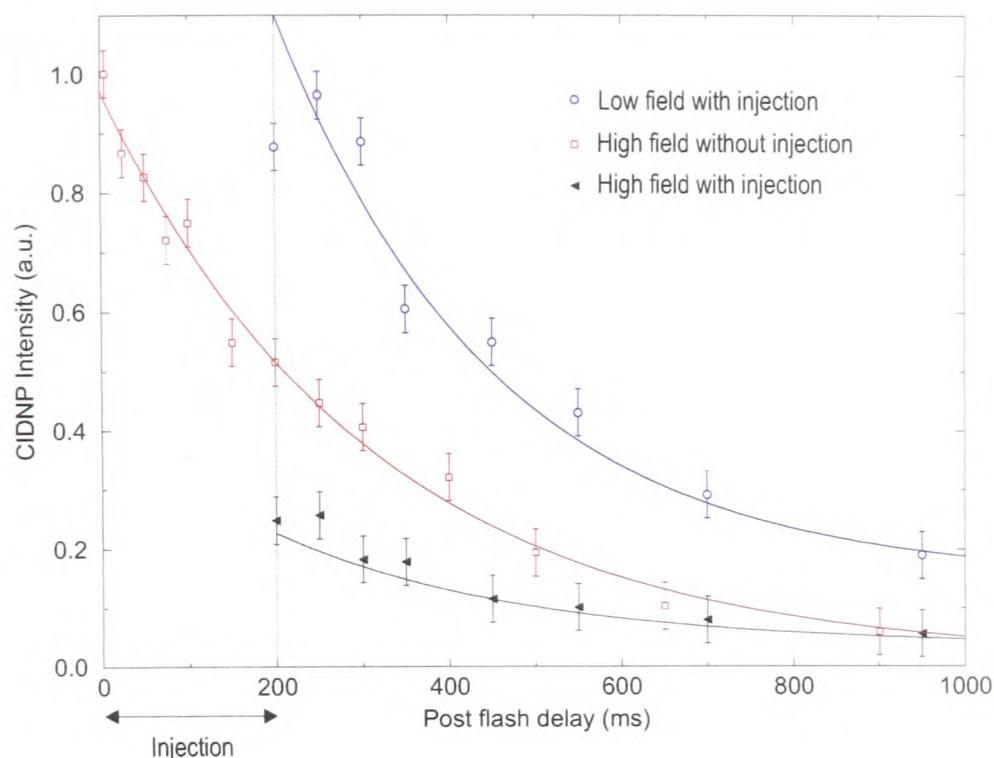


Figure 7.2: Comparison of the CIDNP intensity of the A-state of bovine α -lactalbumin (1.5 mM, pH 2) detected after a 500 ms 4 W laser flash and generated at “low” field (circle), high field *without* an injection (square) and high field *with* an injection (black triangle). Flavin concentration in the low field experiment was 0.8 mM and at high field was 0.2 mM.

polarization decays by spin-lattice relaxation (e.g. the results without injection), and the injection itself causes a further loss of 30%. CIDNP is generated within a $\sim 100 \mu\text{l}$ portion of the $300 \mu\text{l}$ sensitive region of the NMR coil (Chapter 2) and hence mixing caused by the injection forces polarized sample out of this sensitive region into a larger final volume of $700 \mu\text{l}$ leading to a ~ 2.5 fold (i.e. $300 \mu\text{l}/700 \mu\text{l}$) “dilution” of the signal. These results would suggest that injection of smaller volumes of refolding buffer should reduce this loss by both shortening the injection time and reducing the “dilution” factor, however no significant improvement was observed experimentally.

In this context, the results obtained in Chapter 6 with the “low” field stopped-flow device are of particular interest. Experiments in which CIDNP was generated in the native states of bovine α -lactalbumin and hen lysozyme at ~ 4 T and then transferred to high field for detection, showed roughly identical signal intensities to an experiment performed at high field without injection. Whilst the “low field” approach is therefore of no great use in its present form for equilibrium studies, it provides a dramatic signal enhancement for the CIDNP pulse labeling experiment. To illustrate this, the CIDNP intensity of the A-state of bovine α -lactalbumin generated at ~ 4 T with a 500 ms laser flash and transferred to high field for detection via a 200 ms injection pulse was measured as a function of the post laser flash delay and is also shown in Fig.7.2. The benefit over the high field approach is striking, and suggests a 5 fold increase in signal intensity.

In an attempt to increase the sensitivity of the experiment further, the effects of removing O_2 from samples prior to irradiation was assessed. As was described in the introduction to this thesis, excited triplet flavins are quenched by reaction with O_2 thereby reducing the concentration of flavin available for reaction with exposed aromatic residues. Whilst the oxidative properties of O_2 are welcome in experiments that require several laser flashes (Chapter 4), in “single shot” experiments such as CIDNP pulse labeling, each solution is used only once and the issue of sample degradation does not arise. Fig.7.3a, illustrates the effect of bubbling a sample of histidine (0.5 mM, pH 7) for ~ 5 minutes with either N_2 or O_2 . Whilst saturating the sample

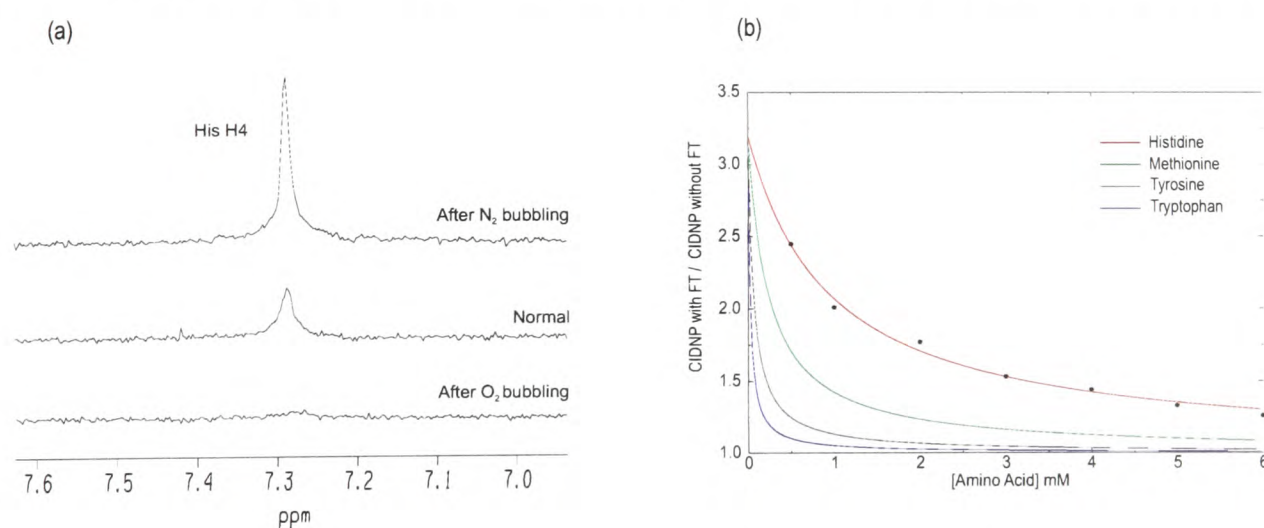


Figure 7.3: (a) Comparison of the normal histidine (0.5 mM, pH 7) H4 CIDNP intensity to that obtained after ~ 5 minutes of bubbling with N_2 or O_2 . Each spectrum was obtained with a single 500 ms 4 W laser flash. (b) Concentration dependence of the relative enhancement with degassing shown in (a) for histidine. The dependence was fitted and simulated for the other three amino acids as described in the text.

with O_2 removes the CIDNP effect completely, degassing with N_2 enhances the signal ~ 3 fold. Degassing *protein* solutions by simple bubbling is inefficient as they tend to “froth” and an alternative freeze thaw procedure [221] was therefore adopted. The protein sample was placed within a Schlenk tube, frozen in ethanol/dry ice ($-77^\circ C$) and then evacuated under a 2×10^{-1} mbar vacuum using a simple rotary pump. The sample was then thawed in warm water to allow degassing and then refrozen. The cycle was repeated three times by which time no further degassing was apparent. In order to put the benefit of freeze thaw on a more quantitative footing, the histidine H4 CIDNP intensity obtained with and without freeze thaw was measured over a range of histidine concentrations and the ratio of these two quantities is shown in Fig.7.3b as a function of the histidine concentration. In the absence of degenerate exchange, the histidine CIDNP intensity can be written (Chapter 1):

$$I_{His} \propto \frac{k_{His}[His]}{k_q + k_{His}[His]} \quad (7.1)$$

where k_q is the (pseudo-) first order rate constant for decay of 3F , e.g. by fluorescence and/or quenching by molecular oxygen and k_{His} is the second order rate constant

for the reaction of histidine with 3F . The ratio of the CIDNP intensity obtained with (I_{His}^{FT}) and without (I_{His}) freeze thaw is therefore given by:

$$\frac{I_{His}^{FT}}{I_{His}} \propto \frac{k_q + k_{His}[His]}{k_q^{FT} + k_{His}[His]} \quad (7.2)$$

The data was fitted as shown in Fig.7.3b using the value previously obtained for k_q/k_{His} in Chapter 6 (i.e. $13 \times 10^{-3} \text{ mol dm}^{-3}$). On this basis, extrapolation to zero concentration yields a ratio for k_q/k_q^{FT} of ~ 3.2 (i.e. a 70% reduction in the quenching rate). As Eqn.7.2 illustrates, the effect is greatest at low amino acid concentrations and for amino acids with low second order rate constants for reaction with triplet flavin such as histidine. To illustrate this the expected benefit for the other three amino acids as a function of concentration was simulated using Eqn.7.2 and a ratio of second order rate constants of 40:15:4:1 for Trp:Tyr:Met:His respectively. For bulky proteins, the rate of reaction should be significantly reduced relative to the free amino acids and in accordance, a ~ 2 fold enhancement was obtained for the exposed tryptophans of native hen lysozyme (1.5 mM, pH 5.2). It would therefore appear that the freeze thaw procedure has great potential for enhancing CIDNP intensities in “single shot” experiments and could therefore also be applied to fast folding proteins in real-time CIDNP experiments (Chapter 5) where protein concentrations are even lower (~ 0.5 mM), and hence the benefit is potentially even greater.

The CIDNP pulse labeling experiment involves long laser flashes (500 ms) and long recovery periods (>200 ms) and hence cross-polarization effects (i.e. NOEs) are likely to play an important part in determining the details of the final spectrum. To estimate the extent of such effects, the time evolution of CIDNP in the native state of bovine α -lactalbumin was measured using the same approach as in the pulse labeling experiment (i.e. 500 ms laser flash and 200 ms injection) but without re-folding. Native bovine α -lactalbumin is a suitable choice as all three residue types are present and well resolved. The results are shown in Fig.7.4 where the spectra obtained (b-d) 0, 100 and 200 ms after transfer to high field are compared to that obtained at high field (a) with a short 50 ms laser flash and immediate acquisition. In contrast to this spectrum in which only directly polarized peaks are observed, at 0 ms (Fig.7.4b) the directly (H4 and H6) and indirectly (H5 and H7) polarized Trp 118 peaks are already of equal magnitude. The H4 signal then disappears very rapidly as a consequence of emissive cross-polarization from the neighbouring β -CH₂ protons whilst H6 decays relatively slowly along with the indirectly polarized H5 and H7. Such differential behaviour for the aromatic protons of tryptophan has also been observed for the exposed Trp 3 of phospholipase A₂ [18]. The H3,5 protons of Tyr 18 also appear to decay relatively slowly and interestingly, it would appear that emissive and absorptive cross-polarization from H3,5 and β -CH₂ respectively to H2,6 cancel each other out and hence very little polarization is observed for these

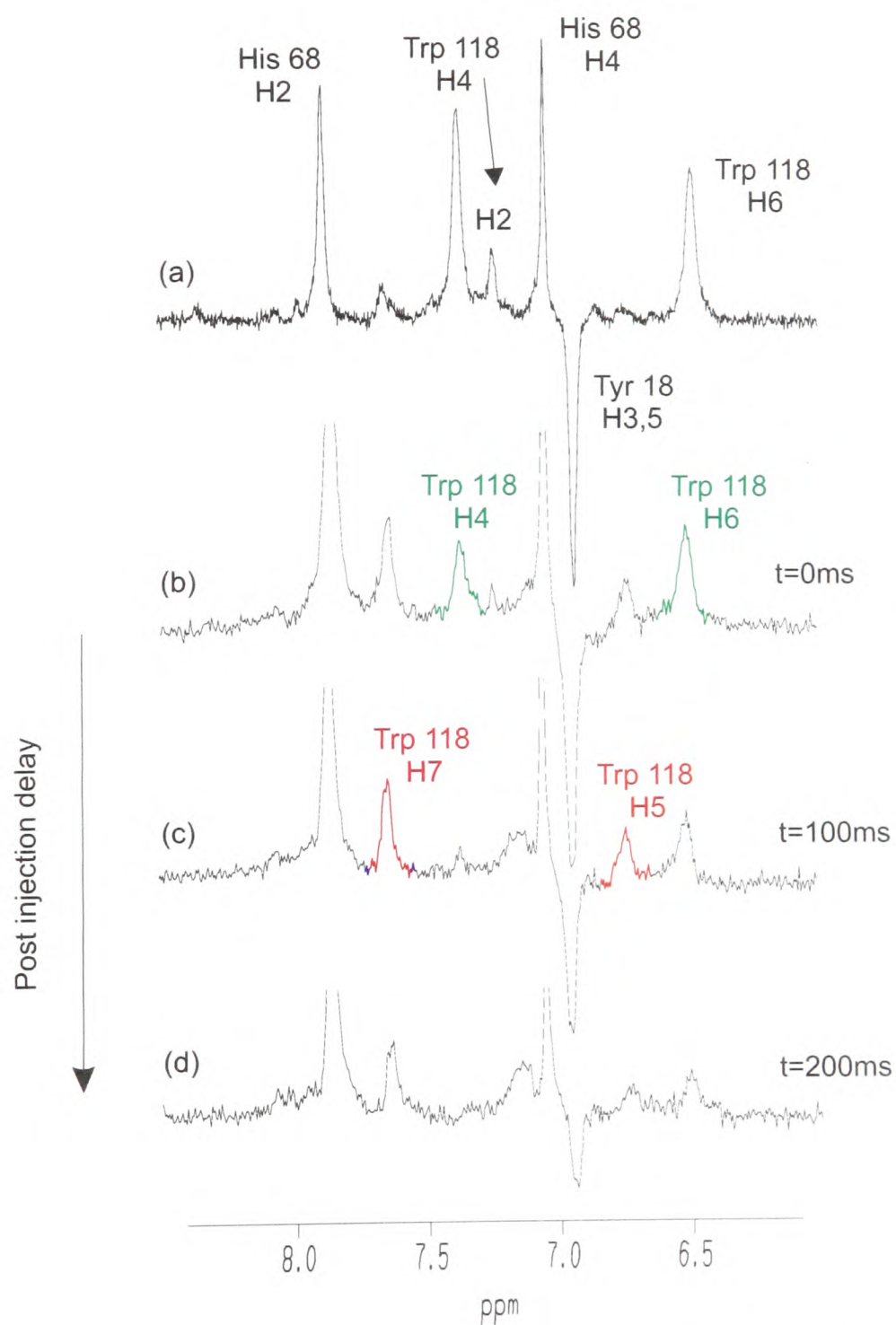


Figure 7.4: Comparison of the 400 MHz ^1H CIDNP spectrum of bovine α -lactalbumin in the native state (1.5 mM, pH 7) generated and detected at high field with a short 50 ms laser flash (a) to those generated at “low” field and detected at high field (b-d) 0, 100 and 200 ms after injection (500 ms laser flash and 200 ms injection). Directly and indirectly polarized Trp 118 peaks are highlighted in green and red respectively.

protons. The protons of His 68 (H2 and H4) lack neighbouring intraresidue protons and hence decay slowly.

In order to minimize such cross-polarization effects and any further loss of polarization subsequent to the initiation of folding, the recovery and refolding delays that follow the injection pulse will need to be kept as short as possible. Some of the preliminary tests that were performed on the “low field” stopped-flow device and were described in Chapter 6 (Fig.6.6) are therefore of particular importance and indicate that both efficient mixing and recovery from the shock of the stopped-flow should be complete within 100 ms of injection. Hence, in order to maximize the fraction of polarization generated in the partly folded state that is successfully transferred to the native state for detection the protein would need to refold to its native state within 100 ms. In the presence of calcium, bovine α -lactalbumin is a fast folding protein, however even under optimized conditions, folding is only complete within around 1 s. Under most conditions, hen lysozyme folds on roughly the same timescale as bovine α -lactalbumin however a recent study [115] has shown that the presence of small amounts of the denaturant 2,2,2-trifluoroethanol (TFE) can accelerate folding by an order of magnitude and hence, before applying the experiment to the A-state of bovine α -lactalbumin (Section 7.2.3), it was tested on the so called “TFE state” of hen lysozyme (Section 7.2.2).

7.2.2 TFE state of hen lysozyme

Investigations into the effect of TFE on the solution conformation and dynamics of hen lysozyme using circular dichroism and NMR spectroscopy have revealed that a cooperative transition to a partly structured TFE state occurs with a midpoint around 20% (v/v) TFE at pH 2, 27 °C [108]. This state has been characterized at 50% and 70% (v/v) TFE and shown to be partially folded with more α -helical structure than that of the native protein but few specific tertiary interactions [108, 113, 114]. As was mentioned earlier, the folding kinetics of hen lysozyme depends sensitively on the final concentration of TFE present in the refolding mixture. At pH 5.2, TFE acts as an accelerant up to a concentration of $\sim$ 7% (v/v) [115] and therefore in order to transfer CIDNP generated at 50% (or 70%) (v/v) TFE to the native state as rapidly as possible, one would need to initiate refolding via a 7 (or 10) fold dilution. With the “low field” stopped-flow device, the maximum final volume is 700 μ l and hence, such a dilution would require injecting 100 μ l of the protein solution into 600 μ l of refolding buffer (or 70 μ l into 630 μ l). However, as was described in Chapter 6, the dead volume of the “low” field device is of the order of 100 μ l and thus with such a small injections, no polarized material would actually reach the NMR tube for detection (Fig.6.7b). Whilst the TFE state has been characterized at 50% and 70% (v/v) TFE, hen lysozyme is essentially unfolded

by 30% (v/v) TFE and an alternative strategy involving a 4.7 fold dilution (150 μ l into 550 μ l) from 30% TFE pH 2 with a simultaneous pH jump to 5.2 was therefore adopted.

Real-time refolding

Figure 7.5 shows the refolding kinetics of hen lysozyme obtained with the same final conditions as in the pulse labeling experiment (6.4% TFE, pH 5.2, 20 °C) as measured by both tryptophan fluorescence and real-time CIDNP (Chapter 5). As

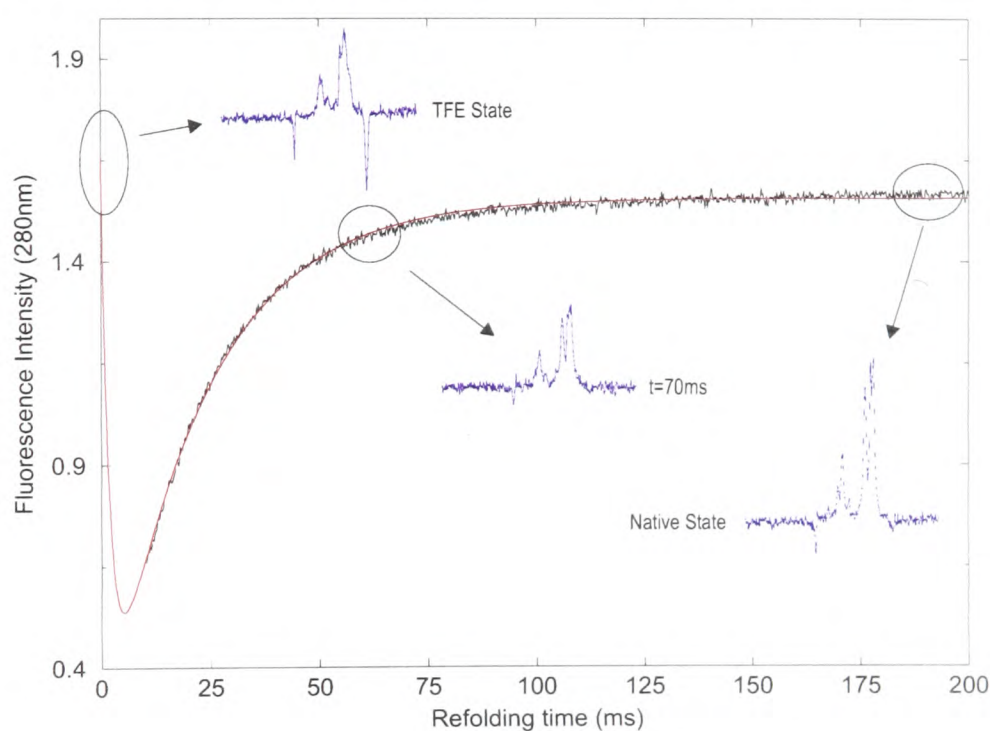


Figure 7.5: Kinetic progress curve of hen lysozyme refolding monitored by tryptophan fluorescence at 20 °C. Refolding was initiated from 70.4% (v/v) TFE, pH 2 by an 11 fold dilution and pH jump. The final conditions were 0.32 mM hen lysozyme, 6.4% (v/v) TFE, pH 5.2. The experimental dead time was $\sim$ 10 ms. Refolding kinetics were recorded with an Applied Photophysics SX17-MV stopped-flow fluorimeter. Excitation was at 280 nm and the fluorescence was recorded for wavelengths longer than 320 nm. Also shown are real-time photo-CIDNP spectra (Chapter 5) obtained with identical final conditions but initiated by 4.7 fold dilution and pH jump within the NMR tube (i.e. as in the pulse labeling experiment). Folding is essentially complete within the dead time of the CIDNP experiment.

expected folding is very fast and essentially complete within the dead time of the CIDNP experiment, confirming that transfer of polarization to the native state from the TFE state is likely to be efficient in the pulse labeling experiment.

Pulse labeling

The results of the pulse labeling experiment are shown in Fig.7.6a. For each refolding delay, 4 “light” and 4 “dark” free induction decays were recorded, subtracted and Fourier transformed to give photo-CIDNP difference spectra. Note that as folding is complete within the dead time of the experiment, the time development of peaks after injection is in theory determined solely by cross-relaxation effects within the native state. For comparison, the decay of CIDNP in the TFE state *without*

refolding over the same timescale (obtained with a single light and dark spectrum) is shown in Fig.7.6b. The difference between the two columns is striking, confirming

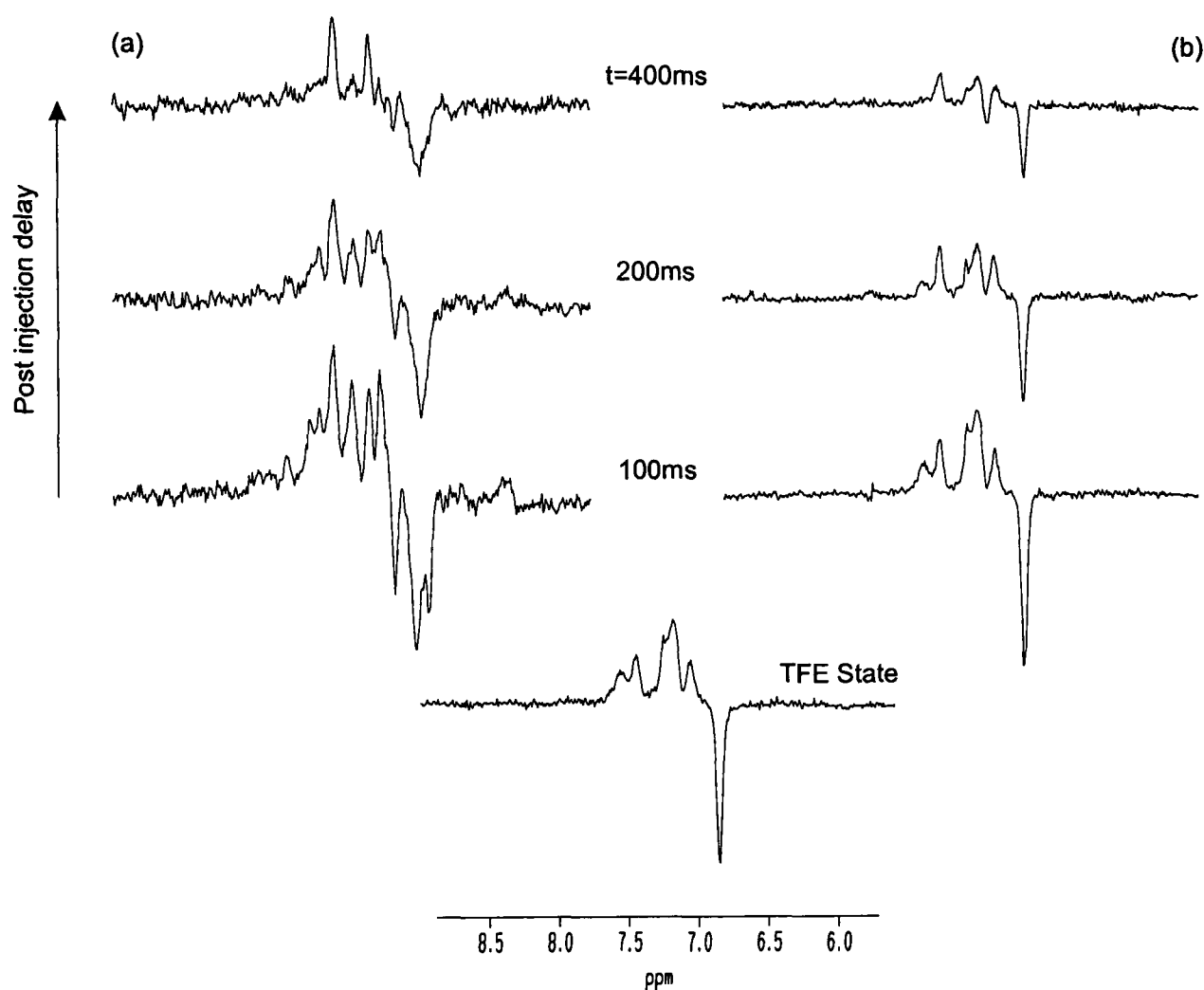


Figure 7.6: ^1H CIDNP spectra of hen lysozyme in the TFE state generated at “low” field and detected at high field 100, 200 and 400 ms after injection (a) *with* and (b) *without* refolding to the native state. Folding was initiated after a 500 ms laser flash by injecting 150 μl of 1.5 mM hen lysozyme in 30% (v/v) TFE, pH 2 into 550 μl sodium acetate buffer 20 mM, pH 5.2.

that successful transfer of polarization to the native state has occurred. The single emissive tyrosine peak in the TFE state has been split into three and a number of resolved tryptophan peaks are also apparent (the absence of histidine polarization is likely to be a simple consequence of their inability to compete for excited flavin). A comparison of the chemical shifts obtained experimentally to those of the native state of hen lysozyme [222] are shown in Table 7.1 and labeled in the expanded $t=100$ ms spectrum in Fig.7.7. The assignment of tyrosine peaks appears unambiguous and suggests that all three tyrosines (Tyr 20, Tyr 23 and Tyr 53) are exposed in the TFE state (only a very weak signal from Tyr 23 has been observed in the native state [119]), however, the great deal of overlap and crowded nature of the tryptophan aromatic region leads to a number of ambiguities. Detection of the well resolved tryptophan indole peaks that resonate in a particularly uncrowded region around 10 ppm could potentially remove these ambiguities and would simply involve performing the pulse labeling experiment in H_2O rather than D_2O as described in Chapter 3. A quicker approach was however adopted. Figure 7.8 compares the

Residue	Nucleus	Results ^a	Literature ^b	Residue	Nucleus	Results ^a	Literature ^b
W28	H4	n.i.	6.77	W108	H4	n.i.	7.36
	H5	6.24	6.29		H5	n.o.	6.50
	H6	n.i.	6.82		H6	7.19	7.17
	H7	7.44	7.46		H7	n.i.	6.93
W62	H4	7.11	7.11	W111	H4	7.30	7.28
	H5	n.i.	7.03		H5	n.i.	7.04
	H6	7.19	7.19		H6	n.i.	7.35
	H7	7.44	7.45		H7	7.54	7.51
W63	H4	7.77	7.76	W123	H4	7.54	7.53
	H5	n.i.	6.85		H5	7.11	7.14
	H6	7.11	7.09		H6	7.11	7.11
	H7	7.19	7.22		H7	7.77	7.77
Y20	H3,5	7.00	6.99				
Y23	H3,5	6.76	6.71				
Y53	H3,5	6.82	6.83				

Table 7.1: ¹H assignments at native chemical shifts of aromatic residues exposed in the TFE state of hen lysozyme (1.5 mM, pH 2, TFE 30% (v/v)). n.i., not identified because of overlap, n.o., not observed. ^aChemical shifts taken from Fig.7.7. References were taken from ^bRef.[222].

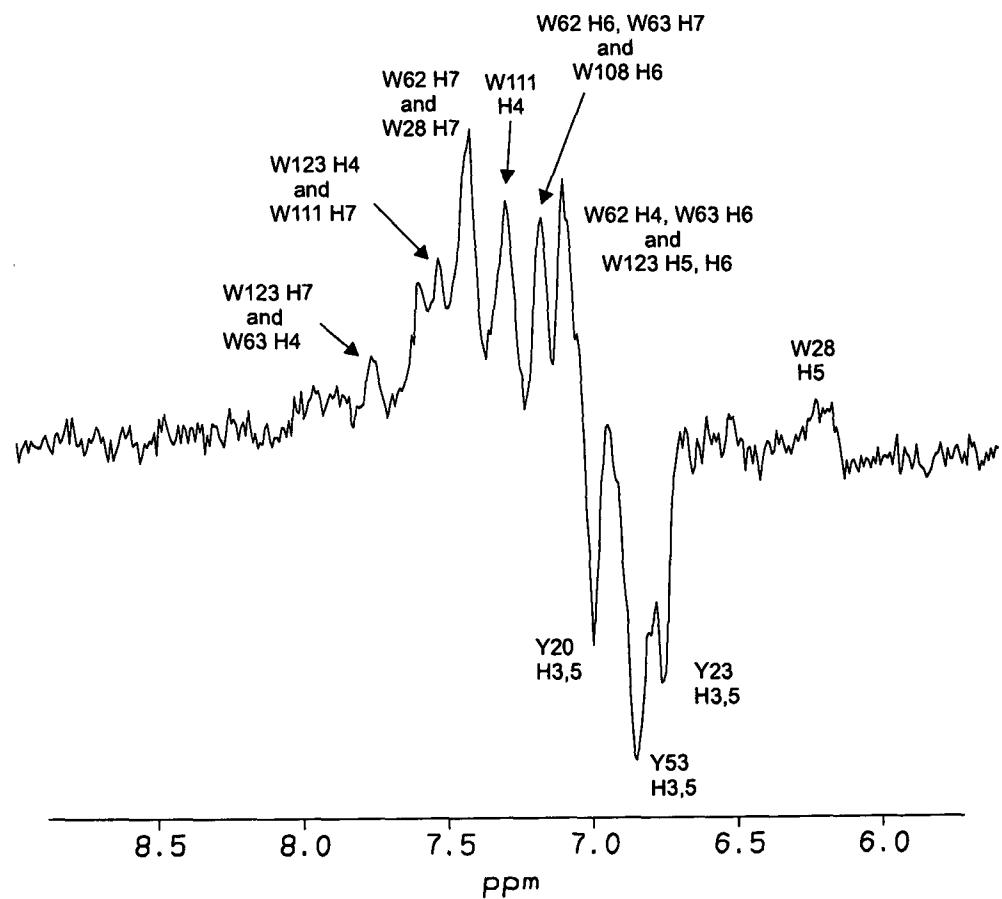


Figure 7.7: Assigned CIDNP pulse labeling spectrum of hen lysozyme in the TFE state taken 100 ms after refolding to the native state (expanded from Fig.7.6). Assignments are described in the text and Table 7.1.

equilibrium NMR and photo-CIDNP spectra obtained for hen lysozyme at pH 2 in (a) 30% (v/v) TFE, 20 °C (i.e. the same initial conditions as in the pulse labeling experiment) and (b) 70% (v/v) TFE, 37 °C. Whilst the spectrum in 30% (v/v)

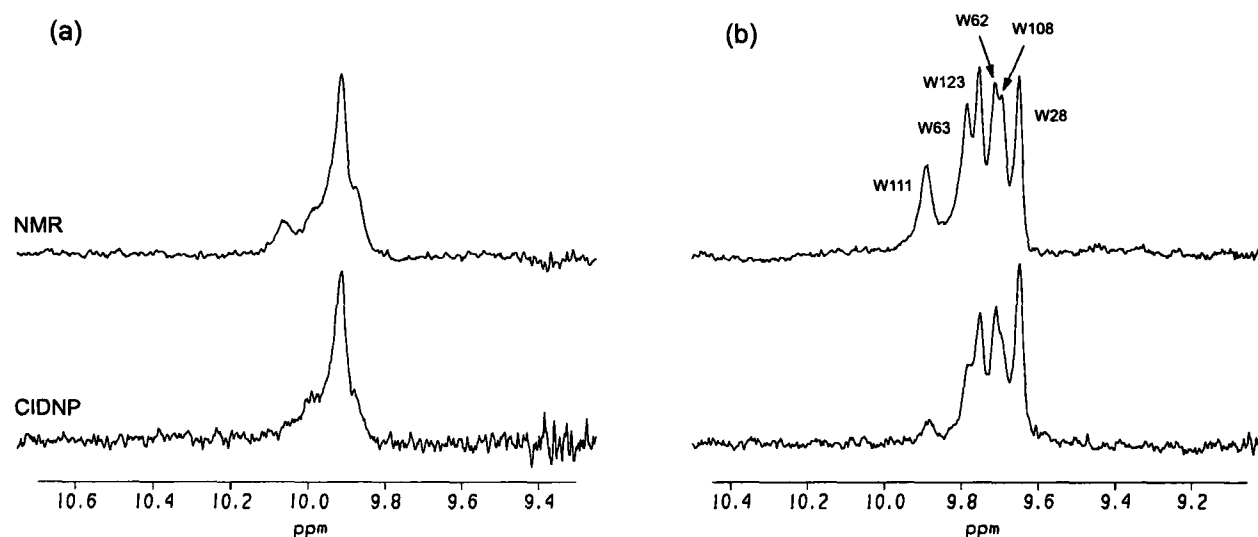


Figure 7.8: A comparison of the indole region of the 600 MHz ^1H NMR and photo-CIDNP spectra of hen lysozyme in (a) 30% (v/v) TFE at pH 2, 20 °C and (b) 70% (v/v) TFE at pH 2, 37 °C. The NMR spectra were averaged over 64 scans whilst the photo-CIDNP spectra were obtained with 16 laser flashes (100 ms, 4 W). Assignments were taken from Ref.[113].

TFE is poorly resolved, at higher temperature and concentration of denaturant all six tryptophans can just be resolved and have been assigned [113]. A comparison of the NMR and photo-CIDNP spectra suggests that the six tryptophans are exposed in the following order Trp 28 > Trp 62, Trp 63, Trp 108, Trp 123 $\gg$ Trp 111 (only Trp 62 and to a lesser extent Trp 123 are exposed in the native state [119]). Whilst the structure under these conditions may differ slightly from the initial state in the pulse labeling experiment these results suggest that the native structure of hen lysozyme is significantly disrupted in high concentrations of TFE. However, the observation of residual side-chain dispersion [108, 113] and the apparent burial of Trp 111 would indicate the presence of some residual structure in regions involving the hydrophobic tryptophan residues. Interestingly, whilst little structure is induced by TFE in a 32-residue peptide fragment of lysozyme corresponding to the C-, D-, and C-terminal 3_{10} -helices in the native enzyme [223] the same region is significantly protected against hydrogen exchange in the TFE state [108]. This has been thought to indicate that long-range interactions contribute to the stability of this region of the polypeptide chain in the TFE state. In accord with this, a periodicity in the magnitude of protection has been observed for the amides of the C-helix in both the native state [106] and TFE state [108], and has been attributed to the amphipathic nature of the helix and docking of this helix against the hydrophobic core that involves Trp 28, Trp 108 and Trp 111 [107, 224].

7.2.3 A-state of bovine α -lactalbumin

Whilst the fast folding of hen lysozyme from the TFE state makes it an ideal test system for the pulse labeling experiment, it remains a somewhat artificial non native state and lacks the all important connection with a kinetic refolding intermediate. As described earlier, the similarity between the stable A-state of bovine α -lactalbumin and transient intermediates of both bovine α -lactalbumin and hen lysozyme is well established however, although addition of TFE to the refolding buffer does accelerate refolding in the same way as for hen lysozyme [225] it does so to a lesser extent and it therefore lacks fast folding conditions. As refolding from the A-state to the native state simply requires a pH jump from pH 2 to 7 and not dilution of denaturant as in the previous section, a more favourable transfer of 300 μ l of polarized material from “low” field into 400 μ l of refolding buffer containing an excess of Ca^{2+} was adopted.

Real-time refolding

Figure 7.9 shows the refolding of bovine α -lactalbumin obtained with the same final conditions as in the pulse labeling experiment (pH 7, 37 mM Ca^{2+} , 20 °C) as

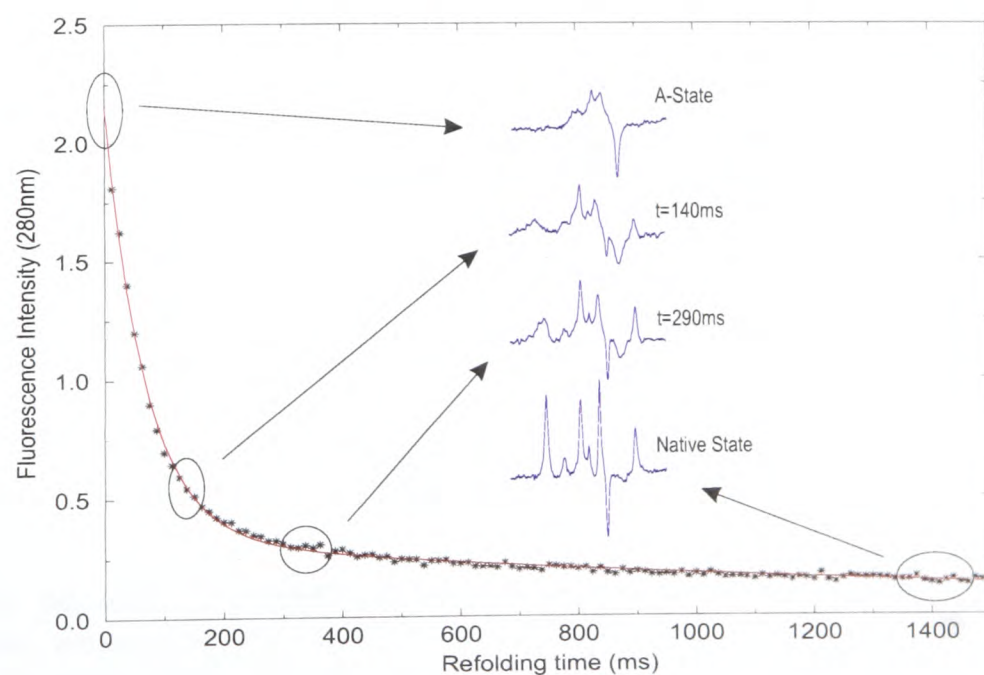


Figure 7.9: Kinetic progress curve of holo-BLA refolding monitored by tryptophan fluorescence (excitation at 280 nm) at 20 °C. Refolding was initiated from pH 2 by an 11 fold dilution and pH jump. The final conditions were 0.64 mM bovine α -lactalbumin, pH 7. Refolding kinetics were recorded as described in Fig.7.5. Also shown are real-time photo-CIDNP spectra (Chapter 5) obtained with identical final conditions but initiated by 2.3 fold dilution and pH jump (i.e. as in the pulse labeling experiment) within the NMR tube.

measured by both tryptophan fluorescence and real-time CIDNP (Chapter 5). As expected refolding is much slower than for hen lysozyme in TFE. Fortunately the exponential nature of folding means that whilst 100% folding may take up to 1 s, folding is $\sim$ 70% complete within 100 ms and hence partial transfer is still possible.

A significant amount of polarization still trapped in unfolded material will however be expected at early refolding times (i.e. 100 ms).

Pulse labeling

The results of the pulse labeling experiment are shown in Fig.7.10a. For each refolding delay, 8 “light” and 8 “dark” free induction decays were recorded, subtracted and Fourier transformed to give photo-CIDNP difference spectra. For comparison, the decay of CIDNP in the A-state *without* refolding over the same timescale (obtained with a single light and dark spectrum) is also shown in Fig.7.10b. The difference

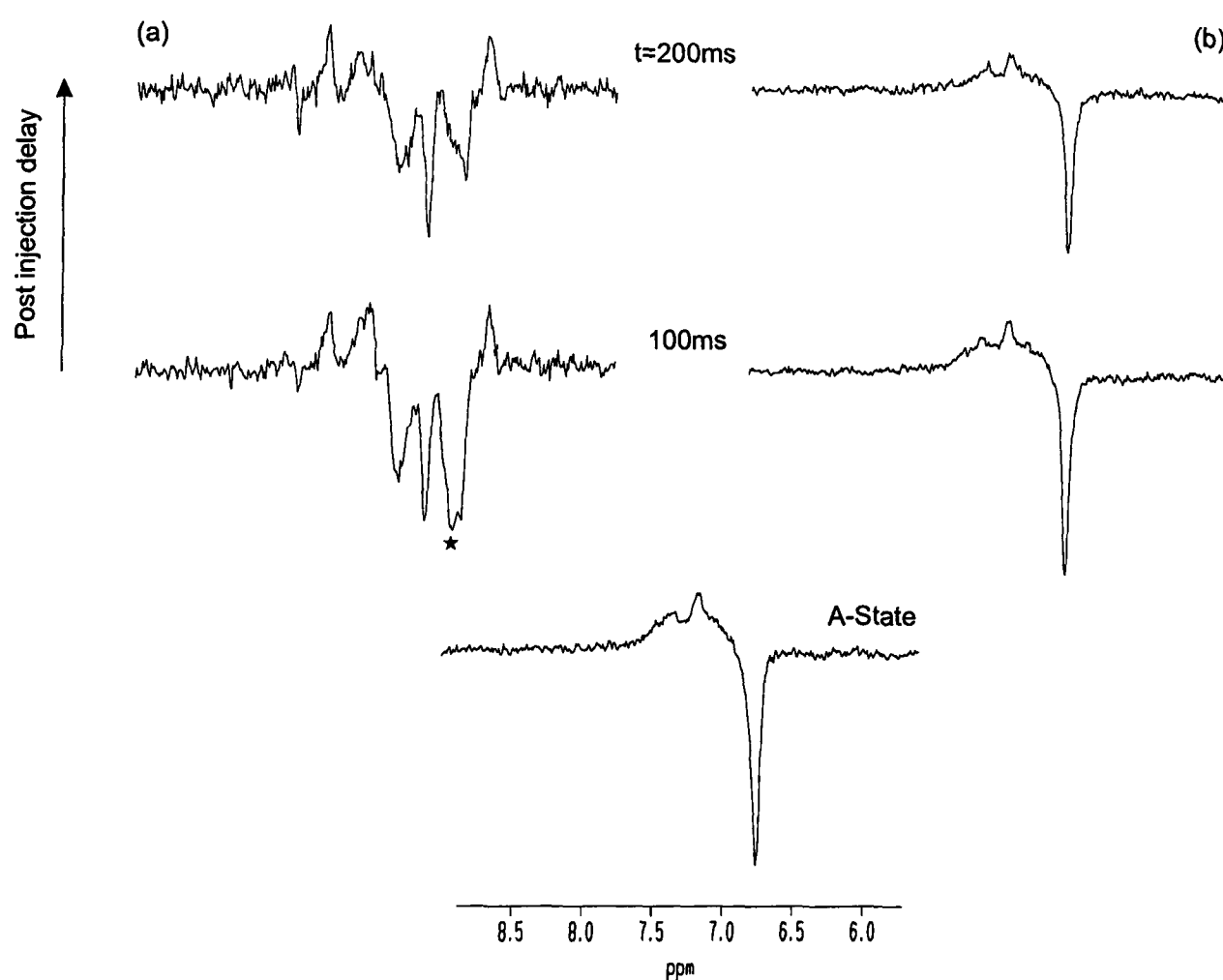


Figure 7.10: 400 MHz ^1H CIDNP spectra of bovine α -lactalbumin in the A-state generated at “low” field and detected at high field 100 and 200 ms after injection (a) *with* and (b) *without* refolding to the native state. Folding was initiated after a 500 ms laser flash by injecting 300 μl of 1.5 mM bovine α -lactalbumin, pH 2 into 400 μl sodium cacodylate buffer 200 mM, pH 7 and 65 mM Ca^{2+} . An asterisk has been used to indicate emissive tyrosine polarization that remains “trapped” in the A-state at early refolding times.

between the two columns is again striking, confirming that transfer of polarization to the native state is occurring. Note that as a consequence of the slower folding of bovine α -lactalbumin as compared to hen lysozyme in Section 7.2.2, the time development of certain peaks after injection is now potentially determined by both cross-relaxation effects *and* refolding. In particular, whilst the strong broad unfolded tyrosine peak at 6.75 ppm (labeled with an asterisk in Fig.7.10a) is fairly strong after 100 ms, suggesting that a significant concentration of protein remains in the A-state

at short refolding times, it is already considerably weaker by 200 ms. A number of resolved tyrosine and tryptophan peaks are apparent in the pulse labeling spectra. A comparison of chemical shifts obtained experimentally to those of the native state of bovine α -lactalbumin [31] are shown in Table 7.2 and labeled in the expanded $t=100$ ms spectrum in Fig.7.11. As compared to the TFE state of hen lysozyme, there

Residue	Nucleus	Results ^a	Literature ^b	Ambiguities
W118	H4	7.42	7.42	
	H6	6.52	6.50	
	H7	7.65	7.63	W60 H4 (7.64)
Y18	H3,5	6.94	6.94	
	H2,6	7.12	7.10	Y36 H3,5 (7.17)
Y36	H3,5	7.12	7.17	Y18 H2,6 (7.10)
Y50	H3,5	6.68	6.72	Y103 H3,5 (6.55)
YMG	H3,5	6.75	6.75 ^c	

Table 7.2: ^1H assignments at native chemical shifts of aromatic residues exposed in the A-state of bovine α -lactalbumin (1.5 mM, pH 2). ^aChemical shifts taken from Fig.7.11. References were taken from ^bRef.[31] and ^cRef.[226].

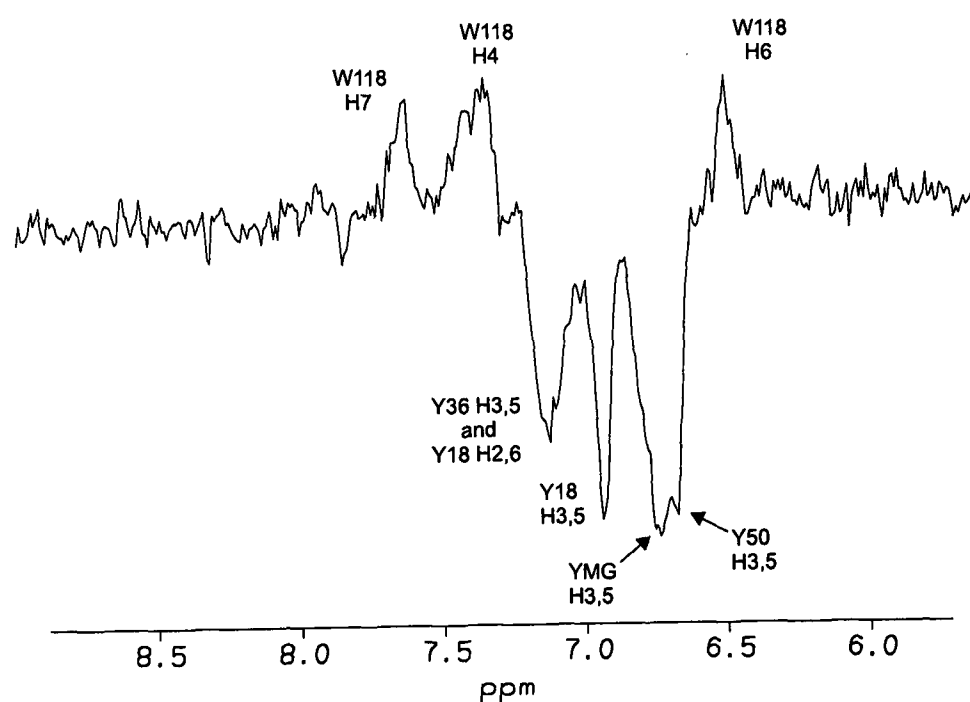


Figure 7.11: Assigned CIDNP pulse labeling spectrum of bovine α -lactalbumin in the A-state taken 100 ms after refolding to the native state (expanded from Fig.7.10). Assignments are described in the text and Table 7.2.

are fewer tryptophan peaks and hence there are fewer ambiguities. Assignments of tyrosine peaks are however less confident as the great deal of overlap and crowded nature of the region leads to a number of ambiguities.

The results of the pulse labeling experiment suggest that three of the four tyrosines

(Tyr 18, Tyr 36 and Tyr 50) and one of the four tryptophans (Trp 118) are exposed in the A-state (the absence of histidine polarization is again likely to be a simple consequence of their inability to compete for excited flavin). In the native state of bovine α -lactalbumin only Tyr 18 and Trp 118 are exposed [31]. These results would therefore indicate that one of the hydrophobic clusters that lies at the core of the native state of bovine α -lactalbumin and involves the three unpolarized tryptophan residues (Trp 26, Trp 60 and Trp 104) and the remaining tyrosine (Tyr 103) [31, 158] remains intact in the A-state.

A variety of lines of evidence suggest that some form of hydrophobic core in the helical domain of the native structure persists in the A-state. An assignment of resonances in the A-state of guinea pig α -lactalbumin based on equilibrium magnetization transfer with the native state found the resonances of Trp 26, Trp 104, and Tyr 103 (Trp 60 is absent in guinea pig α -lactalbumin) to be amongst the most strongly perturbed from random coil values [219]. A number of well-defined interresidue NOE effects have also been detected between the side-chains of Tyr 103, Trp 104 and His 107 in the A-state of bovine α -lactalbumin suggesting some form of aromatic cluster [227]. Whilst peptides corresponding to the isolated B- and C-helices (residues 24-36 and 88-99 respectively) appear to be entirely unfolded in water [228], in the A-state the most protected amides are located in these two hydrophobic segments [229]. Trp 26 and Trp 104 are known to play a key role in the packing between these two helices in the native conformation and the involvement of such hydrophobic interactions in stabilizing the secondary structure in the A-state has therefore been suggested [229]. Note that very effective protection from exchange of the *indole* NH of Trp 26 has also been observed in the A-state of both bovine [227] and guinea pig [229] α -lactalbumin. Experiments using paramagnetic surface probes [226] have also suggested the burial of Trp 26 and Trp 104 in bovine α -lactalbumin. Tryptophan fluorescence (attributed to the buried Trp 26 and Trp 104 in guinea pig α -lactalbumin [230]) is only marginally more efficiently quenched by small molecule solutes such as iodide in the A-state than it is in the native state and the wavelength of maximal emission is also very similar in the two states [230]. Finally, Kronman and co-workers [231] have shown that two to three of the four tryptophans residues are inaccessible in the A-state to long-range perturbants such as sucrose and glycerol in solvent perturbation experiments.

It is interesting to note that within the overall sequence homology and structure of the c-type lysozymes and α -lactalbumins, the hydrophobic-box motif involving Trp 28, Trp 108 and Trp 111 in hen lysozyme and Trp 26, Trp 60, Trp 104 and Tyr 103 in bovine α -lactalbumin are identical [31]. The results presented here would therefore suggest that retention of this hydrophobic core plays an important role in

stabilizing intermediate states, and hence supports the view that during refolding, collapse of the protein structure produces a hydrophobic environment that may help generate or stabilize secondary structure.

7.3 Exchange spectroscopy

As was mentioned in the introduction to this chapter, an alternative approach to the kinetic pulse labeling technique involves monitoring magnetization transfer between the partly folded and native states under *equilibrium* conditions. At the midpoint of a cooperative unfolding transition (e.g. thermal unfolding) both folded and unfolded states coexist and interconvert at rates typically in the range 0.1-10 s⁻¹ corresponding to slow exchange on the NMR timescale. Under such conditions, distinct resonances at native and unfolded chemical shifts should be observed in a one-dimensional NMR spectrum. A two-dimensional EXSY (exchange spectroscopy) experiment [232] can then be used to correlate the chemical shifts of nuclei that are exchanging between chemical environments in the native and partly folded states. At its simplest level, the EXSY pulse sequence involves two chemical shift precession periods (t_1 and t_2) - one on each of the two sites - separated by a mixing time (τ_m) during which exchange occurs between these two sites.

The pulse sequence used for EXSY is identical to that used in a two-dimensional NOESY (nuclear Overhauser spectroscopy) experiment that correlates the chemical shifts of nuclei that are involved in mutual dipolar relaxation processes and hence, in a two-dimensional EXSY/NOESY spectrum taken at the midpoint of a cooperative transition, cross peaks caused by magnetization transfer both *within* (i.e. NOEs) and *between* (i.e. chemical exchange) the two states will be detected. The utility of the experiment for correlating resonances of the different states therefore depends on being able to discriminate between these two kinds of effects. The simplest approach in the case of thermal unfolding involves recording additional spectra at temperatures sufficiently below and above the unfolding transition zone that in each case only one state is appreciably populated (note that the latter is often omitted as NOEs in unfolded states are often too weak to be detected) [105, 219]. By comparison of the three spectra, exchange peaks can then be identified.

Once suitable temperatures above, below and at the midpoint of unfolding have been determined, the success of the experiment then depends crucially on choosing a mixing time that is appropriate for the rate of interconversion between the two states. As was mentioned above, exchange rates tend to occur over a wide range of values and are likely to depend on both the protein and the unfolding conditions. Thus, whilst a short mixing time of 40 ms was used by Baum and co-workers [219]

with guinea pig α -lactalbumin at pH 7.4, 65 °C (exchange rate 7 s^{-1}), Dobson and co-workers [102, 103] set the mixing time to 1 s when studying hen lysozyme at pH 3.8, 77 °C (exchange rate 0.7 s^{-1}). Estimates of exchange rates are conventionally obtained by selective saturation transfer [102] or directly from the magnitude of exchange linebroadening [219]. Alternatively, if the exchange rate has not been determined, EXSY/NOESY spectra can simply be acquired for a range of mixing times. Note that once a suitable mixing time has been determined, typically only a handful of exchange peaks are detected e.g. Phe 31, Trp 26, Tyr 103 and Trp 104 for guinea pig α -lactalbumin in the above example [219]. Note also that for a number of protein systems studied, suitable conditions under which native and partly folded states interconvert on an appropriate timescale could not be found.

In the following section, application of this approach to the molten globule like state of bovine α -lactalbumin obtained by thermal unfolding at pH 7.4 will be discussed.

7.3.1 Thermal unfolding of holo-BLA

Figure 7.12 shows the thermal unfolding of holo-BLA (1.5 mM, pH 7.4) as monitored by near-UV circular dichroism. Unfolding appears highly cooperative with a midpoint around 65 °C and is essentially complete by 75 °C. The unfolded state

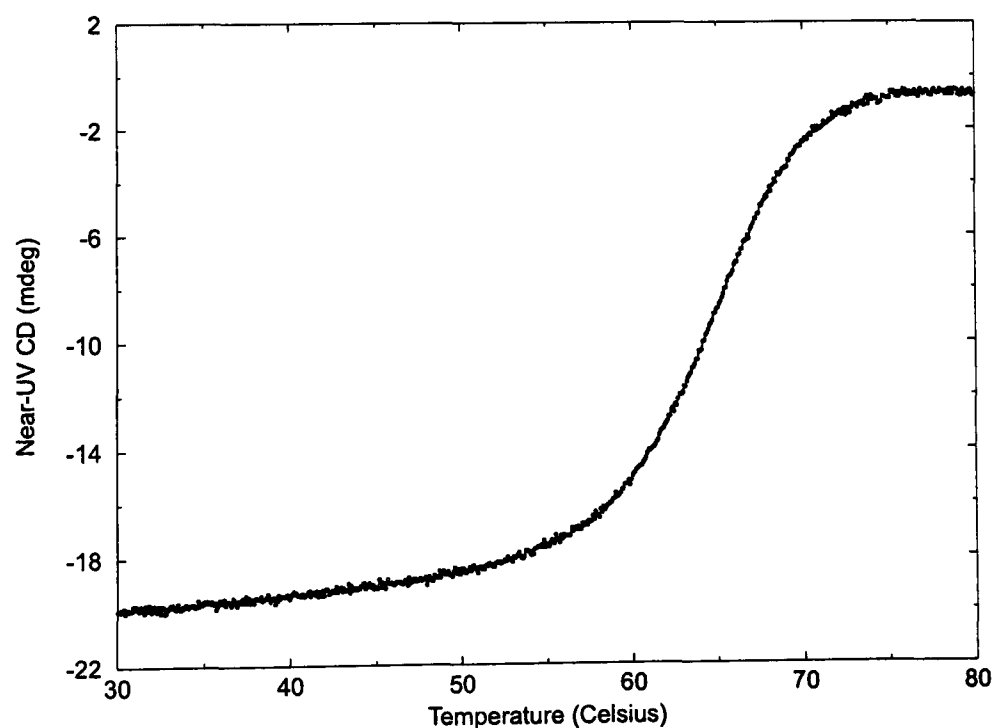


Figure 7.12: Thermal unfolding curve of holo-BLA (1.5 mM, pH 7.4) as judged by near-UV (297 nm) CD. Spectrum was obtained using a Jasco J720 spectropolarimeter and 1 mm path length cell.

under these conditions has been shown to be similar to the A-state at pH 2 by a variety of spectroscopic techniques [233], although perturbations from the fully unfolded state are somewhat less pronounced than those observed at pH 2 and lower temperatures. For comparison, unfolding was monitored over the same temperature range by photo-CIDNP as shown in Fig.7.13. The spectrum at 30 °C is essentially

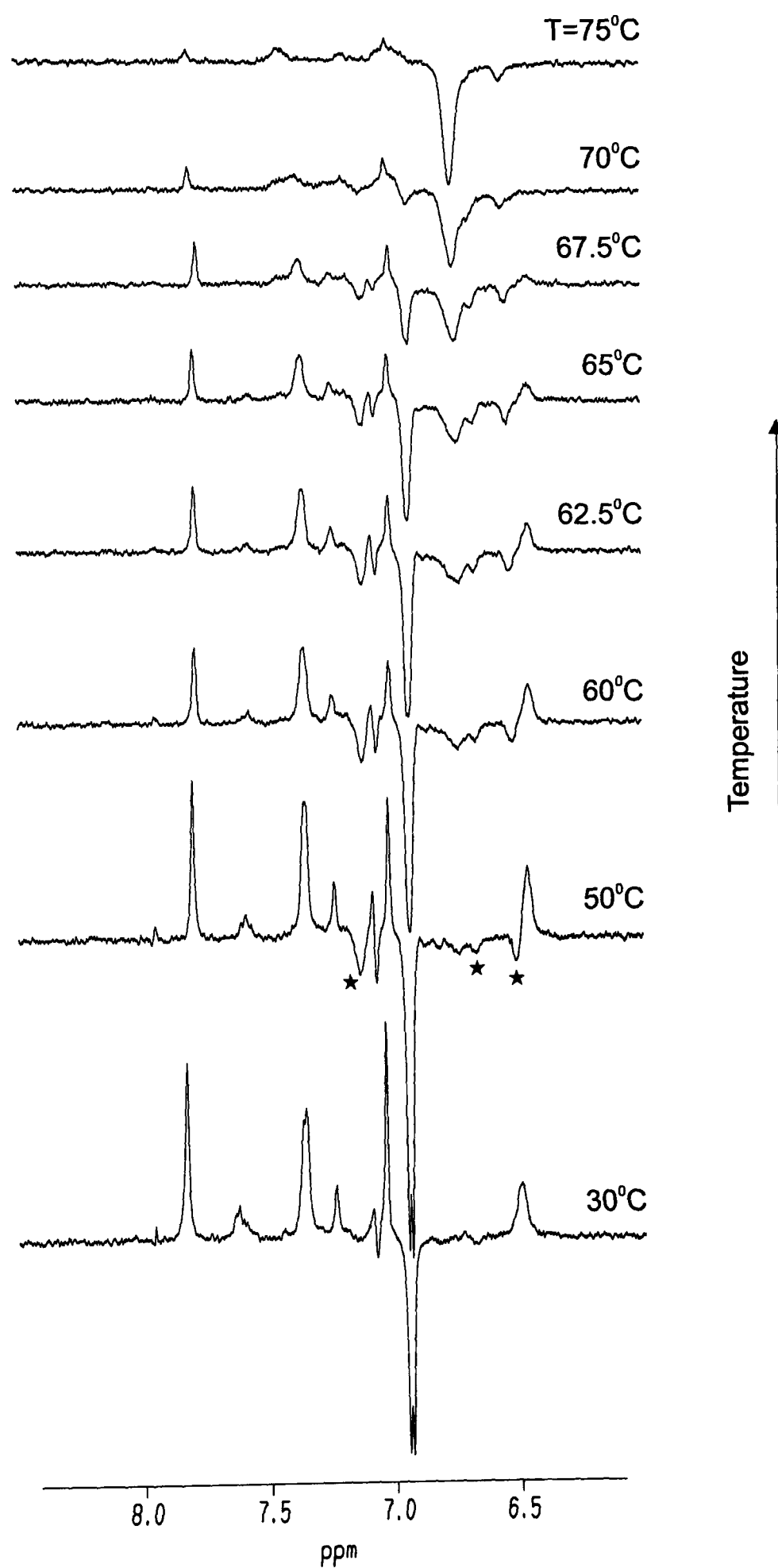


Figure 7.13: 600 MHz ^1H photo-CIDNP spectra of holo-BLA (1.5 mM, pH 7.4) taken at the temperatures shown. Emissive tyrosine peaks labeled with an asterisk in the spectrum taken at 50°C are absent at room temperature and seem to appear prior to the cooperative denaturation around 65°C .

native and by 75 °C molten globule like with characteristic broad lineshapes and a large tyrosine to tryptophan intensity ratio. In between these two extremes, native histidine and tryptophan peaks gradually disappear in what appears to be a cooperative two state transition. Two quite distinct stages are however observed in the emissive tyrosine region. In the first stage (30-60 °C) new peaks at 6.55, 6.70 and 7.16 ppm appear and have been labeled with an asterisk in the spectrum taken at 50 °C. These peaks grow relative to the native Tyr 18 H3,5 peak at 6.97 ppm with increasing temperature and then disappear above 65 °C. Around the transition point as measured by circular dichroism (60-70 °C), a more cooperative step occurs with a sharp decrease of the native Tyr 18 H3,5 peak and a concomitant growth in the broad tyrosine molten globule (YMG) H3,5 peak at 6.77 ppm. Whilst this second step was expected from the circular dichroism data and corresponds to the cooperative unfolding of the native state to a molten globule state, the origin of the first step at temperatures below the transition zone is less clear. Therefore whilst the original aim of the experiment revolved around assigning the residues contributing to the broad molten globule tyrosine peak at 65 °C via exchange to the native state, an additional interest was now placed on assigning the three new tyrosine peaks labeled at 50 °C.

7.3.2 EXSY/NOESY at 40 °C

An interconversion rate between the native and molten globule states of bovine α -lactalbumin of $\sim 1.5 \text{ s}^{-1}$ has previously been estimated at pH 6.9, 62 °C by magnetization transfer experiments [226], and hence a mixing time of 300 ms was chosen for further experiments. The two-dimensional photo-CIDNP EXSY/NOESY spectrum was first obtained below the transition temperature at 40 °C and is shown in Fig.7.14. As was described in Chapter 4, two-dimensional photo-CIDNP experiments are likely to encounter problems with the decay of flavin dye and hence the experiment was performed using the mixing device that was shown to dramatically prolong the lifetime of samples in Section 4.3. At this temperature only the native state is present and one should therefore only observe native NOEs. Note that only directly polarized protons appear in the ω_1 dimension of two-dimensional photo-CIDNP spectra and hence they differ from their conventional NMR counterparts in being asymmetric about the diagonal [97, 98]. A comparison of the chemical shifts obtained experimentally to those of the native state of bovine α -lactalbumin are shown in Table 7.3. As polarized residues must be accessible to the bulky flavin dye they tend to be found in relatively uncrowded regions on the surface of the protein and hence the majority of NOEs are intraresidue. The various pathways for Tyr 18, His 68 and Trp 118 are particularly clear, both within and between the aromatic ring and aliphatic chain. The only interresidue NOEs are between H6 of Trp 118 and both H2,6 and H3,5 of Phe 31 that are separated by distances of 0.31 and 0.28

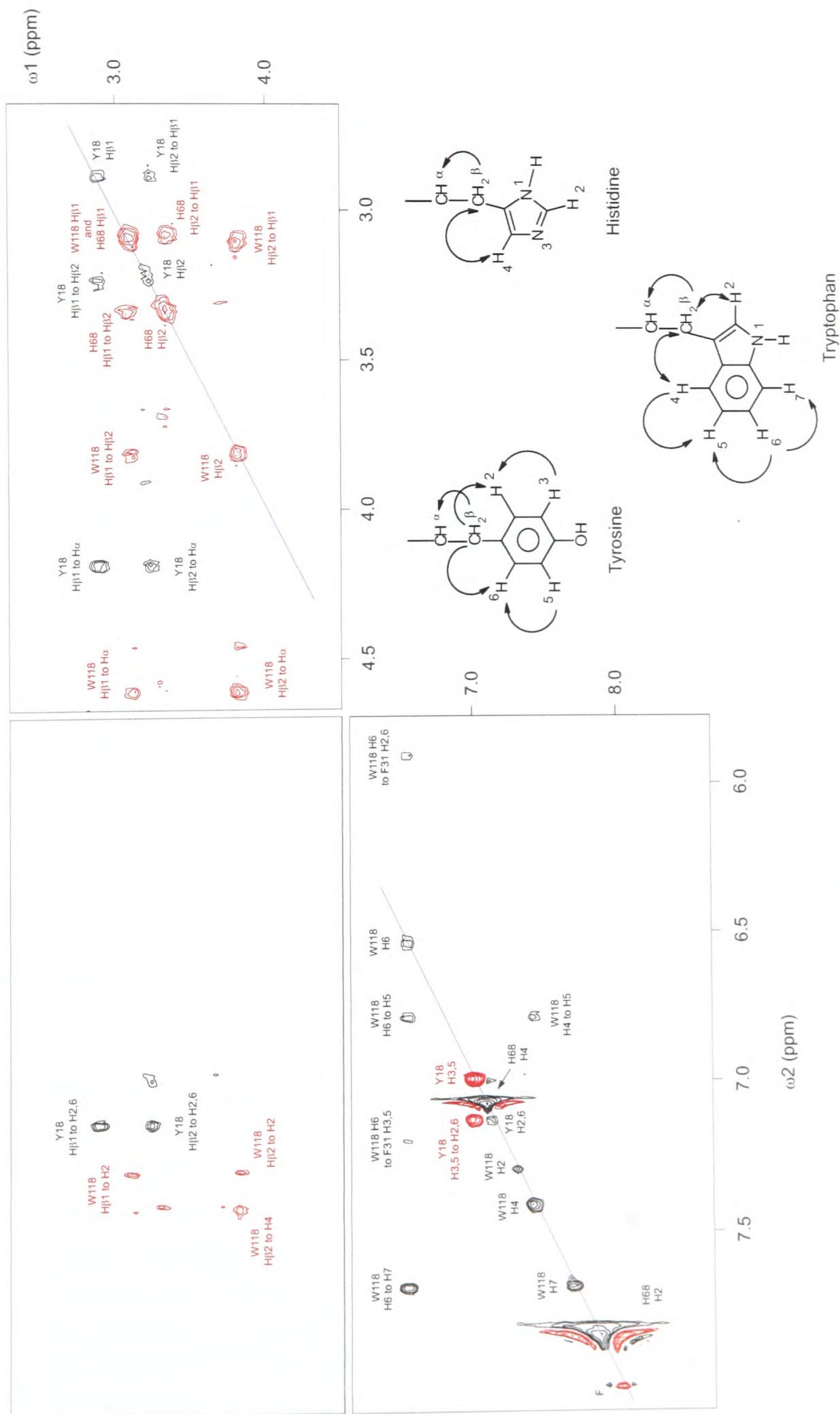


Figure 7.14: 600 MHz CIDNP-EXSY/NOESY spectra of bovine α -lactalbumin (1.5 mM, pH 7.4, 40 °C) obtained with mechanical mixing. Spectral widths are 8000 Hz in both dimensions, with 128 complex points in f_1 (i.e. two irradiations per t_1 increment). To suppress the background magnetization a “dark” spectrum was acquired and automatically subtracted from each “light” spectrum. Mixing was performed by injection of 300 μ l into 500 μ l after every flash. Emissive peaks are in red and absorptive peaks in black. Also shown are the typical intraresidue cross-polarization pathways for tryptophan, tyrosine and histidine.

nm respectively within a hydrophobic cluster that also involves His 32 and Tyr 36 [31, 158].

7.3.3 EXSY/NOESY at 65 °C

The two-dimensional photo-CIDNP EXSY/NOESY spectrum obtained at the midpoint of unfolding is shown in Fig.7.15. As was observed in the one-dimensional spectrum (Fig.7.13) both the native peaks observed at 40 °C and the broad tyrosine molten globule H3,5 peak are apparent at 65 °C confirming that the two states coexist in equilibrium under these conditions. However, in direct contrast with the results of the CIDNP pulse labeling experiment described in the previous section that indicated the exposure of Tyr 18, Tyr 36 and Tyr 50 in the A-state of bovine α -lactalbumin, a *single* tentative exchange cross peak from the broad tyrosine molten globule H3,5 peak at 6.77 ppm to the native Tyr 18 resonance at 6.97 ppm is observed in Fig.7.15. The absence of a cross peak to native Tyr 50 at 6.70 ppm could simply be a consequence of overcrowding due to the proximity of the two peaks, however, native Tyr 36 lies at 7.16 ppm and hence any cross peak would occur in a particularly uncrowded region of the spectrum. As was mentioned in the introduction to this section, suitable conditions for equilibrium magnetization transfer experiments are notoriously difficult to find, and hence the apparent conflict between the two approaches could simply be a consequence of an unsuitable choice of experimental parameters and/or conditions. In order to determine whether a more suitable mixing time for the photo-CIDNP EXSY/NOESY experiment could be found, a series of EXSY/NOESY spectra were obtained by conventional NMR at both 40 and 65 °C using mixing times in the range 40 ms to 1 s. However, whilst a large number of NOE cross peaks were observed, no exchange cross peaks were detected at any of the mixing times. Note that at lower pH the midpoint of unfolding occurs at lower temperature (e.g. at pH 3.5 it is close to 40 °C [219]) where the interconversion rate is slower; however, no alternative conditions were explored.

The emissive tyrosine resonances labeled with an asterisk at 50 °C in Fig.7.13 (6.55, 6.70 and 7.16 ppm) that were mentioned in the previous section are also apparent in Fig.7.15. A comparison of the chemical shifts obtained experimentally for these emissive peaks with those of the native state of bovine α -lactalbumin are shown in Table 7.3 and labeled in the expanded 65 °C one-dimensional spectrum in Fig.7.16. Interestingly the three peaks that appear during the first stage of thermal unfolding all coincide with tyrosine H3,5 resonances at *native* chemical shifts (i.e. Tyr 36, Tyr 50 and Tyr 103) with a number of intraresidue NOEs apparent both within and between the aromatic and aliphatic regions for Tyr 36. The appearance of these peaks would therefore indicate that some form of “breathing” motion occurs within the native protein prior to the cooperative transition to the molten globule

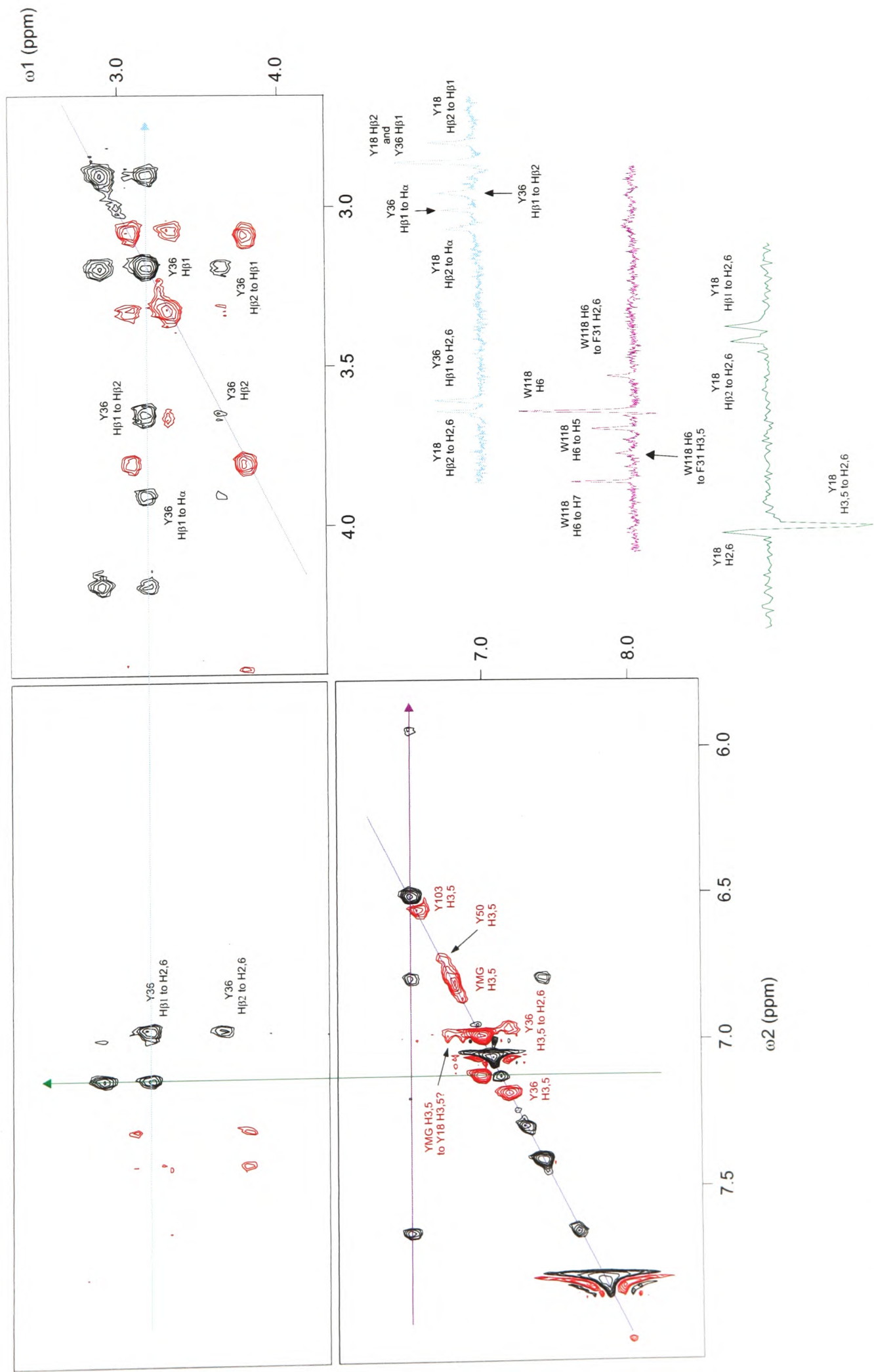


Figure 7.15: 600 MHz CIDNP-EXSY/NOESY spectra of bovine α -lactalbumin (1.5 mM, pH 7.4, 65 °C) obtained with mechanical mixing. Spectral widths are 8000 Hz in both dimensions, with 128 complex points in f_1 (i.e. two irradiations per t_1 increment). To suppress the background magnetization a “dark” spectrum was acquired and automatically subtracted from each “light” spectrum. Mixing was performed by injection of 300 μ l into 500 μ l after every flash. Emissive peaks are in red and absorptive peaks in black. A selection of colour coded traces taken from the two-dimensional spectrum are also shown to illustrate the selectivity of the CIDNP technique and the quality of spectra.

Residue	Nucleus	Results ^a	Literature ^c	Residue	Nucleus	Results ^b	Literature ^c
W118	H2	7.22	7.19	Y36	H2,6	6.94	6.95
	H4	7.41	7.42		H3,5	7.16	7.17
	H5	6.77	6.80		H α	3.89	3.86
	H6	6.50	6.50		H β_1	3.20	-
	H7	7.63	7.63		H β_2	3.62	-
	H α	4.44	4.54	Y50	H2,6	n.o.	7.22
	H β_1	3.05	3.00		H3,5	6.70	6.72
	H β_2	3.78	3.73		H α	n.o.	4.16
Y18	H2,6	7.10	7.10		H β_1	n.o.	-
	H3,5	6.97	6.94		H β_2	n.o.	-
	H α	4.16	4.11	Y103	H2,6	5.45	5.45
	H β_1	2.88	2.79		H3,5	6.55	6.55
	H β_2	3.17	3.17		H α	n.o.	3.77
H68	H2	7.80	7.73		H β_1	n.o.	2.49
	H4	7.04	7.02		H β_2	n.o.	2.49
	H α	3.31	3.34	YMG	H3,5	6.77	6.75 ^d
	H β_1	3.04	3.04				
	H β_2	3.31	3.34				
F31	H2,6	5.94	5.92				
	H3,5	7.20	7.19				

Table 7.3: ¹H assignments of aromatic residues in the photo-CIDNP EXSY/NOESY spectrum of bovine α -lactalbumin (1.5 mM, pH 7.4) observed at both 40 and 65 °C (left) and at 65 °C only (right). n.o., not observed. Chemical shifts were taken from ^aFig.7.14 and ^bFig.7.15 respectively. References were taken from ^cRef.[31, 234] and ^dRef.[226].

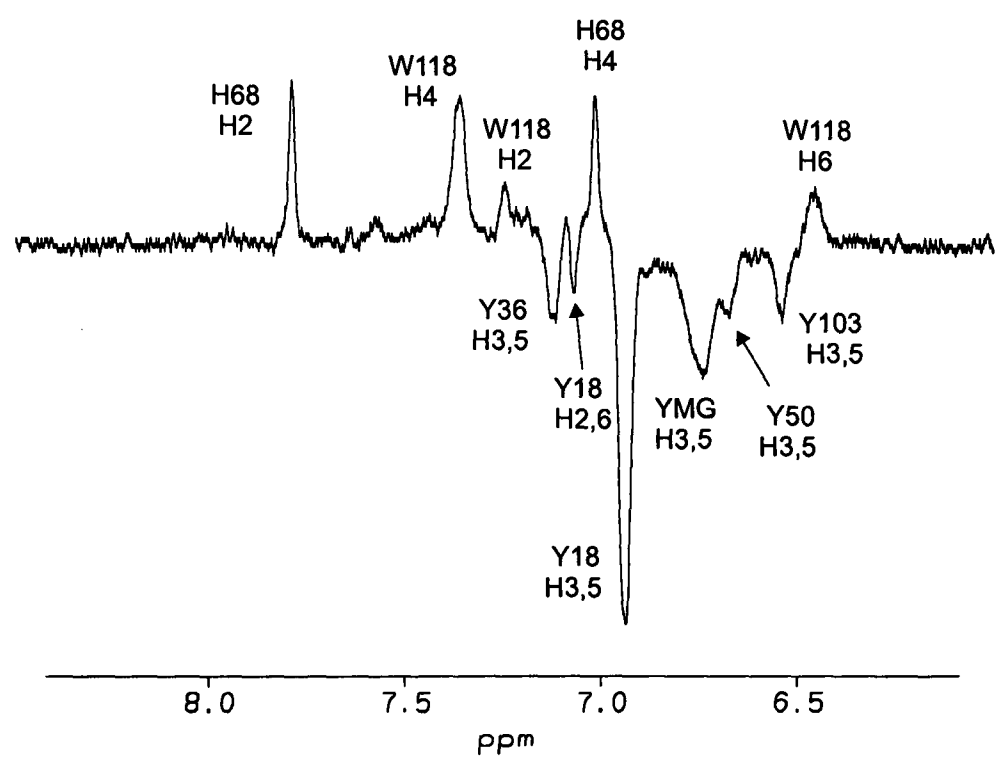


Figure 7.16: Assigned 600 MHz ¹H photo-CIDNP spectra of holo-BLA (1.5 mM, pH 7.4) taken at 65 °C (expanded from Fig.7.13). Assignments are described in the text and Table 7.3.

state. In this context it is interesting to note that generation of CIDNP in tyrosine residues by flavins proceeds via hydrogen abstraction and hence requires an accessible OH group. Although the effects are not entirely clear cut, hydrogen bonding has therefore been used on several occasions to account for missing tyrosine CIDNP in otherwise exposed groups [12]. Hence, whilst Tyr 103 shows no CIDNP enhancement in native bovine α -lactalbumin (e.g. the spectrum taken at 30°C in Fig.7.13), it has been shown to be partially accessible to the spin label TEMPOL [134] and a weak CIDNP enhancement has also been detected in human α -lactalbumin [31]. Improta *et al.* [134] noted that the hydroxyl group of Tyr 103 is involved in hydrogen bonding with the backbone oxygen of Gln 54 in the X-ray crystal structure of baboon α -lactalbumin [157] and used this observation to explain the discrepancy between photo-CIDNP and TEMPOL data. It is therefore possible that some form of partial weakening of hydrogen bonds caused by a temperature dependent increase in dynamics within the native state could explain the appearance of these various tyrosine peaks.

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