Journal of Biomolecular NMR, **16:** 235–244, 2000.
KLUWER/ESCOM

Improved photo-CIDNP methods for studying protein structure and folding

Kiminori Maeda[a,*], Charles E. Lyon[a], Jakob J. Lopez[a], Masa Cemazar[a], Christopher M. Dobson[b] & P.J. Hore[a,**]
Oxford Centre for Molecular Sciences, [a]*Physical and Theoretical Chemistry Laboratory, and* [b]*New Chemistry Laboratory, Oxford University, Oxford OX1 3QZ, U.K.*

Received 21 October 1999; Accepted 21 December 1999

Abstract

Two new techniques offering considerable improvements in the quality of ^{1}H photo-CIDNP spectra of proteins are demonstrated. Both focus on the problem of progressive photo-degradation of the flavin dye used to generate polarization in exposed tryptophan, tyrosine and histidine side-chains. One approach uses rapid addition and removal of protein/flavin solution between light flashes to mix the NMR sample and introduce fresh dye 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. The techniques are demonstrated by 600 MHz CIDNP-NOESY spectroscopy of bovine holo-α-lactalbumin, and by real-time CIDNP observation of the refolding of bovine apo-α-lactalbumin following rapid dilution from a high concentration of chemical denaturant.

Introduction

Chemically induced dynamic nuclear polarization (CIDNP), a phenomenon traditionally used to investigate the mechanisms of free radical reactions (Muus et al., 1977; Salikhov et al., 1984), has been extensively exploited as a surface probe of protein structure (Kaptein, 1978, 1982; Kaptein et al., 1978; Hore and Broadhurst, 1993). In the presence of a suitable photosensitiser – usually a flavin – laser irradiation generates non-equilibrium nuclear polarization in the side-chains of exposed aromatic amino acid residues. Although the resulting sensitivity enhancements and spectral simplifications are often welcome, the real attraction of the method is that only the histidine, tryptophan and tyrosine side-chains that are *physically accessible* to the photoexcited flavin are polarizable (Kaptein, 1978, 1982; Kaptein et al., 1978; Hore and Broadhurst, 1993). A recent example of the use of CIDNP in this way is a stopped-flow experiment in which differential changes in the exposure of tyrosine and tryptophan residues in hen lysozyme were observed as the denatured protein folded to its native state (Hore et al., 1997). (The investigation of protein folding using real-time NMR has been reviewed by van Nuland et al. (1998) and Dobson and Hore (1998).)

Several extensions of the original one-dimensional (1D) 'laser flash–radiofrequency pulse–acquire' ^{1}H CIDNP experiment have been devised. Kaptein and co-workers have developed COSY and NOESY versions in which each repetition of the pulse sequence is preceded by a light flash (Scheek et al., 1984, 1985). More recently, Lyon et al. (1999) have measured 2D ^{15}N-^{1}H heteronuclear CIDNP correlation spectra to reveal the accessibility of tryptophan side-chains in a denatured protein. A serious problem encountered in these experiments, which require prolonged laser irradiation, is the progressive decay of the polarization generated by successive light flashes. The photochemical reactions used to produce CIDNP in proteins are cyclic, so that polarization is observed in the intact

*Permanent address: Department of Chemistry, Graduate School of Science, Tohoku University, Sendai 980-8578, Japan.
**To whom correspondence should be addressed. E-mail: hore@physchem.ox.ac.uk

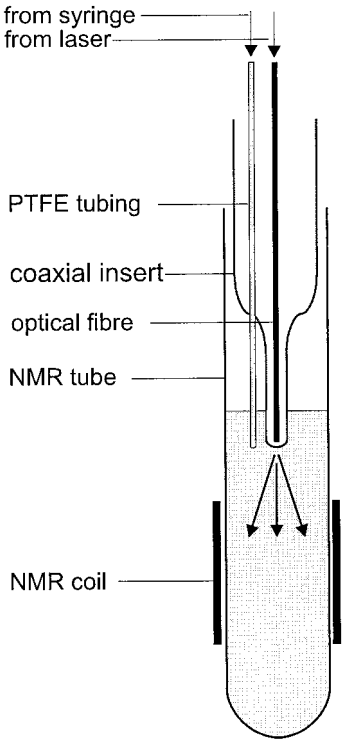

Figure 1. Schematic diagram of the positioning of the optical fibre, coaxial pyrex insert and PTFE transfer tube inside a 5 mm NMR tube. The transfer tube is used for slow injection of flavin solution or for rapid mixing of the solution (see text). This method of sample illumination is taken from Scheffler et al. (1985).

protein rather than a chemically modified form; but they are not *perfectly* cyclic. An investigation into the reaction of lumiflavin with tryptophan (Connolly and Hoch, 1991) 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 flavohydroquinine FH_2 absorbs weakly in the visible region), and to degradation of the tryptophan. The former is likely to be the more serious problem because the CIDNP intensities depend strongly on the optical density of the sample, and the concentration of the flavin is usually an order of magnitude smaller than that of the polarizable histidine, tyrosine and tryptophan side-chains in a protein. The most probable cause of photoreduction is the thermodynamically favourable disproportionation of flavosemiquinone radicals ($2FH^{\cdot} \rightarrow F + FH_2$) (Hore et al., 1981; Müller, 1987).

The progressive loss of polarization in the course of a multi-flash experiment causes unwanted line-broadening 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. Several attempts have been made to overcome the problem. As bleaching occurs predominantly in the irradiated region of the NMR tube, sample mixing can be used to replenish the flavin (McCord et al., 1981; Scheek et al., 1984). Manipulation of the molecular oxygen concentration is also helpful: O_2 efficiently oxidises FH_2 to F (Müller, 1991), but it is consumed in the process; indeed, removal of O_2 by degassing accelerates photo-bleaching (Connolly and Hoch, 1991). However, too high a concentration of O_2 dramatically attenuates the initial polarization, probably by rapid quenching of the photoexcited triplet, ^3F (Connolly and Hoch, 1991). Hitherto, the most successful technique has been to spin the NMR tube rapidly between scans so as to create a vortex in the solution (Scheek et al., 1984). 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 (Winder, 1997).

Here we demonstrate two new techniques for combatting the photo-degradation problem, which promise greatly to extend the range of possible photo-CIDNP experiments.

Results and discussion

Photo-CIDNP measurements were performed essentially as originally described by Kaptein (Kaptein, 1978; Kaptein et al., 1978). Blue-green light (4 W) from a continuous-wave argon ion laser (Spectra Physics Stabilite 2016-05) was chopped into 100 ms pulses by a mechanical shutter controlled from a home-built 600 MHz NMR spectrometer. The light was introduced into the NMR tube via an optical fiber (diameter 1 mm), the end of which was held 4 mm above the top of the NMR coil, inside a coaxial pyrex insert (Wilmad WGS 5BL) dipping into the solution (Figure 1) (Scheffler et al., 1985). Flavin mononucleotide (FMN) was used as photosensitiser in all experiments.

To assess the degree of photodegradation, series of CIDNP spectra were recorded with a 14 s delay during which the complete ^1H spectrum was presaturated, prior to each light flash, and a 5 ms delay between the light and the acquisition pulse. Fully relaxed NMR spectra were obtained by repeating the

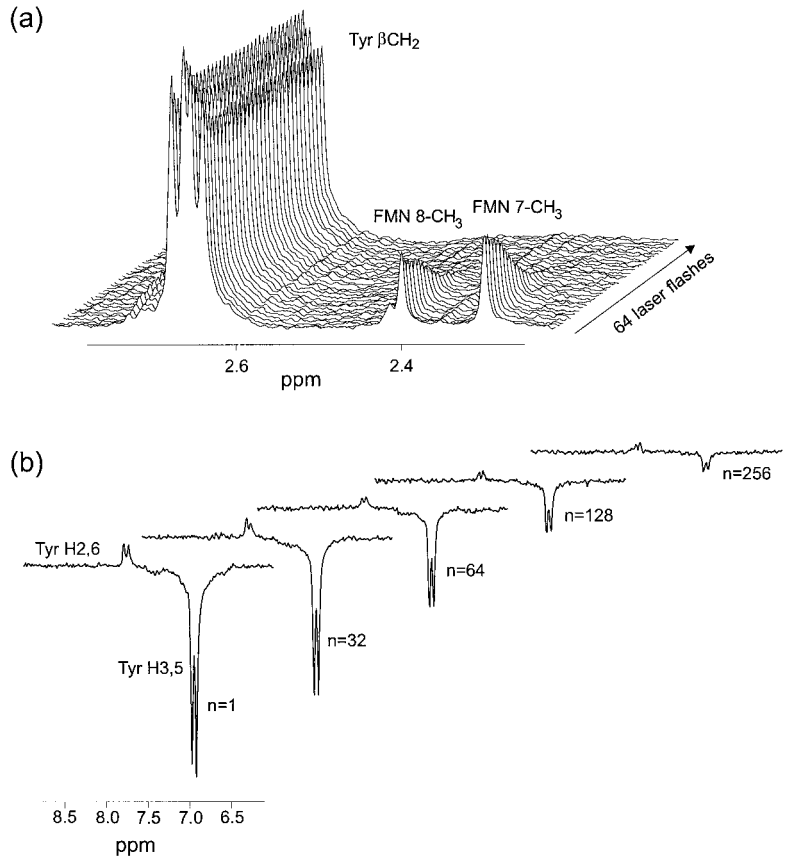

Figure 2. (a) A series of NMR spectra showing the 7-CH_3 and 8-CH_3 resonances of FMN (0.2 mM) and one of the βCH_2 protons of tyrosine (4 mM in D_2O at pH 4.5). Each spectrum was recorded after a 4 W argon ion laser pulse (100 ms) with a delay to allow the CIDNP to relax completely prior to the radiofrequency pulse. Alternate spectra from a series of 64 are shown. (b) Photo-CIDNP spectra recorded under similar conditions to (a) showing the polarization of the aromatic protons of tyrosine. Spectra obtained from the 1st, 32nd, 64th, 128th and 256th light flashes are shown. The decay of the flavin peaks in (a) and the tyrosine resonances in (b) are caused principally by flavin photo-degradation.

measurement with a delay between the light flash and the radiofrequency pulse of sufficient length to allow the CIDNP to relax to equilibrium. The FMN concentration (0.2 mM) that gave the optimum initial enhancement was used unless otherwise stated.

Figure 2a shows the change in the NMR intensities of the amino acid tyrosine (4 mM) and FMN (0.2 mM) in D_2O in a series of spectra of the kind described above. Unlike the tyrosine resonances, which are hardly affected by the light flashes that precede each scan, the FMN peaks decay rapidly. The corresponding photo-CIDNP experiment (Figure 2b) shows the steady loss of tyrosine polarization generated by each flash. There seem to be two principal reasons why this decay is slower than observed for FMN in Figure 2a. First, the flavin concentration at the start of the experiment and the dimensions of the sample result in an optical density for the illuminated part of the NMR sample in the sensitive region of the NMR coil that is considerably greater than unity. A very high proportion of the light should therefore be absorbed, and a significant reduction in the flavin concentration can take place before this ceases to be true and the number of radical pairs generated, and hence the observed polarization, begins to fall. Second, as soon as a small quantity of reduced flavin has been produced, the comproportionation reaction $F + FH_2 \rightarrow 2FH^{\cdot}$ (Müller, 1987) leads to a small concentration of stable flavosemiquinone radicals. Degenerate exchange reactions of the radical with the parent flavin molecule, either by hydrogen atom transfer or, after deprotonation of the radical, by electron transfer, will lead to paramagnetic broadening of the flavin resonances which will further attenuate their intensity in spectra such as Figure 2a, without affecting the tyrosine CIDNP.

At the end of each series of CIDNP measurements, obvious bleaching of the yellow FMN colour could be seen in the irradiated region of the NMR tube (~50% of the 500 μl sample). $FMNH_2$ resonances were evident in 'dark' spectra recorded after irradiation, together with a variety of unassigned peaks apparently due to photo-products derived from FMN, and possibly $FMNH_2$, reflecting the complex photochemistry (Heelis, 1991). The fractional loss of polarization could be attenuated somewhat by using a larger initial FMN concentration, 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. These results indicate that the progressive loss of CIDNP is principally photo-degradation of the flavin rather than of the amino acid.

Proceeding on this basis, we have investigated two 'mechanical' solutions to the problem. The simpler approach is to add small quantities (typically 1.0 μl) of e.g. 10 mM FMN solution to the NMR sample between scans, in an attempt to replace that lost by reduction (Hincke et al., 1981). This is conveniently done by introducing a thin (0.5 mm internal diameter) PTFE tube into the sample via a small hole in the coaxial insert (Figure 1). The FMN solution was slowly injected using a syringe driven by a stepper motor controlled from the spectrometer. Although a variety of FMN concentrations and injection volumes were tried, it proved difficult to find conditions which gave a significant reduction in the rate of loss of polarization. On the contrary, an acceleration of the CIDNP decay was much easier to achieve. The difficulty lies in the inhomogeneous concentration of dye in the sample tube, problems with the optical density if too much dye is added, the reliance on diffusion to distribute flavin throughout the coil region and, possibly, formation of absorbing photo-products from the high local concentration of flavin. It is also difficult to know in advance how much flavin to add. Attempts to inject FMN directly into the sensitive region of the probe gave similar results.

However, the same sample tube configuration (Figure 1) can be used in a different way with considerably greater success, as follows. The idea now is to inject as rapidly as possible ~300 μl of solution containing the sample under investigation into ~500 μl of the same solution in the NMR tube, draw it back up again, and wait ~10 s before each light flash and signal acquisition. If done sufficiently briskly (the syringe was driven pneumatically 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, the PTFE transfer line and the syringe. This technique, though undoubtedly crude, is surprisingly effective, as shown in Figure 3. Mixing dramatically prolongs the lifetime of the polarization in a solution of tyrosine; for a sample of the protein lysozyme, a similar result was found for the two exposed tryptophan residues. The scatter in the CIDNP intensities for the mixed lysozyme solution is no worse than for a static sample, nor were there systematic changes in linewidths during the course of the measurements. The presence of the coaxial insert and optical fibre 4 mm from the NMR coil presented no significant problems with shimming.

An alternative approach to the problem of FH_2 formation is 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 below. Figure 4 shows the effect of 10 mM hydrogen peroxide (an oxidant) and, for contrast, sodium dithionite (a reductant) on the decay of the CIDNP signal of tyrosine. 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 Figure 2a, but with 10 mM H_2O_2, are shown in Figure 5. The deceleration in the disappearance of the FMN is pronounced.

The CIDNP intensities in solutions of tyrosine and of hen lysozyme after 128 light flashes relative to the respective initial intensities are shown in Figure 6 as a function of H_2O_2 concentration. Evidently, a concentration of 10 mM is sufficient to reduce the loss of signal quite markedly, especially in the case of the protein. The presence of such amounts of H_2O_2 seems to have no adverse effect on hen lysozyme or its CIDNP spectrum. The circular dichroism, 1D and 2D NMR spectra of lysozyme are not changed by the presence of 100 mM H_2O_2, indicating that no chemical degradation has occurred. No unexpected CIDNP peaks are observed, nor is there any other evidence for side reactions. Mass spectra of three-day old solutions of lysozyme containing 10 mM H_2O_2 are indistinguishable from those of lysozyme alone; only small differences were found following exposure for similar times to 100 mM H_2O_2.

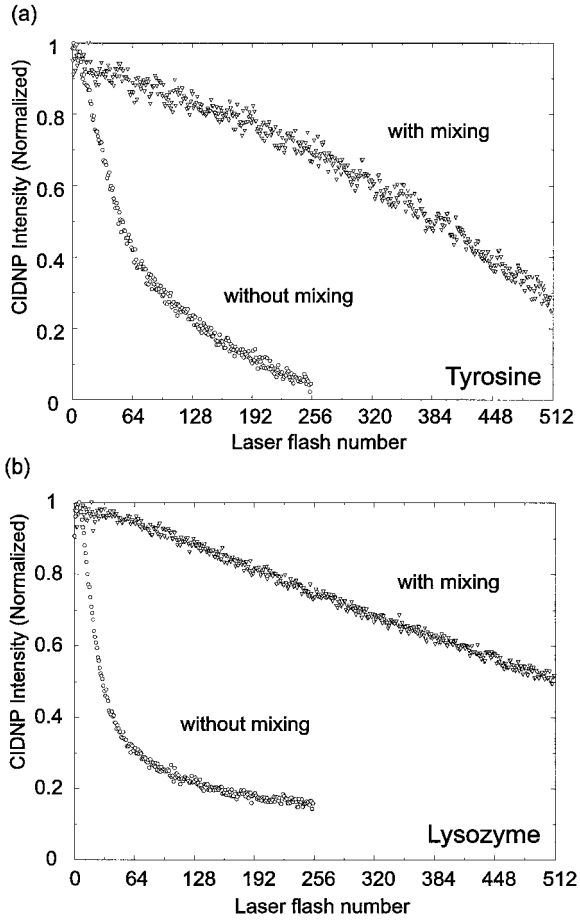

Figure 3. (a) CIDNP intensities of the H3,5 protons of tyrosine (4 mM) with FMN (0.2 mM) in D_2O at pH 4.5 with (triangles) and without (circles) mixing the NMR sample (as described in the text). (b) A similar experiment on hen lysozyme (1.5 mM, pH 5.2) in which the combined CIDNP intensity of the H2 and H4 protons of Trp-62 is plotted. Note the prolonged lifetime of the polarization for the mixed solutions.

The improvement in the CIDNP intensities after 128 laser flashes afforded by 10 mM H_2O_2 is summarized in Table 1 for tyrosine, tryptophan, histidine and lysozyme. The effect of the H_2O_2 on tryptophan is similar to that of tyrosine (Figure 6). For histidine, the increase in the enhancement is smaller for a 1 mM concentration of the amino acid than for a 50 mM sample, supporting the idea that degradation of the amino acid can also occur in some cases. For lysozyme, even at 0.7 mM, the presence of H_2O_2 gives a marked improvement, suggesting that photo-damage to side-chains in the protein is much less pronounced than it would be for the same concentration of a free amino acid.

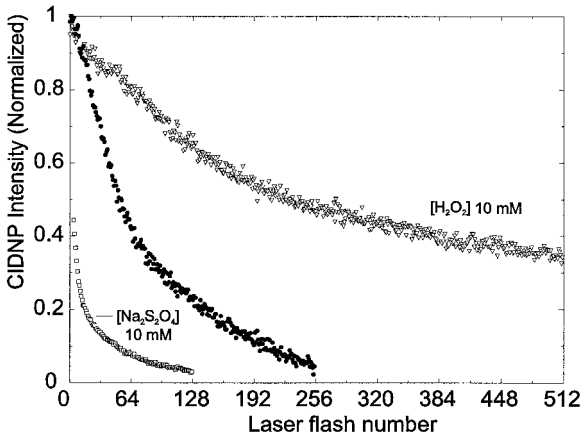

Figure 4. CIDNP intensities of the H3,5 protons of tyrosine (4 mM) with FMN (0.2 mM) in D_2O at pH 4.5 (circles), with 10 mM H_2O_2 (triangles), or with 10 mM sodium dithionite (squares).

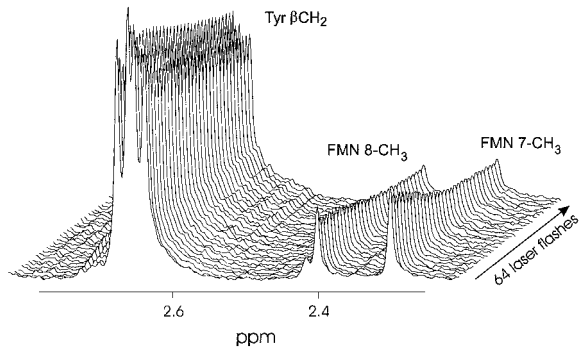

Figure 5. A series of NMR spectra recorded under the same conditions as Figure 2a except for the presence of 10 mM H_2O_2. The FMN methyl group resonances decay much less rapidly than in the absence of H_2O_2, reflecting the re-oxidation of reduced flavin by the H_2O_2.

Despite the encouraging results for lysozyme, there are obvious limitations to the use of a potent oxidant like peroxide. Exposed methionine residues are susceptible to oxidation (to sulphoxides), as are the thiol groups in reduced cysteine residues, especially in the presence of small quantities of metal ions as may come from the syringe hardware (Creighton, 1984). Nevertheless, if such irreversible damage occurs slowly, as is likely to be the case, it would probably not interfere significantly. CIDNP samples are routinely prepared immediately prior to use, subjected to a small number of relatively quick measurements ($\sim$1 h), and discarded afterwards. Clearly, control experiments, as reported above for lysozyme, are needed before using H_2O_2 in the way proposed here.

In the remainder of this paper we illustrate the potential benefits of adding H_2O_2 and of rapid mixing,

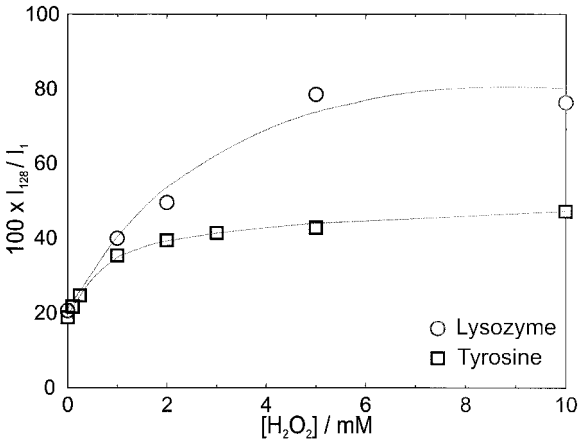

Figure 6. Effect of different concentrations of H_2O_2 on the loss of CIDNP intensity for 2 mM tyrosine (squares) and 1.5 mM hen lysozyme (circles). The vertical axis is the scaled CIDNP signal generated by the 128th laser pulse relative to that of the first, expressed as a percentage. The protection against FMN photoreduction reaches a plateau at ~10 mM H_2O_2, and is more effective for lysozyme than for tyrosine.

using spectra of holo- and apo-bovine α-lactalbumin, one of the first proteins to have been studied by photo-CIDNP (Berliner and Kaptein, 1980, 1981; Berliner et al., 1987; Improta et al., 1995a, b), and one whose structure and folding have been extensively studied by NMR (Alexandrescu et al., 1992; Balbach et al., 1995; Forge et al., 1999).

Two-dimensional CIDNP. Although 1D ^{1}H CIDNP spectra are generally much less crowded than their NMR counterparts, overlap often makes assignment difficult, especially if signals from several residues of the same type (histidine, tyrosine or tryptophan) are present. For this reason, Kaptein et al. have developed CIDNP versions of some of the simpler 2D NMR experiments (Scheek et al., 1984, 1985). Loss of flavin by photoreduction can present severe problems for such experiments in which each scan is preceded by a light flash. Even with rapid sample spinning between scans, the number of t_1 increments has hitherto been restricted to 64 or 128, with just one transient per increment, with no F_1 quadrature detection, and with a spectral width in F_1 just wide enough to cover the aromatic region of the ^{1}H spectrum (Scheek et al., 1984, 1985; Redfield et al., 1985; Stob et al., 1988).

Figure 7 shows parts of a 600 MHz CIDNP-NOESY spectrum of native bovine holo-α-lactalbumin, recorded using the sample mixing procedure described above, with 128 increments in t_1, two scans per t_1 value for quadrature detection in F_1, and a t_1 increment small enough (125 μs) to avoid folding in the F_1 dimension. The assignments are taken from previous NMR studies (Alexandrescu et al., 1992; Forge et al., 1999). The spectrum is particularly simple to interpret as only a few surface residues are directly polarized. It contains clearly resolved intra-residue cross peaks for histidine, tyrosine and tryptophan side-chains. There are also inter-residue NOEs between H6 of Trp-118 and both H2,6 and H3,5 of Phe-31; these protons are separated in the crystal structure by distances of 0.31 and 0.28 nm respectively within a hydrophobic cluster that also involves His-32 and Tyr-36 (Alexandrescu et al., 1992). These results show the ability of this type of experiment to provide information about the structural environment of the exposed residues.

Spectra measured without mixing had such large linewidths in F_1 that the cross peaks were almost or completely lost in the noise. Figure 8a shows two CIDNP-NOESY interferograms (at the chemical shift of the H2,6 protons of Tyr-18) recorded with and without mixing, but under otherwise identical conditions. The effect of mixing on the signal decay and the quality of the cross sections (Figure 8b) through the two-dimensional spectra is striking.

Real-time re-folding. We have recently demonstrated the use of photo-CIDNP in combination with a stopped-flow technique to monitor in real time the changes in side-chain accessibility that occur as a protein refolds (Hore et al., 1997). The time dependence of the refolding of hen lysozyme, essentially complete within a second, was studied in a series of 'one-shot' experiments in which folding was initiated by a pH jump together with rapid dilution of chemical denaturant. As each solution is used only once, to record a single CIDNP spectrum at a different time after the start of refolding, the issue of sample degradation does not arise. However, it is likely to be a significant problem when trying to record multiple spectra during the course of a somewhat slower reaction (as in a recent real time NMR study of the folding of α-lactalbumin in the absence of calcium ions (Balbach et al., 1995)).

As a preliminary demonstration of the value of the presence of H_2O_2 in a kinetic refolding experiment, we have studied the refolding of apo-α-lactalbumin. Figure 9 shows CIDNP spectra of apo-α-lactalbumin without (a) and with (b) 20 mM H_2O_2. In each case, the sum of the first four spectra (below) is compared with the sum of four spectra recorded after the sample had received 64 light flashes (above). The two initial spectra are essentially identical, showing that, as in

Table 1. The effect of added hydrogen peroxide on CIDNP intensities

	Tyrosine	Tryptophan	Histidine		Lysozyme
Concentration (mM)	4	4	1	50	0.7
$100\times I_{128}/I_1$ (no H_2O_2)	23	34	3	11	21
$100\times I_{128}/I_1$ (10 mM H_2O_2)	67	66	5	55	76

The entries in the table are experimental values of the CIDNP intensity for the 128th laser pulse relative to that for the first, expressed as a percentage.

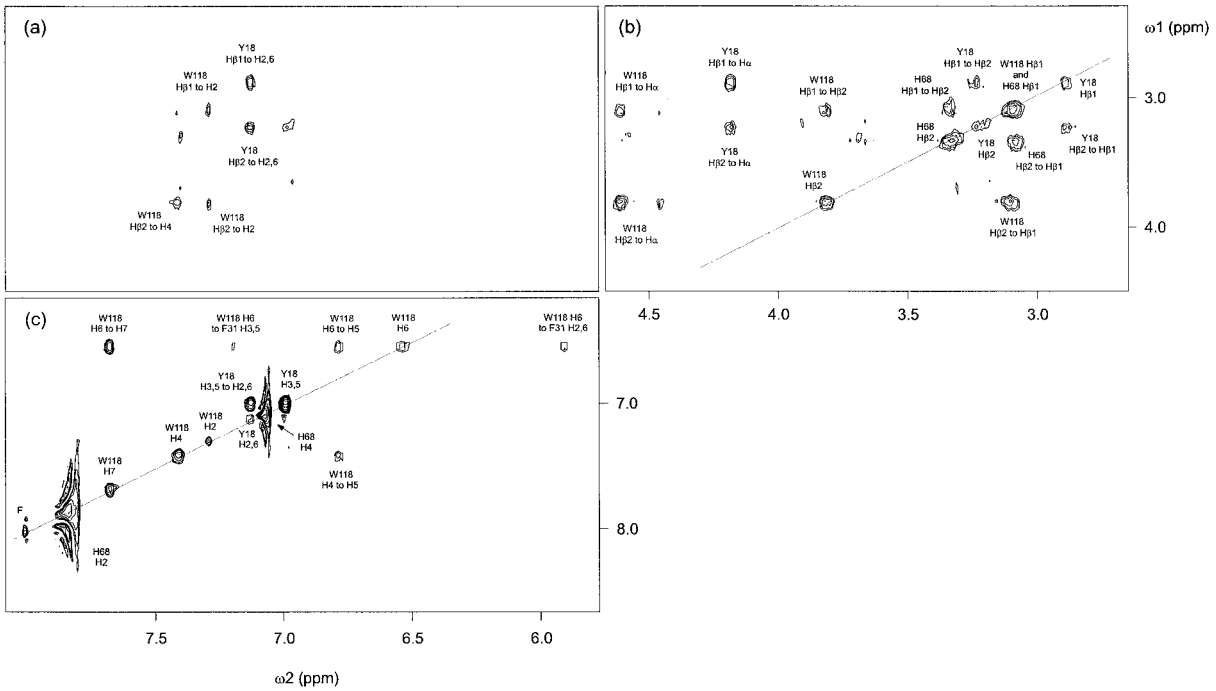

Figure 7. (a–c) Portions of a 600 MHz CIDNP-NOESY spectrum of 1.5 mM bovine holo-α-lactalbumin (0.2 mM FMN, in D_2O at pH 7.4, 40 °C) recorded with the sample mixing technique described in the text. The lack of symmetry about the diagonal reflects the non-uniform polarization of the aromatic and β-CH_2 protons in each histidine, tryptophan and tyrosine residue of the protein. Each of the 256 repetitions of the pulse sequence was preceded by a 100 ms, 4 W light flash. No attempt has been made to distinguish absorptive and emissive polarizations in this figure.

the case of lysozyme, the peroxide has a negligible effect on the CIDNP. The final spectra in the two cases have significantly different amplitudes, reflecting the photo-degradation of the flavin in the absence of the H_2O_2. The two spectra in the presence of H_2O_2 have similar relative intensities for the tyrosine, tryptophan and histidine signals, supporting once again the idea that flavin photo-reduction is responsible for the loss of signal, rather than chemical modification of reactive side-chains.

Figure 10a shows selected CIDNP spectra from a series recorded at 7 s intervals after 14-fold dilution of apo-α-lactalbumin in 6 M guanidinium chloride by rapid injection into a refolding buffer containing 10 mM H_2O_2. For comparison, Figures 10b–d show the CIDNP spectra of the native state (b), the low pH 'molten globule' A-state (c), and the unfolded state in 6 M guanidinium chloride (d). As expected, the final transient spectrum in (a) is very similar to the spectrum of fully native apo-α-lactalbumin (b) (except that the histidine resonances are stronger relative to those of Tyr-18 and Trp-118, and slightly shifted, both probably as a result of small pH differences between the two solutions).

The first transient spectrum bears a strong resemblance to the CIDNP spectrum of the A-state of the protein at low pH. This is consistent with the idea that a rapid collapse of the polypeptide chain from the

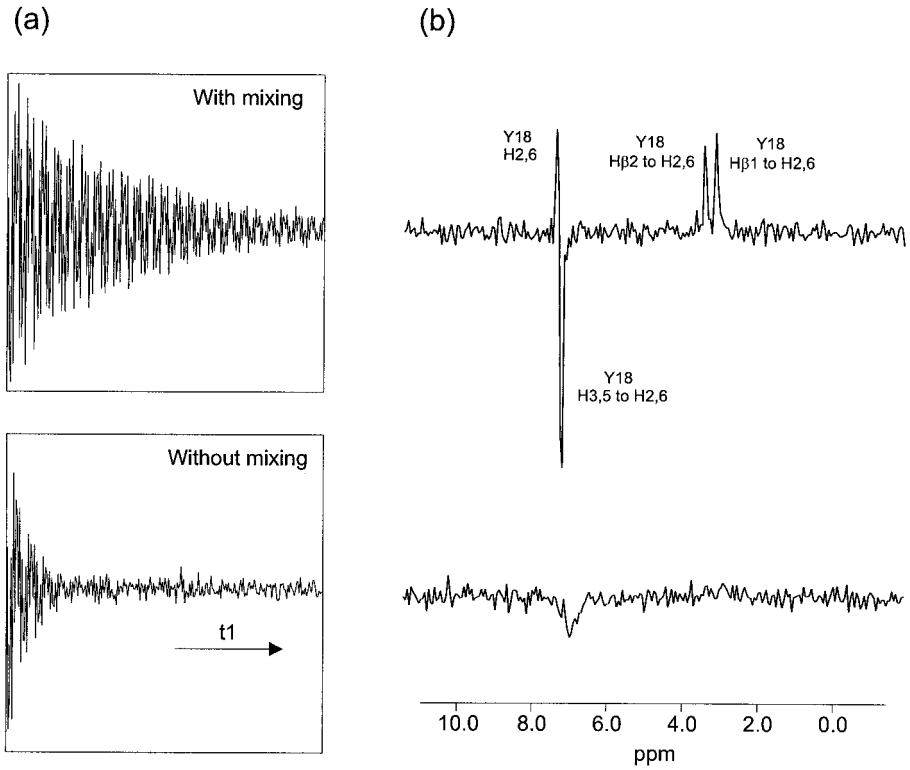

Figure 8. (a) CIDNP-NOESY interferograms at the chemical shift position of the H2,6 protons of Tyr-18 in bovine holo-α-lactalbumin with and without the sample mixing technique described in the text. (b) The corresponding cross sections showing the diagonal peak of Tyr-18 H2,6 and cross peaks arising from polarization transfer to H2,6 from H3,5 and the βCH_2 protons of the same residue.

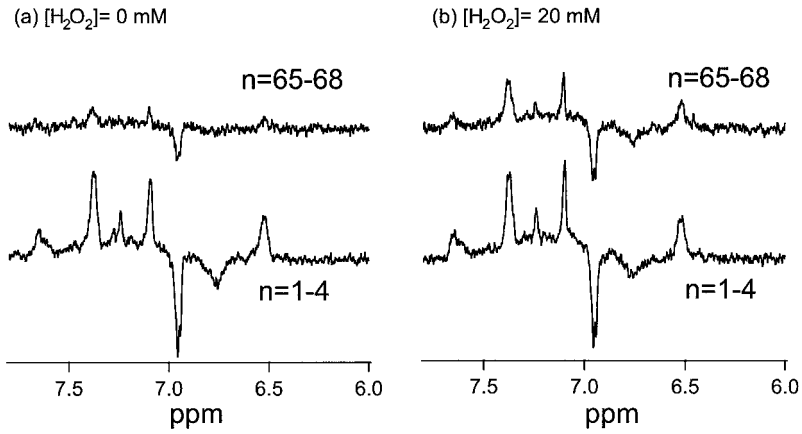

Figure 9. 600 MHz ^1H photo-CIDNP spectra of bovine apo-α-lactalbumin (0.9 mM, 0.2 mM FMN, in D_2O at pH 7.0) without (a) and with (b) 20 mM H_2O_2. In each case, the lower spectrum is the sum of the signals for the first four light flashes; the upper spectrum corresponds to flashes 65–68.

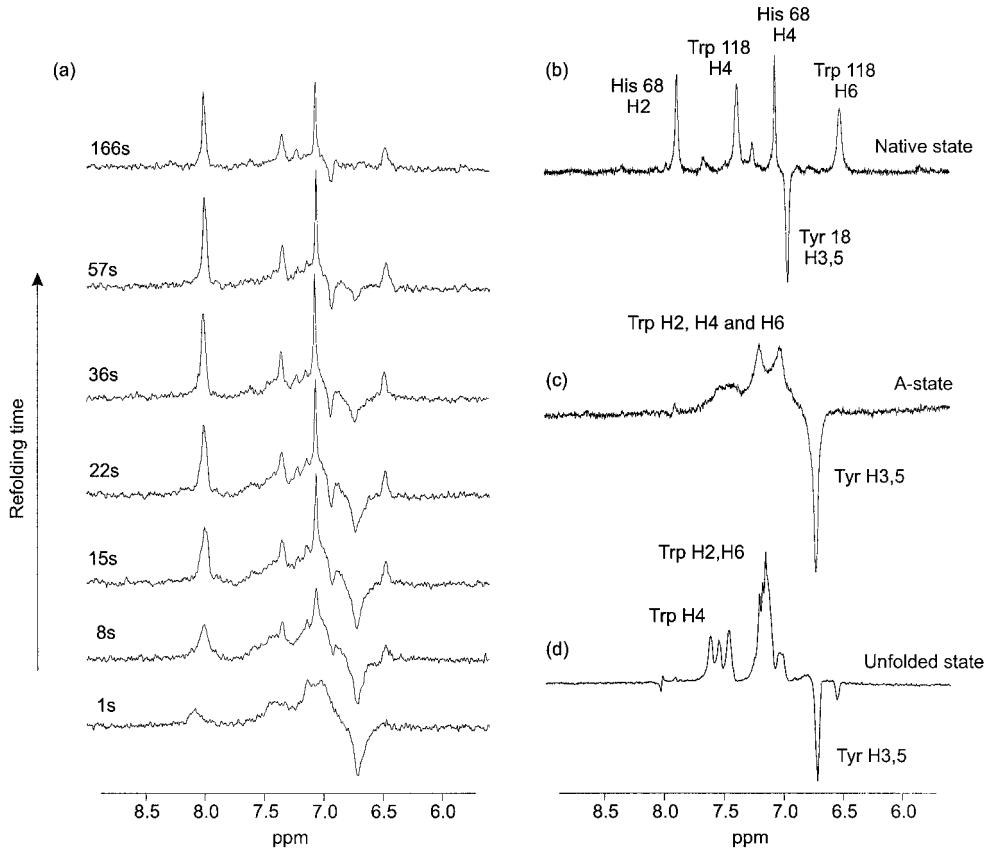

Figure 10. (a) Real-time 600 MHz photo-CIDNP spectra of the refolding of bovine α-lactalbumin following the injection of 40 μl of 7 mM apo-protein in 6 M guanidinium chloride into 510 μl of 200 mM cacodylic acid buffer at pH 7.2 with 10 mM H_2O_2. The final guanidinium chloride concentration was 0.44 M. Each spectrum is the result of a single laser pulse at 7 s intervals during the refolding reaction. CIDNP spectra of (b) native (0.44 M guanidinium chloride, pH 7.2), (c) A-state (no guanidinium chloride, pH 2), and (d) unfolded (6 M guanidinium chloride, pH 7.2) states of α-lactalbumin.

highly unfolded (U) state present in 6 M guanidinium chloride to a compact molten globule state occurs in a time much less than the dead-time of the measurement (∼100 ms) (Forge et al., 1999). The partial burial of hydrophobic side-chains that this would entail is in agreement with the tryptophan:tyrosine intensity ratio in the early transient spectra being smaller than in the spectrum of the U-state (d). The later spectra in Figure 10a reflect the structural reorganisation that takes place during the refolding process. Hydrophobic groups (e.g. tryptophan side-chains) that are partially buried in the A-state become exposed for functional reasons as the N-state is formed. At the same time, tyrosine side-chains that are accessible to flavin in the A-state are incorporated into the close-packed native structure. Similar conclusions have been drawn from a real-time photo-CIDNP investigation of hen lysozyme (Hore et al., 1997; Dobson and Hore, 1998) and demonstrate the potential of NMR studies in attempts to describe protein folding at a molecular level.

Conclusions

We have shown that the problem of photodegradation in photo-CIDNP experiments on proteins can be substantially reduced by mixing the sample using a rapid extraction/injection technique, or by addition of low concentrations of hydrogen peroxide as an oxidising agent. Both approaches have the potential to extend the range of systems that can be studied and the type of experiments that can be performed. The ability to record many free induction decays from a single sample will be valuable in applications that demand extensive signal averaging to obtain satisfactory signal-to-noise, where repeated signal acquisitions are needed e.g. in 2D or real-time measurements, or where

several spectra under different conditions are required from a precious sample.

Acknowledgements

We are pleased to acknowledge support from the Engineering and Physical Sciences Research Council, the Biotechnology and Biological Sciences Research Council, and the Medical Research Council through the Oxford Centre for Molecular Sciences. Further support was provided by INTAS (Project 96-1269). We are grateful to Dr. D. Hamada, Dr. J.A. Jones, Dr. C. Redfield and Mr. C. Jones for valuable advice and assistance. K.M. was supported by Grants-in-Aid for Scientific Research 70229300 and 11740304 while in Oxford as an Overseas Researcher of the Japanese Ministry of Education. The research of C.M.D. is supported in part by the Howard Hughes Medical Institute and the Wellcome Trust.

References

Alexandrescu, A.T., Broadhurst, R.W., Wormald, C., Chyan, C.L., Baum, J. and Dobson, C.M. (1992) *Eur. J. Biochem.*, **210**, 699–709.

Balbach, J., Forge, V., van Nuland, N.A.J., Winder, S.L., Hore, P.J. and Dobson, C.M. (1995) *Nat. Struct. Biol.*, **2**, 865–870.

Berliner, L.J. and Kaptein, R. (1980) *J. Biol. Chem.*, **255**, 3261–3262.

Berliner, L.J. and Kaptein, R. (1981) *Biochemistry*, **20**, 799–807.

Berliner, L.J., Koga, K., Nishikawa, H. and Scheffler, J.E. (1987) *Biochemistry*, **26**, 5769–5774.

Connolly, P.J. and Hoch, J.C. (1991) *J. Magn. Reson.*, **95**, 165–173.

Creighton, T.E. (1984) *Proteins. Structures and Molecular Properties*, W.H. Freeman, New York, NY, pp. 17–20.

Dobson, C.M. and Hore, P.J. (1998) *Nat. Struct. Biol.*, **5**, 504–507.

Forge, V., Wijesinha, R.T., Balbach, J., Brew, K., Robinson, C.V., Redfield, C. and Dobson, C.M. (1999) *J. Mol. Biol.*, **288**, 673–688.

Heelis, P.F. (1991) In *Chemistry and Biochemistry of Flavoenzymes*, Vol. 1 (Ed., Müller, F.), CRC Press, Boca Raton, FL, pp. 171–193.

Hincke, M.T., Sykes, B.D. and Kay, C.M. (1981) *Biochemistry*, **20**, 4185–4193.

Hore, P.J. and Broadhurst, R.W. (1993) *Progr. NMR Spectrosc.*, **25**, 345–402.

Hore, P.J., Winder, S.L., Roberts, C.H. and Dobson, C.M. (1997) *J. Am. Chem. Soc.*, **119**, 5049–5050.

Hore, P.J., Zuiderweg, E.R.P., Kaptein, R. and Dijkstra, K. (1981) *Chem. Phys. Lett.*, **83**, 376–383.

Improta, S., Molinari, H., Pastore, A., Consonni, R. and Zetta, L. (1995a) *Eur. J. Biochem.*, **227**, 78–86.

Improta, S., Molinari, H., Pastore, A., Consonni, R. and Zetta, L. (1995b) *Eur. J. Biochem.*, **227**, 87–96.

Kaptein, R. (1978) In *NMR Spectroscopy in Molecular Biology* (Ed., Pullman, B.), D. Reidel, Dordrecht, pp. 211–229.

Kaptein, R. (1982) *Biol. Magn. Reson.*, **4**, 145–191.

Kaptein, R., Dijkstra, K. and Nicolay, K. (1978) *Nature*, **274**, 293–294.

Lyon, C.E., Jones, J.A., Redfield, C., Dobson, C.M. and Hore, P.J. (1999) *J. Am. Chem. Soc.*, **121**, 6505–6506.

McCord, E.F., Bucks, R.R. and Boxer, S.G. (1981) *Biochemistry*, **20**, 2880–2888.

Müller, F. (1987) *Free Radical Biol. Med.*, **3**, 215–230.

Müller, F. (1991) In *Chemistry and Biochemistry of Flavoenzymes*, Vol. 1 (Ed., Müller, F.), CRC Press, Boca Raton, FL, pp. 1–71.

Muus, L.T., Atkins, P.W., McLauchlan, K.A. and Pedersen, J.B. (1977) *Chemically Induced Magnetic Polarization*, D. Reidel, Dordrecht.

Redfield, C., Dobson, C.M., Scheek, R.M., Stob, S. and Kaptein, R. (1985) *FEBS Lett.*, **185**, 248–252.

Salikhov, K.M., Molin, Yu. N., Sagdeev, R.Z. and Buchachencko, A.L. (1984) *Spin Polarization and Magnetic Effects in Radical Reactions*, Elsevier, Amsterdam.

Scheek, R.M., Stob, S., Boelens, R., Dijkstra, K. and Kaptein, R. (1984) *Faraday Discuss. Chem. Soc.*, **78**, 245–256.

Scheek, R.M., Stob, S., Boelens, R., Dijkstra, K. and Kaptein, R. (1985) *J. Am. Chem. Soc.*, **107**, 705–706.

Scheffler, J.E., Cottrell, C.E. and Berliner, L.J. (1985) *J. Magn. Reson.*, **63**, 199–201.

Stob, S., Scheek, R.M., Boelens, R., Dijkstra, K. and Kaptein, R. (1988) *Isr. J. Chem.*, **28**, 319–327.

van Nuland, N.A.J., Forge, V., Balbach, J. and Dobson, C.M. (1998) *Acc. Chem. Res.*, **31**, 773–780.

Winder, S.L. (1997) Ph.D. Thesis, Oxford University.