Two-Dimensional $^{15}N-^{1}H$ Photo-CIDNP as a Surface Probe of Native and Partially Structured Proteins

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There has been considerable interest recently in using NMR to investigate the structures of partially folded proteins because of their relationship to kinetic intermediates in protein folding and aggregation.[1] These studies are hampered by low spectral resolution arising from the conformational heterogeneity and complex dynamical nature of such species. An NMR method with the potential to avoid some of these difficulties is photo-CIDNP (chemically induced dynamic nuclear polarization) in which a photochemical reaction perturbs NMR intensities and so probes the solvent-exposure of aromatic amino acid side chains.[2] Despite the selectivity of the technique, and the availability of two-dimensional (2D) variants,[3] the limited spectral resolution of ^{1}H NMR is still a severe drawback.[4] Here we demonstrate that ^{15}N CIDNP, in particular when combined with the 2D $^{15}N-^{1}H$ HSQC technique,[5] has the resolution and sensitivity to study denatured states of proteins, and should also be capable of extension to stopped-flow investigations of protein folding.[6−9]

The photo-CIDNP technique uses laser-induced photochemistry to enhance the ^{1}H NMR signals of histidine, tryptophan, and tyrosine side chains. A photoexcited dye, usually a flavin, reacts with accessible aromatic groups to generate short-lived radical pairs whose recombination probability depends on the spin configuration of nuclei with significant hyperfine interactions. The result is absorptive or emissive nuclear polarization that can be an order of magnitude larger than that at thermal equilibrium.

Reported photo-CIDNP experiments on proteins appear to have been restricted to ^{1}H NMR, presumably for reasons of sensitivity and convenience. In principle there is no obstacle to looking at other nuclides, in particular ^{13}C and ^{15}N, not least because of the availability of isotopically enriched proteins for NMR sequential assignment and structure determination. ^{15}N CIDNP is attractive for several reasons. Spin density calculations for the tryptophan cation radical (formed by electron transfer to the photoexcited triplet state of flavins) indicate a significant hyperfine interaction for the indole nitrogen, N1, and its directly bonded proton, H1.[10] An absorptive enhancement of H1 has been observed in both H_2O and dimethylsulfoxide.[11] Indirect detection of ^{15}N CIDNP by polarization transfer to H1 would give a single resonance from each tryptophan and none from tyrosine or histidine (imidazole NH protons of exposed histidines normally exchange too rapidly to be observed). Such spectra would be substantially less crowded than the aromatic region of ^{1}H CIDNP spectra which contain three to five multiplets from each exposed tryptophan, and two from each tyrosine and histidine. A further improvement in resolution should be possible using 2D heteronuclear correlation techniques.

Exploratory experiments[12] were carried out on ^{15}N-enriched $^{15}N_2$-L-tryptophan. The existence of spin density on N1 in the $Trp^{\bullet+}$ radical, and the possibility of N↔H coherence transfer were confirmed in three ways: by direct detection of the H1 enhancement; by transferring the H1 polarization to N1 and back (H→N→H); and by observing the N1 CIDNP by transfer to H1 (N→H). The enhancements in NMR intensity in the "light" spectrum relative to the identical experiment without laser irradiation ("dark") were 6.5 ± 0.3, 6.0 ± 0.3, and 100 ± 15, respectively.[13] The much larger enhancement in the N→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 N→H experiment had the greatest signal-to-noise ratio (by a factor of 2) suggesting that the N1 hyperfine coupling constant in $Trp^{\bullet+}$ is larger than that of H1. A similar sensitivity improvement can be expected for tryptophan residues in proteins where the hyperfine interactions should be little changed.

Hen lysozyme was chosen to illustrate the potential of the method for studying proteins. In the native state just two of the six tryptophan residues—Trp-62 and to a lesser extent Trp-123—can be polarized by flavins,[14] reflecting their accessibility. By contrast, the denatured states in aqueous solution, produced thermally or by addition of urea or by reduction of the four disulfide bonds, were found to have, on average, polarization corresponding to just one exposed tryptophan (in addition to strong enhancements for some or all of the three tyrosines).[4] However the ^{1}H spectral resolution and sensitivity are insufficient to determine whether this arises from a single completely accessible residue or from a number of partially exposed residues.

Figure 1 shows the indole N−H region of two $^{15}N-^{1}H$ HSQC spectra of native hen lysozyme. The H→N→H dark spectrum

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(12) CIDNP experiments were performed on a home-built 600 MHz NMR spectrometer of the Oxford Centre for Molecular Sciences. Light from a continuous wave argon ion laser (principal wavelengths 488 and 514 nm) was introduced into the nonspinning 5 mm NMR tube by means of a 1 mm diameter optical fibre, the end of which was positioned at the bottom of a Wilmad coaxial Pyrex insert (catalogue no. WGS 5BL), 4 mm above the top of the NMR coil (Scheffler, J. E.; Cottrell, C. E.; Berliner, L. J. *J. Magn. Reson.* **1985**, *63*, 199−201). With this arrangement no modifications to the NMR probe were needed. All spectra were recorded using 0.2 mM flavin mononucleotide. The solvent was 95% H_2O/5% D_2O.
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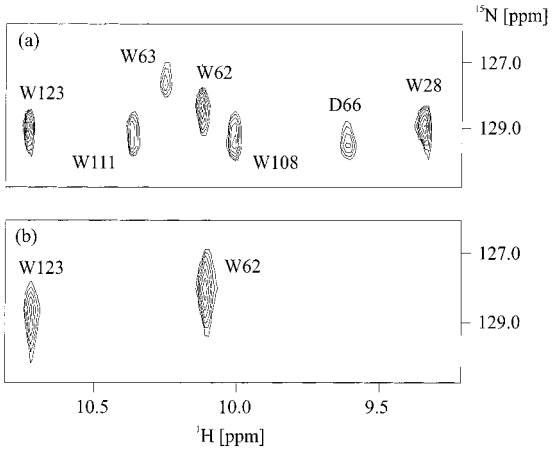

Figure 1. Indole NH region of 600 MHz 2D gradient-enhanced ^{15}N−^{1}H HSQC spectra[5] of ^{15}N-labeled native hen lysozyme (1.5 mM, pH 5.2, 40 °C) consisting of 32 complex points in t_1, and spectral widths of 2500 Hz and 8000 Hz in the ^{15}N (f_1) and ^{1}H (f_2) dimensions, respectively. (a) Dark H→N→H spectrum (without laser irradiation) with four scans per t_1 increment. The assignments were taken from ref 15; the seventh peak is the amide NH of Asp-66. (b) Light N→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→N polarization transfer and so detects the ^{15}N CIDNP.

(a) has resonances from all six tryptophans, while the N→H light spectrum (b) displays just two peaks above the noise; as expected from the ^{1}H spectra, these are Trp-62 and Trp-123.[14] The enhancements in the (N→H) light spectrum relative to (H→N→H) signals detected in the dark,[16] 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. Detection of ^{15}N rather than ^{1}H CIDNP has the advantage that the N→H dark spectrum is so weak (less than 1% of the light spectrum) that subtraction to remove unpolarized peaks, with its $\sqrt{2}$ signal-to-noise penalty, is unnecessary.

Figure 2 shows the same experiments for lysozyme denatured in 10 M urea at pH 3.6 and 45 °C. The dark (H→N→H) spectrum (a) shows four resolved indole NH 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. As expected for this largely unstructured state, the chemical shift resolution, particularly in the ^{1}H dimension, is greatly reduced in contrast to the native state where the ^{1}H resolution alone is sufficient to distinguish the six resonances. In the light (N→H) spectrum[17] (b), all of the tryptophans are polarized with approximate enhancements[16]

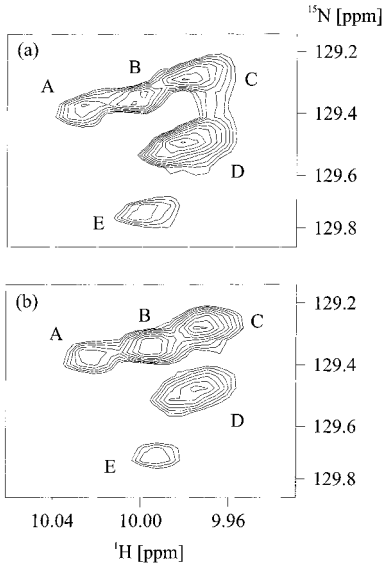

Figure 2. Indole NH region of 600 MHz 2D gradient-enhanced ^{15}N−^{1}H HSQC spectra of ^{15}N-labeled hen lysozyme (1.0 mM, pH 3.6, 45 °C) denatured with 10 M urea, consisting of 32 complex points in t_1, and spectral widths of 156 and 7017 Hz in the ^{15}N (f_1) and ^{1}H (f_2) dimensions, respectively. (a) Dark H→N→H spectrum with four scans per t_1 increment. (b) Light N→H spectrum obtained as described in the caption to Figure 1, with *one* scan per t_1 increment, in the presence of 10 mM H_2O_2.[17]

(defined as above) of 1.4, 2.0, 1.9, 0.9, 1.3 for resonances A−E, implying partial exposure of all six side chains and varying degrees of involvement in local regions of structure. This conclusion is consistent with NMR studies in which the most pronounced deviations from random coil predictions are found in regions of the protein involving aromatic residues, and the tryptophans in particular.[18]

A number of applications of ^{15}N−^{1}H CIDNP can be envisaged. The 1D indirect detection of ^{15}N polarizations would lend itself well to stopped-flow studies of protein folding as recently demonstrated for ^{1}H CIDNP using lysozyme.[8] In this experiment, rapid dilution of a denatured protein into a refolding medium allows the time dependence of side chain accessibility to be monitored on a 10−100 ms time scale (limited at present by the duration of the laser pulse, and the time required for mixing the solutions). The advantage of ^{15}N over ^{1}H would be that one could follow the kinetics of *individual* tryptophans as the molecule regained its native conformation. For more slowly folding proteins (∼30 min), it should be possible to record a complete 2D CIDNP heteronuclear correlation spectrum as refolding takes place, to give insight into changes in side chain exposure, in addition to the kinetic information available from the line shapes.[7]

The present results suggest that other heteronuclear CIDNP experiments could be viable. It may be possible to detect ^{15}N polarization of histidines via the small spin−spin coupling to the aromatic CH protons. Moreover, stopped-flow NMR experiments on ^{19}F-tryptophan labeled proteins[9] could be adapted and extended to other amino acid residues; for example both 3-^{19}F-tyrosine[19] and 6-^{19}F-tryptophan[20] show ^{19}F CIDNP.

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(16) The ^{15}N polarizations in the light (N→H) spectrum relative to those in the corresponding (N→H) dark spectrum would be larger by a factor of $\sim\gamma(^{1}\mathrm{H})/\gamma(^{15}\mathrm{N}) \cong 10$.

(17) To combat the problem of progressive signal attenuation caused by the 64 laser pulses (two per t_1 increment) needed to record the light spectrum (an effect visible in Figure 1b as an increased line width in the ^{15}N dimension), Figure 2b was recorded in the presence of 10 mM hydrogen peroxide. This addition was found to prolong the lifetime of the sample, probably by reoxidizing reduced flavin, without otherwise affecting the NMR, CIDNP, or circular dichroism spectra (K. Maeda, unpublished). Experimental conditions were varied to optimise the ^{15}N polarizations, especially for denatured lysozyme where the signals are inherently weak. Optimal FMN and protein concentrations, for both native and denatured states, were, respectively, 0.2 mM and 1.0−1.5 mM. Experiments using lower pH (∼2) and urea concentrations (∼8 M) and lower temperatures (∼20 °C) to denature lysozyme gave much weaker polarizations.

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