Nitrogen-15 Detection of Broad Amide Protons in Paramagnetic Proteins

Maruse Sadek,*[a] Sergio D. B. Scrofani,[b] Robert T. C. Brownlee[a] and Anthony G. Wedd[b]

[a] *School of Chemistry, La Trobe University, Bundoora 3083, Australia*
[b] *School of Chemistry, University of Melbourne, Parkville, Victoria 3052, Australia*

Nitrogen-15 detected NMR experiments allow identification of the broadened ^{1}H resonances of amide hydrogens adjacent to paramagnetic centres in an electron transfer protein, the 2[Fe_4S_4] ferredoxin from *Clostridium pasteurianum*.

A major difficulty in the study of paramagnetic proteins by NMR methods is the observation of ^{1}H resonances associated with the hydrogen atoms located close to the paramagnetic active sites.[1] Identification of these protons is important to establish secondary and tertiary structure and to address mechanism. Interesting examples include the 2[Fe_4S_4] ferredoxins from *Clostridium pasteurianum* (*Cp*Fd) and other organisms: *each* cluster features up to eight NH···S hydrogen bonds between backbone NH functions and cluster thio and cysteinyl sulfur atoms (Fig. 1).[2]

These interactions, together with protein folding and access of solvent water, appear to modulate redox potentials, the electronic state of the iron–sulfur clusters and the mechanism of electron transfer.[2,3] However, the relevant protons relax efficiently *via* nuclear spin–electron spin interactions, resulting in broad (*ca.* 100 Hz) resonance lines which are very difficult to observe when overlapped with narrow resonances arising from protons not affected by the paramagnetic relaxation. The problems of overlap are solved normally by heteronuclear experiments which offer better dispersion of chemical shifts. Methods utilising proton detection are favoured because of superior sensitivity.[4] However, these methods generally give poor results for protons with short relaxation times. This communication reports that ^{15}N-detected NH-correlation experiments are able to identify such protons.

An important contributing factor to the relaxation times[1] is the magnetogyric ratio γ and so ^{15}N resonances are inherently narrower than ^{1}H resonances. In fact, *all* 61 possible ^{15}N resonances of ^{15}N labelled *Cp*Fd† (Fig. 2) can be detected in a ^{1}H-decoupled spectrum. In addition to side-chain (3 Asn, 2 Gln, 1 Lys) and N-terminus resonances, the 51 backbone secondary amide and the three Pro tertiary amide nitrogens are observed.

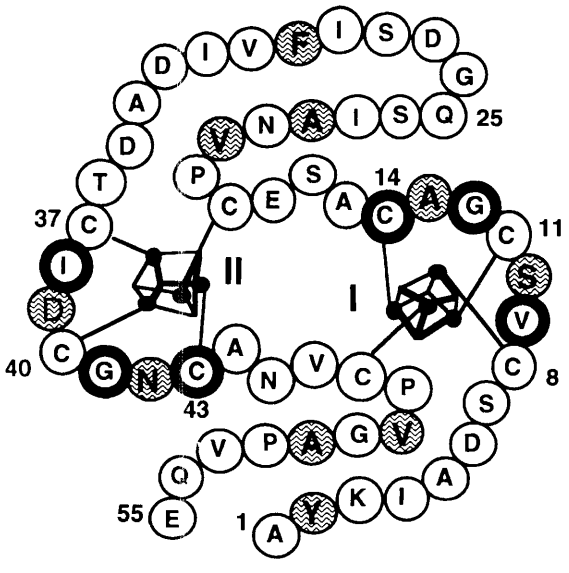

Fig. 1 Simple two-dimensional representaton of *Cp*Fd (M = 6203; 55 amino acids; $[Fe_4S_4(cys)_4]^{2-}$ centres; $\mu_{eff} \approx 2\ \mu_B$ per cluster). The amino acid residues likely to be involved in NH···S hydrogen bonding with (*i*) cluster sulfides are outlined; (*ii*) with cysteinyl S$^\gamma$ atoms are shaded. Single-letter amino acid residue notation is used for clarity.

On the other hand, only 41 of the possible 51 backbone amide protons have been identified in ^{1}H spectra, despite study under a range of conditions.[5,6] In particular, many of the protons likely to be involved[2] in the NH···S interactions (Fig. 1) appear to be missing.

In diamagnetic proteins, the expected ^{15}N chemical shift ranges are δ 105–125 for primary and secondary amides and δ 120–130 for proline residues.[7] Fig. 2 shows that many ^{15}N resonances in *Cp*Fd are shifted downfield from δ 130 and as far as δ 168. Those from the three Pro residues show significant downfield shifts in the range δ 137–153 and are easily identified by the absence of proton coupling (resonances 3, 4 and 11 in Fig. 2).

*Cp*Fd and related proteins exhibit a C_2 rotational pseudo-symmetry, which is reflected in structural and NMR properties.[2,8,9] In particular, the ^{1}H and ^{13}C chemical shifts and relaxation times associated with the eight cysteinyl ligands (four to each cluster) occur in pairs.[9] The ^{15}N resonance pair (3,4) (Fig. 2) is assigned to the pseudo-symmetry related prolines 19 and 48 (Fig. 2), allowing the remaining proline residue, Pro 52, to be assigned to ^{15}N resonance 11. The eight other ^{15}N resonances in the region δ 140–170 region also pair according to their NMR properties, (1,2), (5,7), (6,8) and (9,10), and are expected to be associated with the four symmetry-related pairs of amino acid residues. Backbone NH's of a number of residues, including the cysteinyl ligands, are shown to be hydrogen bonded to the thio and Cys S$^\gamma$ atoms of the clusters[2] and are thus expected to be affected by hyperfine interactions.[10] Detection of the proton resonances associated with these nitrogens is important to illuminate the role of NH···S hydrogen bonds on the characteristics of the active sites and on the protein as a whole.

Proton-detected HMQC and HSQC experiments have been applied widely,[4] but while providing valuable information,‡

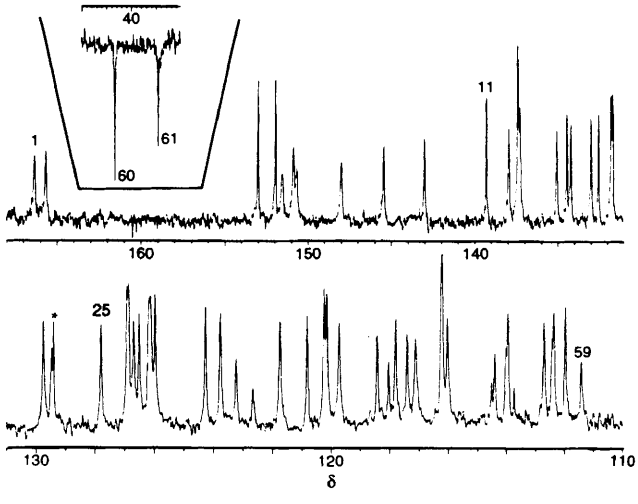

Fig. 2 ^{15}N NMR spectrum of *Cp*Fd (5 mmol dm^{-3}, in 50 mmol dm^{-3} phosphate buffer, $H_2O:D_2O$ = 90:10 (v:v), pH 7.0, 5 mm sample tube, 303 K) obtained on a Bruker AM-400 spectrometer with 10 mm broad band probe tuned to 40.55 MHz and using gated proton decoupling. * Denotes an electronic spike. The ^{15}N chemical shifts were referenced indirectly to $^{15}NH_4Cl$ (δ 24.93, 2.9 mol dm^{-3}).

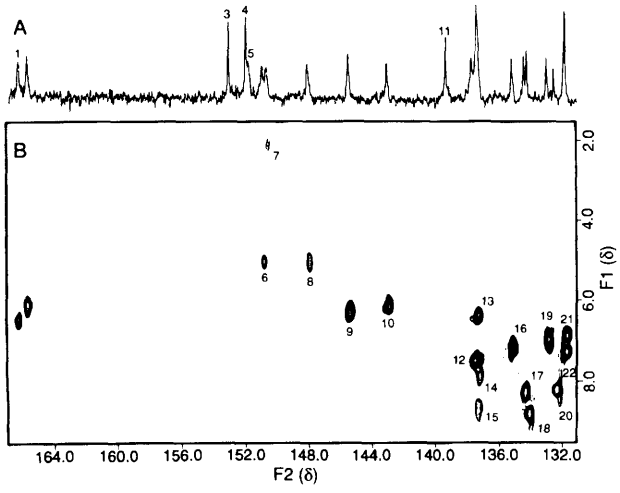

Fig. 3 Hyperfine shifted amide resonance region of ^{15}N detected two-dimensional INEPT spectrum§ of *Cp*Fd [4 mmol dm^{-3}, in 50 mmol dm^{-3} phosphate buffer, $H_2O:D_2O$ = 90:10 (v:v), pH 6.2, 5 mm sample tube, 303 K]

were unable to detect many of the protons of interest in *Cp*Fd. However, a proton-coupled INEPT experiment[4] indicates that, although all {^1H–^{15}N} couplings are *ca.* 80–95 Hz, some nitrogen resonances broaden to 30–40 Hz when proton decoupling is not applied during the acquisition. Presumably, the predominant relaxation mechanism of these nitrogens is a scalar relaxation of a second kind[11] through the efficiently relaxing protons bound to them. Such influences is removed by a proton decoupling which reduces the nitrogen linewidth to usual 2–8 Hz. This suggests that the classic ^{15}N-detected experiments may be superior to the modern inverse experiments for the detection of broadened ^1H resonances.

The hyperfine-shifted region of a ^{15}N-detected two-dimensional INEPT experiment§ is presented in Fig. 3. All crosspeaks are present, except one corresponding to ^{15}N resonance 5. They are of variable intensity but are all relatively sharp. The ^1H dimension shows that some of the proton resonances fall outside the normal range of chemical shifts of amide protons (δ 6–10). In particular, the proton resonance associated with resonance 7 exhibits an unusually high-field chemical shift of δ 2.2 and a very short T_2/T_1 relaxation time, deduced from the weak intensity of its {^{15}N–^1H} crosspeak. There would be no possibility of identifying amide proton resonances in the region δ 2–3 of a protein spectrum by homonuclear two-dimensional ^1H experiments due to spectral overlap with aliphatic resonances. The proton resonances at δ 5.0 and 5.1 associated with the ^{15}N pair (6,8) also appear at higher field than usual. Except for (5), *all* crosspeaks are clearly identified in this *single* experiment. The ^{15}N resonances 51, 52, 54, 56, 57 were identified as those corresponding to $CONH_2$ side-chains.

The present results clearly demonstrate that ^{15}N-detected INEPT experiments are superior in identification of fast-relaxing amide protons in paramagnetic proteins. In *Cp*Fd, both ^{15}N and ^1H chemical shifts of a number of amides fall outside the usual regions. Their assignment, an understanding of the basis of the shifts and their variation in mutant proteins will provide insight into the functioning of this electron transfer protein. A detailed assignment of the nitrogen and proton resonances will be presented elsewhere.

The authors acknowledge the Australian Research Council for financial support.

Received, 20th September 1994; Com. 4/05739B

Footnotes

† ^{15}N labelled protein was isolated from bacteria grown on $(^{15}NH_4)_2SO_4$ as nitrogen nutrient.

‡ Proton-detected HMQC and HSQC experiments[4] were unsuccessful in detecting all NH crosspeaks. These experiments were conducted at both 400 and 500 MHz, with or without ^{15}N decoupling. The shortest possible versions of these experiments were employed to minimise the loss of signal intensity due to the efficient relaxation of nuclei. The same results were obtained for *Cp*Fd at pH 7.0 and 6.2. Apart from the amine and tertiary amide nitrogens, the crosspeaks from ^{15}N resonances 1,2,5–8,14,15 in the hyperfine-shifted region, and from ^{15}N resonances 24,28,30,31,36,46,50 in the diamagnetic region, were completely missing at pH 7.0. The change of pH from 7.0 to 6.2 results in resonance ^{15}N resonance 28 appearing as a broad crosspeak, and ^{15}N resonances 36,46,50 as sharp crosspeaks. Obviously the observation of the latter three is predominantly determined by the rate of proton exchange and not by efficient proton relaxation. The ^{15}N resonances 51,52,54,56,57 were confirmed as those corresponding to $CONH_2$ side-chains. The crosspeaks corresponding to the ^{15}N resonances 9,10 and 13,28 were broad and those associated with the ^{15}N resonances 12,19,41 were very broad at both pH values, but clearly present on spectra processed to enhance the resonances with short T_2/T_1 relaxation times.

§ The two-dimensional INEPT experiment was acquired on a Bruker AM-400 spectrometer with 10 mm broad-band probe tuned to 40.55 MHz using a 5 mm NMR tube. The ^{15}N and ^1H pulse widths were 21 μs. 2 K data points were acquired by Bruker sequential mode in the F2 dimension, and 300 datapoints (1024 transients each) in the F1 dimension using TPPI for quadrature detection. Spectral widths of 3086 Hz and 11 904 Hz were used in the F2 and F1 dimensions, respectively. The recycle time for each transient was about 1 s. The coupling related evolution delays were set to 1.5 ms to optimise the coherence transfer by consideration of both the magnitude of coupling, and the fast relaxation times of protons of interest. Composite pulse proton decoupling was applied during the acquisition. Data were processed using Felix NMR processing software running on Sparc2 workstation. Data in both dimensions were multiplied by 80° shifted square sine-bell window functions over the 300 data points in each dimension, zero-filled and Fourier transformed in both dimensions to produce a 1 K by 1 K real matrix. Linear baseline correction was applied before the second Fourier transform. The ^{15}N chemical shifts were referenced indirectly to $^{15}NH_4Cl$ (δ 24.93, 2.9 mol dm^{-3}) and ^1H chemical shifts were referenced to the residual water resonance measured indirectly with respect to DSS (δ 4.72, 303 K).

The two-dimensional INEPT spectrum presented was collected in 84 hours. An HMQC experiment obtained in 20 hours detected adequately the sharp resonances, but it was unable to detect any of the broad resonances, even if acquired over the same time as the two-dimensional INEPT experiment. The two-dimensional INEPT experiment has the additional advantage of not requiring water presaturation and this also greatly contributes to the quality of the data.

References

1 I. Bertini, P. Turano and A. J. Vila, *Chem. Rev.*, 1993, **93**, 2833.
2 G. Backes, Y. Mino, T. M. Loehr, T. E. Meyer, M. A. Cusanovich, W. V. Sweeney, E. T. Adman and J. Sanders-Loehr, *J. Am. Chem. Soc.*, 1991, **113**, 2055.
3 R. Langen, G. M. Jensen, U. Jacob, P. J. Stephens and A. Warshel, *J. Biol. Chem.*, 1992, **267**, 25625.
4 S. W. Homans, *A. Dictionary of Concepts in NMR*, 2nd edn., Clarendon Press, Oxford, 1992.
5 J. Gaillard, I. Quinkal and J.-M. Moulis, *Biochemistry*, 1993, **32**, 9881.
6 S. D. B. Scrofani, R. T. C. Brownlee, M. Sadek and A. G. Wedd, manuscript in preparation, 1994.
7 D. S. Wishart, B. D. Sykes and F. M. Richards, *J. Mol. Biol.*, 1991, **222**, 311.
8 I. Bertini, F. Capozzi, C. Luchinat, M. Piccioli and A. J. Vila, *J. Am. Chem. Soc.*, 1994, **116**, 651.
9 S. D. B. Scrofani, P. S. Brereton, A. M. Hamer, M. J. Lavery, S. G. McDowall, G. A. Vincent, R. T. C. Brownlee, N. J. Hoogenraad, M. Sadek and A. G. Wedd, *Biochemistry*, 1994, in the press.
10 Y. K. Chae, F. Abildgaard, E. S. Mooberry and J. L. Markley, *Biochemistry*, 1994, **33**, 3287.
11 T. C. Farrar and E. D. Becker, *Pulse and Fourier Transform NMR: Introduction to Theory and Methods*, Academic, New York, 1971.