J|A|C|S
COMMUNICATIONS

Published on Web 03/09/2002

# $^{13}C\{^{13}C\}$ 2D NMR: A Novel Strategy for the Study of Paramagnetic Proteins with Slow Electronic Relaxation Rates

Timothy E. Machonkin, William M. Westler, and John L. Markley*

*National Magnetic Resonance Facility at Madison, Department of Biochemistry, University of Wisconsin−Madison, Madison, Wisconsin 53706*

Received December 11, 2001

NMR is a powerful tool in the study of paramagnetic proteins.[1] Solution structures have been determined from NMR data for members of several classes of paramagnetic proteins (for examples, see ref 2). Hyperfine-shifted NMR signals, which report on electron−nuclear interactions, have proven useful for evaluating the electronic structures of paramagnetic centers. For example, in Fe−S proteins, hyperfine data have demonstrated valence trapping in plant [2Fe-2S] ferredoxins,[3] spin delocalization across H-bonds in rubredoxin,[4] and spin state equilibria in [4Fe-4S] proteins.[5]

Nuclei close to the paramagnetic center frequently relax too rapidly to be studied by conventional 2D and 3D NMR techniques. This prevents the observation of what usually is the most interesting part of the protein. Absent metrical information for the metal center and its surrounding region, some NMR investigators have resorted to modeling that region with approximations derived from X-ray crystallography (for example, ref 6). The problems are exacerbated in systems with slow electronic relaxation rates $\tau_s$, owing to the inverse correlation between nuclear and electronic relaxation. In such cases, the hyperfine-shifted resonances may be so broad and/or severely overlapped as to be unobservable.

Directly detected $^{13}C$ NMR offers advantages for the study of paramagnetic centers in proteins. Because the three contributions to paramagnetic relaxation (dipolar, Curie, and contact) are each proportional to $1/\gamma_N^2$, $^{13}C$ offers as much as a 16-fold improvement in line width compared to $^1H$.[7] This has been applied before in 1D $^{13}C$ and 1D $^{13}C\{^{15}N\}$ decoupling experiments on Fe−S proteins.[8] This indicates that $^{13}C\{^{13}C\}$ 2D NMR experiments should be superior to $^1H$-detected 2D experiments for observing and assigning paramagnetically broadened signals.

We have tested this approach with oxidized human [2Fe-2S] ferredoxin ($HuFd_{ox}$). The uninformative hyperfine-shifted region of the 1D $^1H$ NMR spectrum (not shown) consists of an unresolved envelope of signals at 20−50 ppm and a single peak at 13 ppm ($T_2^* \sim 0.8$ ms),[9] assigned respectively to the Cys $H^\beta$ and $H^\alpha$ resonances.[10] 3D NMR spectra, which yielded assignments for other parts of the molecule, failed to provide signals from 17 residues in the [2Fe-2S] cluster binding loops that apparently are affected by the paramagnetism.[11]

The $^1H$-decoupled$^{13}C\{^{13}C\}$ constant time COSY (CT-COSY)[12] approach investigated here offers several advantages: (1) in the indirect dimension, the signals are all in-phase absorptive and broadband homonuclear $^{13}C$-decoupled; (2) the constant time period $\tau_e$ can be shortened to allow optimal coherence transfer for fast-relaxing signals; and (3) the signal-to-noise is improved relative to other experiments such as DQ-COSY (INADEQUATE). Because one-bond $^{13}C-^{13}C$ scalar coupling constants fall in the relatively narrow range of 35−55 Hz, the use of constant time does not pose any difficulties.

Figure 1 shows the region of the $^{13}C\{^{13}C\}$ CT-COSY spectrum of [U-$^{13}C$,$^{15}N$]-$HuFd_{ox}$ that contains $C^\alpha-C^\beta$ Ser and Thr cross-peaks. In Figure 1A, the acquisition parameters have been set to values ($\tau_e = 10$ ms, recycle time = 1.27 s) appropriate for detection

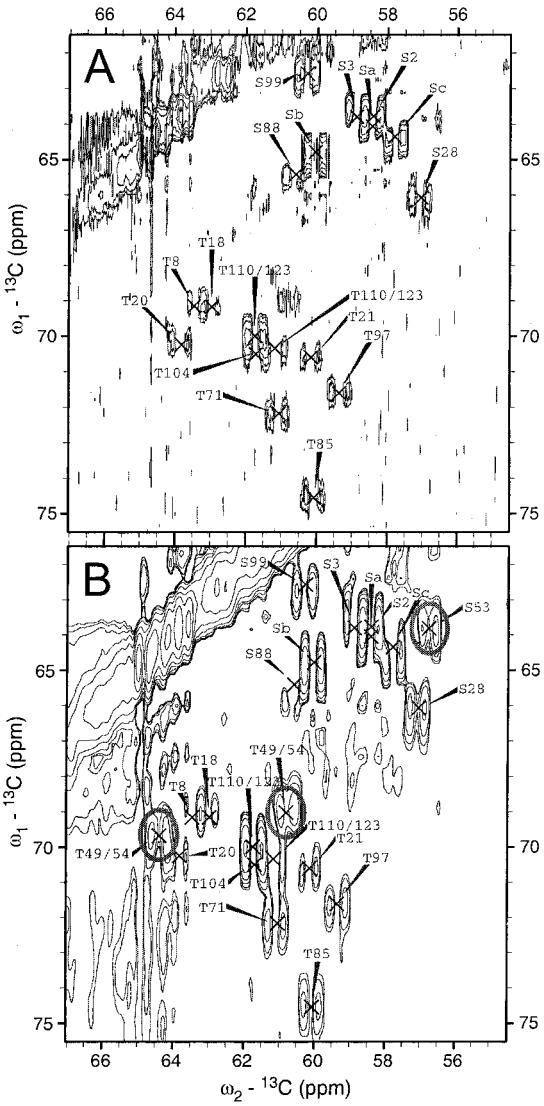


***Figure 1.*** $^{13}C\{^{13}C\}$ CT-COSY data displaying the $C^\alpha-C^\beta$ cross-peaks of Ser and Thr residues of 4.4 mM [U-$^{13}C$, $^{15}N$]-$HuFd_{ox}$ (90% $H_2O$/10% $D_2O$, 20 °C, pH 7.35, 50 mM potassium phosphate buffer), recorded on a Bruker DMX500 NMR spectrometer with a 5 mm QNP probe under two sets of acquisition parameters. (A) Complex points, 460 ($t_1$) × 32768 ($t_2$); $\tau_e$, 10 ms; recycle time, 1.27 s; number of scans, 96. (B) Complex points, 256 ($t_1$) × 4096 ($t_2$); $\tau_e$, 5.6 ms; recycle time, 0.138 s; number of scans, 1600. Spectra were acquired as phase-sensitive data with time proportional phase incrementation (TPPI) and phased such that, in the direct dimension, diagonal peaks are in-phase absorptive and cross-peaks are antiphase dispersive.



10.1021/ja017733j CCC: $22.00 © 2002 American Chemical Society

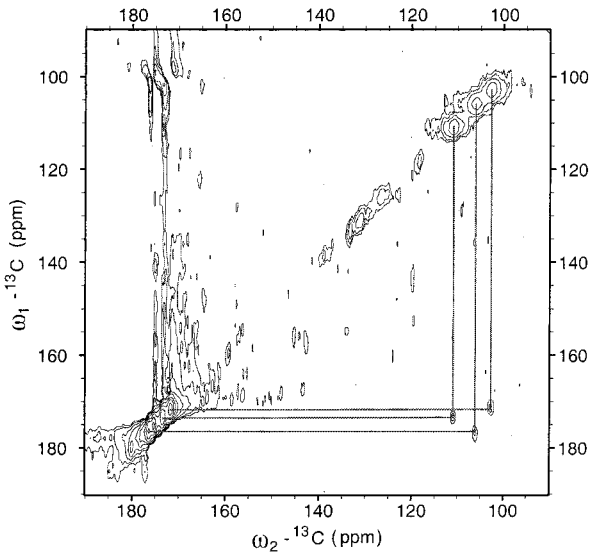


***Figure 2.*** $^{13}C\{^{13}C\}$ CT-COSY data showing the C′−•C$^{\alpha}$ region of the hyperfine-shifted Cys residues of 6.1 mM [$^{13}C$,$^{15}N$-Cys]-HuFd$_{ox}$ (90% $H_2O$/10% $D_2O$, 20 °C, pH 7.35, 50 mM potassium phosphate buffer), recorded on a Bruker DMX 500 NMR spectrometer with a 5 mm QNP probe. The spectrum was acquired and phased as in Figure 1. Acquisition parameters: complex points, 115 ($t_1$) × 2048 ($t_2$); $\tau_e$, 2.5 ms; recycle time, 55 ms; number of scans, 8192.

of "diamagnetic" resonances, those from nuclei remote from the [2Fe-2S] cluster; while in Figure 1B, the parameters have been optimized for the detection of rapidly relaxing signals ($\tau_e$ = 5.6 ms, recycle time = 0.138 s). The diamagnetic cross-peaks (Figure 1A) are assigned by comparison with the 3D backbone tracing experiments[11] and 3D HCCH-COSY.[10c] Five peaks appear that were not observed in the backbone tracing experiments but were observed by HCCH-COSY. These are assigned by amino acid type to connectivities in Ser and Thr residues in the flexible N- and C-termini (S1/S117/S124, T110/T123), although not sequence specifically. In the paramagnetic optimized version (Figure 1B), three new cross-peaks are observed, one in the Ser region and two in the Thr region. These are readily assigned to S53, T49, and T54, which are near the [2Fe-2S] cluster and whose signals were not seen in the 3D experiments. From the X-ray structure of a truncated form of oxidized bovine ferredoxin[13] the C$^{\alpha}$ of S53 is only 5.3 Å from the Fe−Fe midpoint. The intensities of these three cross-peaks are comparable to those of diamagnetic cross-peaks. C$^{\beta}$−C$^{\gamma}$ cross-peaks were also observed for T49 and T54.

Figure 2 shows the $^{13}C\{^{13}C\}$ CT-COSY spectrum of [$^{13}C$,$^{15}N$-Cys]-HuFd$_{ox}$. The acquisition parameters have been further adjusted to allow detection of signals from nuclei that relax very rapidly: $\tau_e$ = 2.5 ms, recycle time = 55 ms. Cross-peaks were observed that correspond to Cys C′−C$^{\alpha}$ connectivities from three of the four Cys that ligate the cluster. From the line widths measured from a 1D $^{13}C$ spectrum (not shown), the $T_2$* values for Cys C′ and C$^{\alpha}$ are 3.4−4.3 and 0.64−0.80 ms,[14] respectively. Thus, despite the very short nuclear relaxation rates for these signals, connectivity information could be obtained from a properly optimized $^{13}C\{^{13}C\}$ CT-COSY experiment.

Reported cross-peaks in paramagnetic $^{1}H\{^{1}H\}$ COSY spectra[15] subsequently have been shown to arise from cross correlation between dipole−dipole and Curie spin relaxation rather than *J*-coupling.[16] For the following reasons, cross correlation is unlikely to account for the $^{13}C-^{13}C$ cross-peaks observed here: (1) $^{13}C-^{13}C$ dipolar relaxation is slow; (2) Curie relaxation is not expected to be significant, owing to the relatively low molecular mass (13 kDa) and small $\langle S^2 \rangle$ of the protein; (3) $T_2$ is dominated by electron−nuclear dipolar and contact relaxation, owing to the slow electronic relaxation rate; and (4) $^{13}C-^{13}C$ scalar couplings are much larger than $^{1}H-^{1}H$. Also, the observed line shapes of the $^{13}C-^{13}C$ cross-peaks are those expected for scalar coupling (dispersive antiphase doublets), rather than those expected for cross correlation (absorption antiphase doublets). Thus, the observed cross-peaks reflect true coherence transfer through *J*-coupling.

In summary, we have demonstrated that $^{13}C\{^{13}C\}$ CT-COSY is effective at identifying connectivity information for fast relaxing resonances near a paramagnetic center in a case where $^{1}H$ detection yielded little usable information. The approach is effective in systems with slow electronic relaxation rates and little dispersion in the paramagnetically perturbed signals and can provide connectivity information even for ligating residues. Much work remains to develop tools that will allow extraction of geometric and electronic structure information from data of this kind. Nonetheless, in combination with other 2D NMR experiments and selective labeling methods, this strategy should be of great utility in obtaining more information from nuclei near the active sites of paramagnetic proteins than previously possible.

**Acknowledgment.** We thank Dr. R. Fredrick for advice in protein expression and Drs. M. Anderson, E. Mooberry, and F. Abildgaard for spectrometer assistance. This work was supported by NIH grant R01 GM58667; data were collected at the National Magnetic Resonance Facility at Madison, which has operating support from NIH grant P41 RR02301. T.E.M. is supported by NIH postdoctoral fellowship F32 GM20497.

**References**

(1) (a) Bertini, I.; Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems*; Lever, A. B. P., Gray, H. B., Eds.; Physical Bioinorganic Chemistry Series 3; Benjamin/Cummings: Menlo Park, CA, 1986. (b) Bertini, I.; Turano, P.; Vila, A. J. *Chem. Rev.* **1993**, *93*, 2833−2932. (c) Cheng, H.; Markley, J. L. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 209−237.
(2) Bertini, I.; Luchinat, C.; Rosato, A. *Prog. Biophys. Mol. Biol.* **1996**, *66*, 43−80.
(3) Dugad, L. B.; La Mar, G. N.; Banci, L.; Bertini, I. *Biochemistry* **1990**, *29*, 2263−2271.
(4) Wilkens, S. J.; Xia, B.; Weinhold, F.; Markley, J. L.; Westler, W. W. *J. Am. Chem. Soc.* **1998**, *120*, 4806−4814.
(5) Banci, L.; Bertini, I.; Ciurli, S.; Ferretti, S.; Luchinat, C.; Piccioli, M. *Biochemistry* **1993**, *32*, 9387−9397.
(6) Lelong, C.; Sétif, P.; Bottin, H.; André, F.; Neumann, J.-M. *Biochemistry* **1995**, *34*, 14462−14473.
(7) Banci, L.; Bertini, I.; Luchinat, C. *Nuclear and Electronic Relaxation*; VCH: Weinheim, Germany, 1991. Calculations assuming realistic ranges for molecular size, spectrometer frequency, spin, and electronic relaxation rates yield $T_2(^{13}C)/T_2(^{1}H)$ = 11−16 due to the interplay between the Larmor frequency and $\tau_s$ in the dipolar contribution to $T_2$.
(8) (a) Chan, T.-M.; Markley, J. L. *Biochemistry* **1983**, *22*, 6008−6010. (b) Cheng, H.; Grohmann, K.; Sweeney, W. *J. Biol. Chem.* **1992**, *267*, 8073−8080. (c) Cheng, H.; Weslter, W. M.; Xia, B.; Oh, B.-H.; Markley, J. L. *Arch. Biochem. Biophys.* **1995**, *316*, 619−634. (d) Jain, N. U.; Pochapsky, T. C. *J. Am. Chem. Soc.* **1998**, *120*, 12984−12985. (e) Jain, N. U.; Pochapsky, T. C. *Biochem. Biophys. Res. Commun.* **1998**, *258*, 54−59.
(9) $T_2$* is the transverse relaxation rate as determined from the line width, which for fast relaxing resonances should be close to the true $T_2$, since inhomogeneous broadening should be only a minor contribution.
(10) (a) Skjeldal, L.; Markley, J. L.; Coghlan, V. M.; Vickery, L. E. *Biochemistry* **1991**, *30*, 9078−9083. (b) Xia, B.; Jenk, D.; LeMaster, D. M.; Westler, W. M.; Markley, J. L. *Arch. Biochem. Biophys.* **2000**, *373*, 328−334. (c) Machonkin, T. E.; Westler, W. M.; Markley, J. L. Unpublished data.
(11) Xia, B.; Volkman, B. F.; Markley, J. L. *Biochemistry* **1998**, *37*, 3965−3975.
(12) Rance, M.; Wagner, G.; Sørensen, O. W.; Wüthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1984**, *59*, 250−261.
(13) Müller, A.; Müller, J. J.; Uhlmann, H.; Berhardt, R.; Heinemann, U. *Structure* **1998**, *6*, 269−280.
(14) This is likely an underestimate of the true $T_2$*, since here a substantial component of the line width may be unresolved *J*-coupling.
(15) de Ropp, J. S.; La Mar, G. N. *J. Am. Chem. Soc.* **1991**, *113*, 4348−4350.
(16) (a) Qin, J.; Delaglio, F.; La Mar, G. N.; Bax, A. *J. Magn. Reson. B* **1993**, *102*, 332−336. (b) Bertini, I.; Luchinat, C.; Tarchi, D. *Chem. Phys. Lett.* **1993**, *203*, 445−449.

JA017733J