J|A|C|S
COMMUNICATIONS

Published on Web 07/11/2002

Rapid Recycle $^{13}C'$,^{15}N and ^{13}C,$^{13}C'$ Heteronuclear and Homonuclear Multiple Quantum Coherence Detection for Resonance Assignments in Paramagnetic Proteins: Example of Ni^{2+}-Containing Acireductone Dioxygenase

Milka Kostic, Susan Sondej Pochapsky, and Thomas C. Pochapsky*

Department of Chemistry, Brandeis University, 415 South Street, Waltham, Massachusetts 02454

Received May 9, 2002

Many proteins incorporate paramagnetic metal ions that are essential for structure and/or function. The increased transverse and longitudinal relaxation rates of nuclear spins that result from the interaction between NMR-active nuclei and unpaired electron spins limit the applicability of NMR spectroscopy for structural and functional investigations in such systems. Paramagnetically induced nuclear spin relaxation rates increase as the square of gyromagnetic ratio,[1] often making sequence-specific resonance assignments based on standard ^{1}H-detected multidimensional NMR experiments difficult or impossible to obtain near paramagnetic centers of proteins. One alternative is to directly detect ^{13}C or ^{15}N, because their lower gyromagnetic ratios leave their relaxation rates proportionately less affected by unpaired electron spins. We have previously used this approach, combining selective labeling and $^{13}C\{^{15}N\}$ difference decoupling to make residue-specific assignments in ferredoxins.[2] Despite our success, we found the procedure to be both expensive and labor intensive. Obviously, a two-dimensional NMR approach to such assignments, which could make use of uniform labeling, would be preferable. Recently, Machokin et al.[3] published the results of applying $^{13}C\{^{13}C\}$ CT-COSY for identifying connectivities between fast relaxing ^{13}C resonances. Here we present a different approach based on multiple-quantum correlations to provide assignments of ^{13}C and ^{15}N backbone resonances in the vicinity of a paramagnetic center.

For the current work, we examined a 20 kDa Ni^{2+}-containing enzyme, acireductone dioxygenase (ARD),[4] from the methionine salvage pathway of *Klebsiella pneumoniae*. The Ni^{2+} in ARD is high-spin, and backbone assignments could not be made for 34 out of 179 residues by standard NMR methods due to paramagnetic broadening.[5] We now describe experimental and hardware modifications that permitted us to make the first backbone ^{13}C and ^{15}N assignments within the paramagnetic region of ARD. The experiments described here were performed on a 14 T Varian Unity Inova NMR spectrometer operating at 599.702, 150.821, and 60.774 MHz for ^{1}H, ^{13}C, and ^{15}N, respectively. The spectrometer is equipped with a specially modified (Varian Inc.) 5 mm $^{13}C\{^1H/^{15}N\}$ triple resonance PFG probe in which the more sensitive inner transceiver coil is doubly tuned to ^{13}C and ^{15}N, while the outer coil is used for ^{1}H and ^{2}H lock detection. A uniformly ^{13}C,^{15}N labeled ARD sample was prepared as described elsewhere.[5] All experiments were performed at 25 °C. A Cu^{2+}-doped 1 mM ^{13}C,^{15}N urea sample in 10% D_2O/90% H_2O was used for pulse calibration.

Figure 1 shows two $^{13}C_\alpha\{^{13}C'\}$ multiple quantum coherence spectra (MQC) obtained using different experimental parameters. In both cases, the pulse sequence used is a standard MQC sequence.[6] The upper spectrum was optimized for detection of diamagnetic resonances (Figure 1A), recycle delay set to 1.064 s and coherence transfer delay, $\Delta = {}^1/_2({}^1J_{CC'})$ set to 9 ms (appropriate for $^1J_{CC} =$ 55 Hz). Optimizing for residues closer to the Ni^{2+}, the recycle delay was set to 114 ms, and Δ was set to 4.5 ms. Spectral widths in

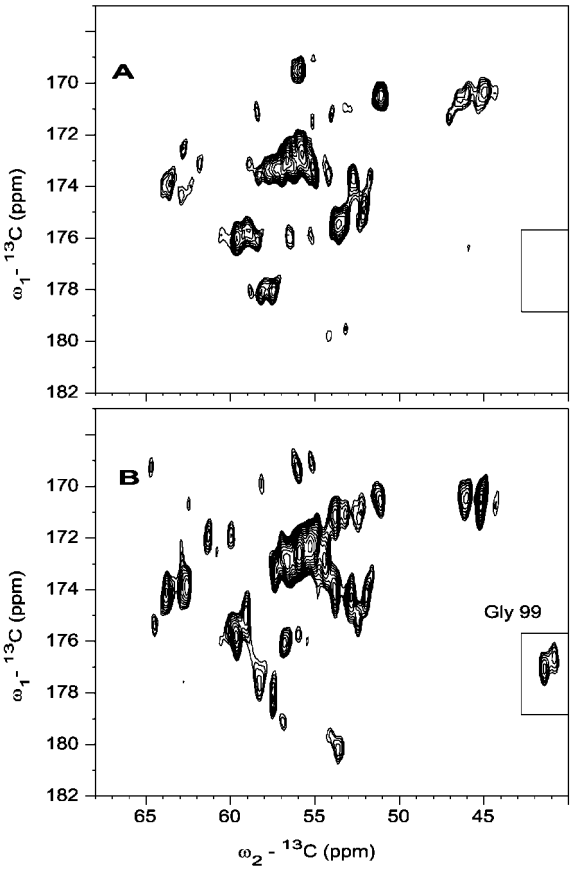

Figure 1. 150.82 MHz $^{13}C_\alpha\{^{13}C'\}$ homonuclear multiple quantum coherence data for 1 mM [U-^{13}C, ^{15}N] ARD (90% H_2O/10% D_2O, 25 °C, pH 7.4, *d*-Tris-HCl buffer) obtained as described in the text with two sets of parameters. $^{13}C_\alpha$ is directly and $^{13}C'$ is indirectly observed. Selective excitations of ^{13}C resonances in carbonyl and C_α regions were achieved using square pulses, positioned in the middle of carbonyl and C_α regions, respectively. Pulses were of appropriate amplitude and duration to provide a 90° pulse on resonance while placing a null at the position of C_α and carbonyl regions, respectively. During t_1 evolution, a $180°_x$ pulse was applied on the ^{1}H channel for refocusing purposes. (A) Parameters optimized for detection of diamagnetic signals: recycle time 1.064 s, evolution delay Δ = 9 ms, 64 (t_1) × 1024 (t_2) complex points and 1024 scans. (B) Parameters optimized for detection of paramagnetic signals: recycle delay 114 ms, evolution delay Δ = 4.5 ms, 64 (t_1) × 1024 (t_2) complex points and 15 000 scans. All data were acquired as phase sensitive with States-TPPI (time-proportional phase incrementation)[7] in t_1. Note appearance of the new peaks corresponding to Gly99 C_α−C' correlation in (B) as outlined.

* To whom correspondence should be addressed. E-mail: pochapsk@brandeis.edu.

10.1021/ja0268480 CCC: $22.00

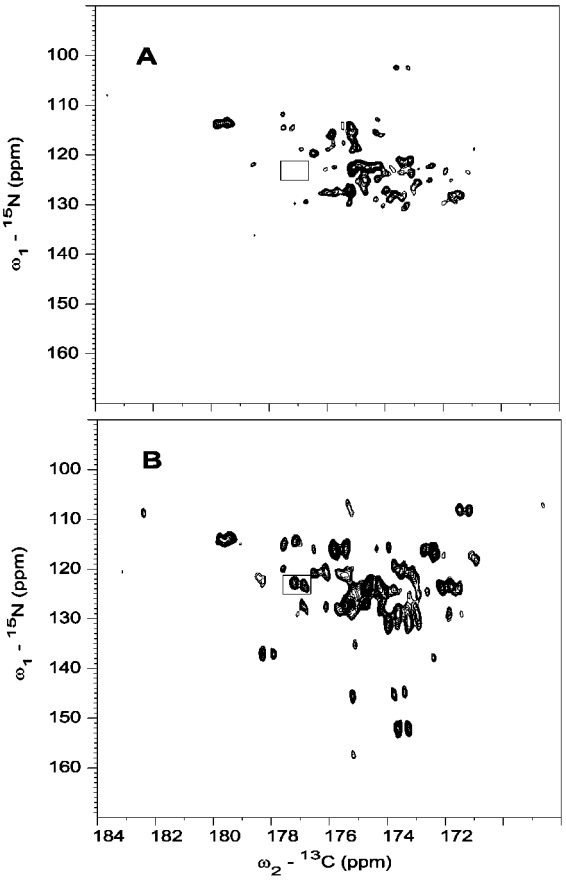

Figure 2. $^{13}C'\{^{15}N\}$ (150.82 MHz ^{13}C) heteronuclear multiple quantum coherence data for 1 mM [U-^{13}C, ^{15}N] ARD (90% H_2O/10% D_2O, 25 °C, pH 7.4, *d*-Tris-HCl buffer) obtained as described in the text using two sets of parameters. All ^{13}C pulses were square pulses designed to have maximum in the middle of the carbonyl region and a null in the C_α region. During t_1 evolution, a $180°_x$ pulse was applied on the ^1H channel for refocusing purposes. (A) Parameters optimized for detection of diamagnetic signals: recycle time 1.128 s, $\Delta = 33$ ms (corresponding to $^1/_2(^1J_{CN}) = 18$ Hz), 64 (t_1) × 1024 (t_2) complex points and 1024 scans. (B) Parameters optimized for detection of paramagnetic signals: recycle delay 82 ms, $\Delta = 12.5$ ms, 64 (t_1) × 1024 (t_2) complex points and 15 000 scans. All data were acquired as phase sensitive with States-TPPI (time-proportional phase incrementation)[7] in t_1. Note appearance of the new peaks corresponding to Gly99 C′−Glu 100 ^{15}N correlation in (B) as outlined.

both dimensions and in both data sets were 8000 Hz. These were set somewhat wider to allow for the the detection of potentially hyperfine-shifted signals. However, all detected peaks were observed in the usual chemical shift regions of the MQC spectrum. The most striking difference between two spectra is the appearance of a new doublet in the region where glycine $^{13}C_\alpha$ resonates. ARD has a single paramagnetically broadened glycine residue, Gly 99. As such, we can assign $^{13}C_\alpha$ and $^{13}C'$ chemical shifts of Gly 99 as 41.1 and 176.8 ppm, respectively.

In the second set of experiments (Figure 2), the MQC sequence was modified to allow heteronuclear (HMQC) correlations to be observed between $^{13}C'$ and ^{15}N. Cross-peaks in the spectra correspond to the correlation between backbone $^{13}C'$ resonance of residue *i* and the amide ^{15}N of residue $i + 1$. Figure 2A is the $^{13}C'\{^{15}N\}$ HMQC spectrum of ARD optimized for detection of diamagnetic resonances, while Figure 2B presents the same experiment optimized for detection of rapidly relaxing spins. Spectral width in the ^{13}C dimension was set to 8000 Hz, as noted above, while ^{15}N spectral width was set to 6000 Hz, with the carrier set at 130 ppm, to allow for the the detection of hyperfine shifted resonances. The two spectra show considerable differences. Most striking is the appearance of several new resonances that are shifted by 10−30 ppm outside the normal ^{15}N shift window in Figure 2B. Although these cross-peaks have not yet been assigned, it is likely that they correspond to residues directly involved in interaction with the Ni^{2+}, possibly as ligands. Also, using the previously assigned carbonyl resonance of Gly 99 (Figure 1B), one can identify the backbone ^{15}N resonance of Glu 100 (see box in Figure 2B), which appears as a doublet in Figure 2B that is absent in Figure 2A, at the $^{13}C'$ shift of Gly 99. Therefore, the Glu 100 ^{15}N shift can be assigned as 122.8 ppm.

Note that in both the MQC and the HMQC experiments described here, the efficiency of coherence transfer is greatly reduced by efficient electron−nuclear spin relaxation. The rapid recycle approach allows more transients to be obtained without making the overall experiment time prohibitive and partially compensates for the reduced efficiency of coherence transfer in the individual experiment.

In conclusion, we present here the results of applying rapid-recycle 2-D $^{13}C'\{^{15}N\}$ and $^{13}C_\alpha\{^{13}C'\}$ HMQC and MQC sequences, on a triple-resonance probe optimized for direct detection of ^{13}C, to paramagnetic proteins. To our knowledge, this represents the first report of using a combination of 2-D ^{13}C detected homonuclear and heteronuclear correlation approaches for assigning backbone resonances around paramagnetic centers in proteins with slowly relaxing unpaired electrons.

Acknowledgment. This work was supported by NIH grant R01-GM44191 (T.C.P.). The 600 MHz NMR was purchased through the NSF MRI program (DBI-9871130, T.C.P., P.I.). We would like to thank Sara Kunz for her technical assistance and Huaping Mo for providing the ARD sample used in this work.

References

(1) Bertini, I.; Luchinat, C. In *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; pp 47−83.
(2) (a) Jain, N. U.; Pochapsky, T. C. *J. Am. Chem. Soc*. **1998**, *120*, 12984−12985. (b) Jain, N. U.; Pochapsky, T. C. *Biochem. Biophys. Res. Commun.* **1998**, *258*, 54−59.
(3) Machonkin, T. E.; Westler, W. M.; Markley, J. L. *J. Am. Chem. Soc.* **2002**, *124*, 3204−3205.
(4) Dai, Y.; Pochapsky, T. C.; Abeles, R. H. *Biochemistry* **2001**, *40*, 6379−6387.
(5) Mo, H.; Dai, Y.; Pochapsky, S. S.; Pochapsky, T. C. *J. Biomol. NMR* **1999**, *14*, 287−288.
(6) Müller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481−4484.
(7) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.

JA0268480