# Completing the Circuit: Direct-Observe 13 C, 15 N Double-Quantum Spectroscopy Permits Sequential Resonance Assignments near a Paramagnetic Center in Acireductone Dioxygenase

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# Completing the circuit: Direct-observe $^{13}C$,$^{15}N$ double-quantum spectroscopy (CAN) permits sequential resonance assignments near a paramagnetic center in acireductone dioxygenase

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Nuclear magnetic resonance (NMR) spectroscopy is an indispensable tool for studying the structure and dynamics of biomolecules in solution. However, paramagnetic centers in proteins present difficulties for NMR in that electron-nuclear (EN) dipolar interactions efficiently relax nuclear spins near the paramagnetic center, short-circuiting coherence transfer between coupled spins and rendering most $^{1}H$-detected multidimensional NMR experiments useless for sequential resonance assignments in affected regions of the protein. As the efficiency of EN dipolar relaxation scales to the square of the gyromagnetic ratio of the nuclear spin, direct detection of heteronuclei such as $^{13}C$ and $^{15}N$ provides a viable alternative for NMR observation of resonances near paramagnetic centers. The lower sensitivity of these nuclei relative to $^{1}H$ can be partially compensated for by using rapid-recycle methods that largely suppress slowly relaxing resonances and permit rapid accumulation of transients.[1-3] We have previously reported [1] rapid-recycle homo- and heteronuclear two-dimensional (2D) double-quantum (DQ) experiments for correlating the carbonyl $^{13}C'$ with bonded amide $^{15}N$ and $^{13}C_{\alpha}$ spins (CON and COCA experiments, respectively) near the paramagnetic $Ni^{+2}$ ion in the active site of acireductone dioxygenase (ARD), an 18 kDa metalloenzyme from the methionine salvage pathway of *Klebsiella oxytoca*, for which a structure has been determined by solution NMR methods. [4] $^{1}H$ resonances are broadened so as to be undetectable within ~10 Å of the bound $Ni^{+2}$ in the ARD active site, and, while the DQ experiments allowed us to correlate isolated C'-N and C'-$C_{\alpha}$ bonded spins in this region, we were unable to obtain correlations between directly bonded $^{15}N$ and $^{13}C_{\alpha}$ spins, making it impossible to obtain continuous sequential assignments via N- $C_{\alpha}$-C'- N connectivity. We now report that using uniformly $^{2}H$, $^{13}C$ and $^{15}N$ labeled ARD in buffered $D_2O$ and more sensitive $^{13}C$ detection electronics, we are able to detect one-bond $^{15}N$-$^{13}C_{\alpha}$ correlations in the vicinity of the $Ni^{+2}$ via a double-quantum correlation experiment (CAN). The CAN experiment, in combination with the single quantum IPAP-CON [5] and CC-COSY [2] experiments allow us to make extensive sequential resonance assignments via the N- $C_{\alpha}$-C'-N pathway in the vicinity of the paramagnetic $Ni^{+2}$.

A 1 mM sample of uniformly $^{2}H$, $^{13}C$, and $^{15}N$-labeled ARD in 100% $D_2O$ (50 mM HEPES, pH 7.45) was purified from a bacterial expression system using previously described methods [6,7]. Perdeuterated $^{13}C$ glucose (CIL, Cambridge, MA) was used as carbon source in deuterated

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minimal growth medium. As a precaution against $^{1}H$ contamination, all additives to the growth medium (salts, antibiotics, vitamins) were either prepared in $D_2O$ or lyophilized and re-dissolved in $D_2O$. All NMR experiments were performed at 298 K on an 18.8T Bruker Avance 800 spectrometer operating at 201.21 and 81.086 MHz for $^{13}C$ and $^{15}N$ respectively. The spectrometer is equipped with a 5 mm TCI cryoprobe and a cryogenically-cooled carbon preamplifier for direct $^{13}C$ detection. The pulse sequence used for CAN is adapted from the original HMQC experiment [8]: $\pi/2_x$ (C) - $\tau$ - $\pi/2_{x,-x}$ (N) - $t_1/2$ - $\pi_x$ (C)-$t_1/2$ - $\pi/2_{x,x,-x,-x}$ (N) - $\tau$ - $t_2$ (receiver phase = x,-x,-x,x). Broadband $^{15}N$ decoupling was applied during the acquisition time $t_2$. $^{13}C$ pulses were rectangular soft pulses with the carrier frequency set in the $C_\alpha$ region of the $^{13}C$ spectrum at 54 ppm. Pulse lengths were chosen such that excitation nulls occurred in the carbonyl region (172 ppm). Quadrature detection in the indirect dimension was obtained using States-TPPI, with 64 $t_1$ increments and 30,000 scans per increment (total experiment time was ~4.5 days). Optimum delays $\tau$ for development of MQ coherence were found to be 20 ms, about 40% of the nominal 1/2J value, while the optimum recycle time was found to be 150 ms (100 ms relaxation delay and 50 ms acquisition time), and are recommended for general implementation. The transformed CAN experiment is shown, annotated with confirmed assignments, in Figure 1. Assignments were made via connectivity from C' to $C_\alpha$ (CC-COSY), $C_\alpha$ to N (CAN) and N to C' of the previous residue (CON). Side chain assignments were made using CC-COSY data. The spin closest to the $Ni^{+2}$ that was definitively assigned is His 98 N (5.9 Å from the metal). All other newly assigned spins lie between 6 and 10 Å from the metal, in the region of ARD for which no assignments could be made using $^{1}H$-detected methods.[4] Localization of assignments on the ARD structure is shown in Figure 2.

In recent years, a number of $^{13}C$-observe experiments have been optimized for use in paramagnetic systems. Of particular interest are the IPAP (*i*n-*p*hase *a*nti-*p*hase) sequences that have been applied with success to both diamagnetic and paramagnetic proteins.[9] While the IPAP-CON and IPAP-COCA experiments gave good results with ARD under the current conditions, the IPAP-CA(N)CO [10] (which bypasses evolution on $^{15}N$ to make sequential connections) does not. We suspect that the two coherence transfers required by this experiment make the experiment ultimately too long to maintain coherence among the rapidly relaxing spins involved. A Cα-detected DIPAP-CAN experiment has been proposed for use with diamagnetic systems, and includes pulses, delays and phase cycling to remove the effects of one-bond Cα-Cβ and Cα-C' couplings from spectra.[9] However, in the present case, line widths are sufficiently broad that these couplings are unresolved. Furthermore, the greater chemical shift dispersion seen for Cα carbons relative to C' (the spin often detected in $^{13}C$ direct-observe experiments) means that even though correlations are broadened by the unresolved couplings, interpretation remains relatively straightforward. Another potential complication, that of inter-residue Cα-N correlations, did not arise, presumably due to the smaller magnitude of the two-bond $C\alpha_i$-$N_{i+1}$ coupling relative to the one-bond $C\alpha_i$-$N_i$ coupling, and correspondingly slower coherence transfer.

There are two reasons why the CAN experiment, which previously yielded no useful data in our hands, succeeded under the current conditions. Obviously, the improved signal-to-noise available from cryogenically cooled $^{13}C$ detector electronics is helpful. More critical, however, is the complete sample perdeuteration and use of deuterated solvent. Excessive $^{1}H$ line broadening, as seen for ARD, indicates that the electronic relaxation time $\tau_e$ is long enough to permit effective coupling of the electronic and $^{1}H$ spin dipoles. As such, the coupled $^{15}N$ and $^{13}C_\alpha$ spins detect rapidly interchanging (but discrete) $^{1}H$ spin states, broadening the heteronuclear resonances. $^{2}H$, having both a lower $\gamma$ and smaller one-bond couplings to attached heteronuclei than $^{1}H$, is less affected by unpaired electronic spins and in turn is expected to result in narrower lines for attached heteronuclei.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

#### Acknowledgements

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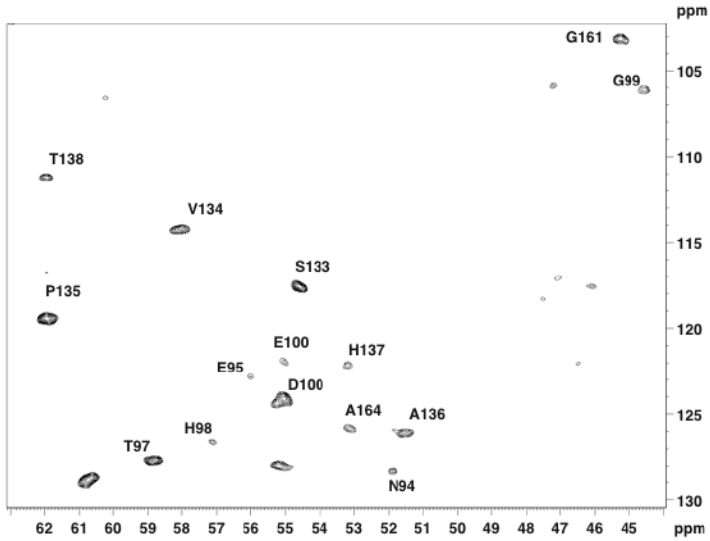


**Figure 1.**

2D CAN spectrum of 1 mM $^{2}H,^{15}N,^{13}C$-labeled ARD, pH 7.4 HEPES in 100% $D_2O$, 298 K, showing sequential $^{13}C\alpha$-$^{15}N$ resonance assignments in the ARD active site. Spectrum was acquired as described in text. Except for Gly 161 (upper right-hand corner of spectrum), all annotated peaks identify previously unassigned resonances relaxed by proximity to the $Ni^{+2}$ in the active site of the enzyme.

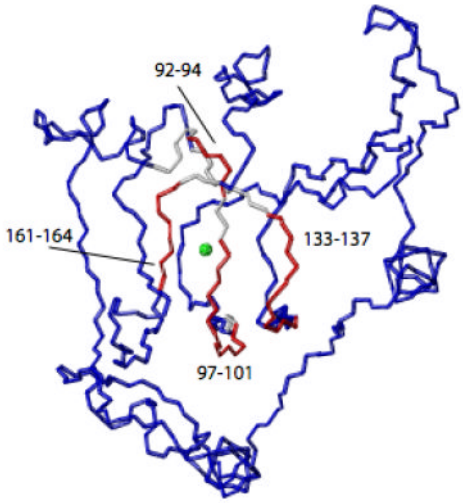


**Figure 2.**
Structure of Ni-bound ARD (PDB entry 1ZRR) [4] showing location of newly assigned residues identified by residue number with backbone atoms in red. Regions shown in blue were assigned by standard methods, those in white are still unassigned. The $Ni^{+2}$ ion is shown as a green sphere.