Coordination Sphere of the Ferric Ion in Nitrile Hydratase[1]

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Nitrile hydratases catalyze the hydration of nitriles to amides.[3] Published studies of the enzyme from *Brevibacterium* sp., strain R312,[4] using EPR,[5] EXAFS, and resonance Raman[6] spectroscopies show that it contains a novel low-spin non-heme ferric ion and suggest at least one oxygen ligand derived from the solvent.[5] We now report 35-GHz continuous-wave (CW)[7] and 9-GHz pulsed[8] electron-nuclear double resonance (ENDOR)[9] studies showing that the iron ligand donor set includes three nitrogens and one oxygen derived from water. Taken together with the previously published EXAFS and resonance Raman work,[6] these data show that the ligand donor set is N_3S_2O. The data also allow us to suggest a probable stereochemistry of the ligands.

The EPR spectrum of nitrile hydratase[10] corresponds to a rhombic g-tensor (g_1 = 2.27, g_2 = 2.14, and g_3 = 1.97). The single-crystal-like 35-GHz CW ENDOR spectrum taken at g_3 of the enzyme in $H_2{}^{17}O$-enriched buffer (approximately 38%) shows remarkably well-resolved ^{17}O resonances that are not present in natural-abundance solutions (Figure 1A).[11] The first-order prediction for single-crystal-like ^{17}O ($I = {}^5/_2$) ENDOR pattern is a pair of equally-spaced quintets described by the equation:

$$\nu_{\pm}(m) = |\nu(^{17}O) \pm A(^{17}O)/2 + 3P(^{17}O)(m - {}^1/_2)|$$

$$-{}^3/_2 \leq m \leq {}^5/_2$$

The ^{17}O resonances in Figure 1A (where $\nu(^{17}O)$ = 7.3 MHz) correspond to the $\nu_+(m)$ branch but have unequal spacings that reflect higher-order terms in the quadrupole interaction. A second-order analysis of this pattern gives $A(^{17}O)$ = 6.6 MHz and $3P(^{17}O)$ = 0.84 MHz; the calculated resonance positions are indicated in the figure.[12] Spectra taken at multiple fields indicate that $A(^{17}O)$ is largely isotropic, consistent with coordination of the ^{17}O nucleus to the iron. Moreover, the hyperfine coupling is quite comparable to that for H_2O bound to Fe_a of the aconitase $[Fe_4S_4]^+$ cluster,[11] and the quadrupole interaction is in excellent agreement as well,[12] confirming the assignment of a solvent-derived ligand to the iron in nitrile hydratase.

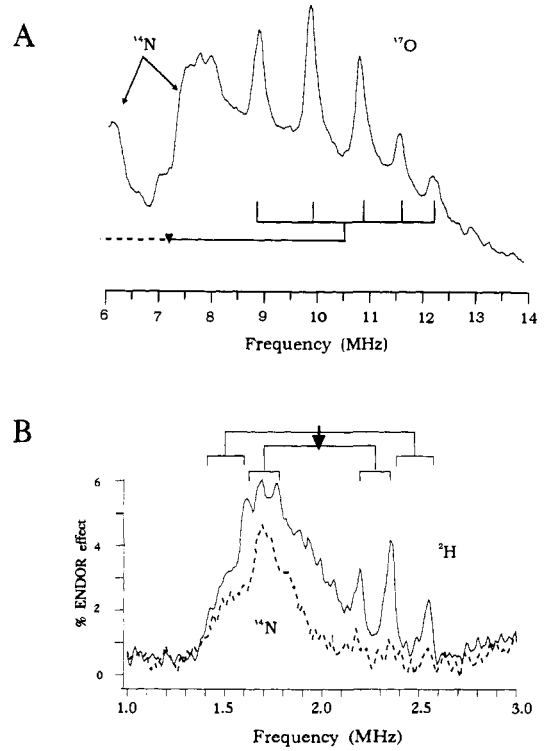

Figure 1. (A) 35-GHz CW ENDOR spectrum of nitrile hydratase in 35%-enriched $H_2{}^{17}O$ taken at the high-field edge of the EPR envelope (g_3 = 1.972). The quintet shown represents the ν_+ branch of the ^{17}O ENDOR ($I = {}^5/_2$) pattern that is centered at the ^{17}O Larmor frequency, 7.3 MHz (▼). The quadrupole splittings and hyperfine values calculated are estimated using second-order perturbation theory in the nuclear quadrupole interaction for an ^{17}O nucleus.[12] The ^{14}N resonances occur at frequencies lower than those observed for the ν_+ branch of the ^{17}O ENDOR pattern and do not interfere with the analysis. Conditions: magnetic field, 1.265 T; microwave frequency, 34.92 GHz; microwave power, 160 μW; modulation amplitude, 0.1 mT; scan speed, 0.5 MHz s^{-1}; time constant, 0.032 s; rf power, 35 W; 75 scans; temperature, 2 K. (B) X-band Mims ENDOR of nitrile hydratase in ^1H_2O buffer (- - -) and ^2H_2O buffer (—) at g = 2.25, near the low-field g_1 = 2.28 edge of the EPR envelope. The spectra are presented as percentage ENDOR and clearly show the additional ENDOR response due to the presence of ^2H_2O bound to Fe. The broader ^{14}N resonance seen in the ^1H_2O sample is easily distinguished from the narrow ^2H lines seen in the ^2H_2O-exchanged sample. The ^2H patterns are centered at the Larmor frequency 1.97 MHz (▼), with first-order splittings from both the hyperfine and quadrupole interactions. Conditions for ^1H_2O: magnetic field, 0.2995 T; microwave frequency, 9.442 GHz; 40 transients. Conditions for ^2H_2O: magnetic field, 0.3020 T; microwave frequency, 9.5100 GHz; 88 transients. All other conditions the same for both samples: microwave pulse length, 16 ns; τ_{12}, 376 ns; τ_{23}, 66.5 μs; rf pulse length, 60 μs; rf pulse power, 100 W; 256 points per spectrum; repetition rate, 5 Hz; T, 2 K.

^1H and ^2H ENDOR spectra of samples in H_2O and ^2H_2O show four classes of exchangeable protons associated with the metal center. As seen in Figure 2B, the Mims pulsed ENDOR spectra[9b] at g_1 of nitrile hydratase in ^2H_2O show ^2H resonances not present in spectra of the enzyme in H_2O. These arise from the two most strongly coupled species, with $A(^2H1)$ = 0.96 and $A(^2H2)$ = 0.6 MHz, corresponding to $A(^1H)$ = 6.25 and 4 MHz; the quadrupole splittings are $3P(^2H)$ = 0.20 and 0.16 MHz, respectively. The

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(2) (a) DuPont. (b) Northwestern University. (c) Current address: Bristol-Myers Squibb Pharmaceutical Research Inst., Princeton, NJ 08543.
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(12) Assuming that the hyperfine and quadrupole axes are coaxial with the g-tensor, a second-order analysis of the single-crystal-like ^{17}O pattern at g_3 leads to a calculated quadrupole tensor of $P(^{17}O) \approx \pm[0.2,-0.5,0.3]$, as compared to $P(^{17}O) = [0.15,-0.5,0.35]$ for H_2O bound to Fe_a in aconitase in the presence of bound substrate or inhibitor.[11]

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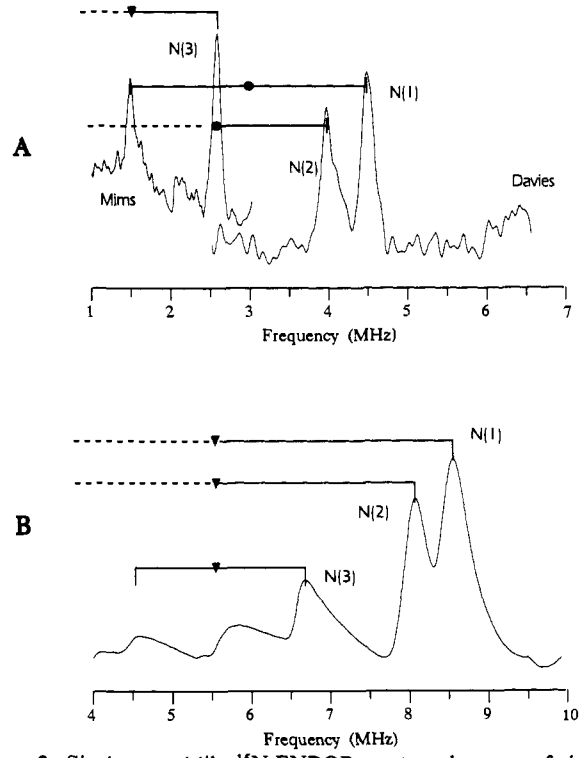


Figure 2. Single-crystal-like ^{15}N ENDOR spectra taken at g_3 of nitrile hydratase grown on $^{15}NH_4Cl$. (A) ^{15}N pulsed ENDOR. The lower frequency spectrum (1–3 MHz) was obtained using a Mims ENDOR sequence, the higher frequency spectrum (2.5–6.5 MHz) using a Davies sequence. N1 and N2 are centered at $A(^{15}N)/2$ (●) and split by $2\nu(^{15}N)$. N3 is centered at $\nu(^{15}N)$ (▼) and split by $A(^{15}N)$. Conditions for Mims ENDOR: microwave frequency, 9.445 GHz; magnetic field, 0.3417 T; $\pi/2$ microwave pulse length, 16 ns; τ_{12} = 0.248 μs; rf power, 100 W; rf pulse length, 40 μs; repetition rate, 6 Hz; T, 2 K; 30 samples/point; 256 points/spectrum. Conditions for Davies ENDOR: microwave frequency, 9.495 GHz; magnetic field, 0.3432 T; π microwave pulse length, 120 ns; τ_{23} = 0.348 μs; rf power, 100 W; rf pulse length, 40 μs; repetition rate, 6 Hz; T, 2 K; 54 samples/point; 256 points/spectrum. (B) 35-GHz ^{15}N CW ENDOR. In the higher field, all three nitrogens give hyperfine-split doublets centered at the ^{15}N ($I = {}^1/_2$) Larmor frequency, 5.5 MHz. The marked centers (▼) of the pattern are offset by +0.3 MHz because of sweep artifacts. Conditions: magnetic field, 1.272 T; microwave frequency, 35.12 GHz; microwave power, 160 μW; modulation amplitude, 0.1 mT; scan speed, 2.5 MHz s^{-1}; time constant, 0.016 s; rf power, 25 W; 75 scans, T, 2 K.

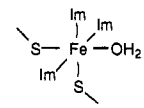
two additional exchangeable species are detected in ^1H ENDOR difference spectra ($^1H_2O - {}^2H_2O$) and have coupling constants of 1 and 2 MHz, respectively (data not shown).

The two largest hyperfine couplings are similar to those of the protons of H_2O bound unsymmetrically to the aconitase cluster, as first proposed on the basis of ENDOR data[11] and substantiated by the crystal structure determination.[13] This suggests that the O-donor ligand in nitrile hydratase is H_2O, not OH^-. Some support for this comes from spectra of samples prepared at higher pH, where only one of these large couplings is seen. The protons associated with the smaller couplings could be associated with N—H···S hydrogen bonds or with the remote N—H of coordinated histidine (vide infra).

^{14}N ENDOR spectra of nitrile hydratase show a rich pattern that is difficult to assign. However, 9.5-GHz pulsed and 35-GHz CW ENDOR spectra of ^{15}N-labeled enzyme,[14] again taken at the high-field g_3-edge of the EPR envelope, demonstrate the presence of three distinct N-donor ligands to the iron. Figure 2A presents the superposition of Davies and Mims pulsed[9b] ^{15}N ENDOR data taken at g_3;[15] one can see both peaks of the expected doublet ($\nu_\pm = |\nu(^{15}N) \pm A(^{15}N)/2|$) for one nitrogen (N1) and the ν_+ peaks for two additional nitrogens; the ν_- peaks for N2 and N3 are calculated to fall at frequencies below those shown in the figure. The center frequency of the Larmor-split N1 doublet corresponds to $A(^{15}N1)$ = 6.0 MHz; for N(^{15}N2) we likewise calculate $A(^{15}N2)$ = 5 MHz using $\nu(^{15}N)$ = 1.48 MHz. The pattern for N3 is a hyperfine-split doublet centered at $\nu(^{15}N)$; here one calculates a much lower coupling, $A(^{15}N3)$ = 2 MHz. Each of these assignments was verified by taking advantage of the suppression effect ("blind spots") of Mims ENDOR.[16] This procedure is corroborated by ^{15}N 35-GHz CW ENDOR spectra that have noticeably better signal:noise but noticeably lower resolution (Figure 2B).[17] In the higher field of the 35-GHz spectrometer, all three of the observed ^{15}N nuclei give hyperfine-split doublets. The spectrum in Figure 2B shows both the $\nu_\pm$ partners for N3 and the ν_+ peaks for N1 and N2; the ν_- partners for N1 and N2 fall at lower frequencies and are of lower intensity. The coupling constants measured by two techniques and at two frequencies are wholly in agreement. Additional pulsed and CW spectra taken across the EPR envelope demonstrate that $A(^{15}N1)$, $A(^{15}N2)$, and $A(^{15}N3)$ are largely isotropic. These data further confirm that N1 and N2 are quite similar but N3 is distinct.

The large, roughly isotropic couplings for N1 and N2 indicate that both are ligands, directly coordinated to Fe. The ratio of $A(^{15}N3)$ to either $A(^{15}N1)$ or $A(^{15}N2)$ is far too large to support the assignment of N3 as the remote nitrogen of a coordinated imidazole, as we have proven in studies of specifically-labeled $^{15}N(\delta)$ histidine bound to the Rieske-type $[Fe_2S_2]^+$ center of phthalate dioxygenase from *Pseudomonas cepacia*.[18] The magnitude of $A(^{15}N3)$ (2 MHz for ^{15}N) is comparable to that assigned to the coordinated nitrogen of the proximal imidazole of the mercaptoethanol complex of ferrimyoglobin (2 MHz for ^{14}N).[19] We therefore assign ligand N3 as *trans* to a cysteinyl mercaptide.

Combination of 35-GHz CW ENDOR and 9-GHz Mims and Davies pulsed ENDOR thus reveals four ligands to iron, three nitrogens, and one oxygen. EXAFS data show a further 2.5 ± 1 sulfur ligands.[6] We therefore conclude that the iron is hexacoordinate where the coordination sphere is N_3OS_2. We have assigned the O-donor ligand as H_2O and logically assume the N-donor ligands are histidine imidazoles. We have assigned N3 to be *trans* to a thiolate sulfur, namely that N3–Fe–S lies along one axis of the coordinating octahedron. This leaves two possible dispositions of the remaining four ligands along the coordinating axes: [(N1–Fe–N2; (H_2O–Fe–S)] or [(N1,2–Fe–OH_2); (N2,1–Fe–S)]. The similarities between N1 and N2 favor the former, and thus the ENDOR data indicate a *mer* geometry of the imidazole ligands:



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(14) Prepared by growing bacteria with 99%-enriched $^{15}NH_4Cl$ (Cambridge Isotopes) as the sole nitrogen source.

(15) The latter is more suitable for small hyperfine couplings, the former for larger couplings.

(16) The Mims ENDOR intensity for a peak corresponding to hyperfine coupling A vanishes when $A(\text{MHz})\tau(\mu s) = n$ (n = 1, 2, ...), where τ is the spacing between pulses 1 and 2.[9b]

(17) Well-known sweep artifacts produce the asymmetry in the CW ENDOR peak shapes and also shift the spectrum ~0.2 MHz in the (positive) sweep direction; this shift is readily corrected.

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