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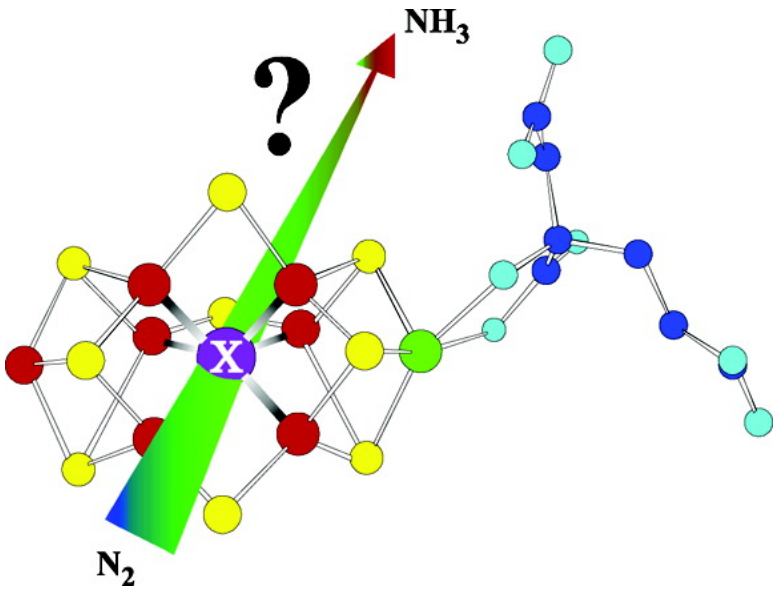

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The Interstitial Atom of the Nitrogenase FeMo-Cofactor: ENDOR and ESEEM Show It Is Not an Exchangeable Nitrogen

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Nitrogenase, which consists of the electron-transfer Fe protein and catalytic MoFe protein, reduces the dinitrogen triple bond under atmospheric pressure and temperature, forming ammonia in a reaction coupled to the hydrolysis of ATP.[1,2] A key step toward an understanding of this process was taken over 10 years ago, with the modeling of the crystal structure of the active site, the [$MoFe_7S_9$: homocitrate] FeMo-cofactor,[3−5] hence the surprise when a recent high-resolution X-ray crystallographic study revealed electron density from a chemical species (denoted X) inside the cofactor, at the center of the structure, at a distance of 2.0 Å from the six "trigonal prismatic" irons and 3.3 Å from all of the sulfur (Figure 1).[6] The identity of X was not established, although the electron density is consistent with a single N, O, or C atom. An attractive proposal suggests that X is a N atom that derives from N_2 and exchanges during catalysis.[6] In the present study, we have tested this possibility by using electron-nuclear double resonance (ENDOR)[7,8] and electron spin-echo envelope modulation (ESEEM)[9] spectroscopies to examine wild-type (WT) and site-specifically altered MoFe proteins that have been turned over with $^{14}N_2$ and $^{15}N_2$.

Previous X-band (9 GHz) ^{14}N ESEEM studies of WT and altered MoFe proteins identified two kinds of ^{14}N signals. One (N1), seen in the WT enzyme, gives strong modulation and was suggested to arise from the side-chain nitrogen of α-359-arginine; the other (N2), whose ESEEM signal is uncovered only in the α-$359^{Arg\rightarrow Lys}$ and α-$381^{Phe\rightarrow Leu}$ variants, gives a weak modulation and was suggested to arise from one or both of the amide nitrogens of α-356-glycine/α-357-glycine.[10] To reliably detect *all* ^{14}N nuclei hyperfine-coupled to the resting-state paramagnetic FeMo-cofactor and check whether *any* of them exchange during turnover, we have (i) re-examined the previous assignments by using X-band ESEEM to examine the WT and altered proteins after turnover with $^{14}N_2$ or $^{15}N_2$, and (ii) for the first time, we have used Q-band (35 GHz) ENDOR and ESEEM to examine the resting-state MoFe protein, to look for additional, previously undetected ^{14}N signals and to test whether they exchange under turnover with $^{15}N_2$.

The FeMo-cofactors in the resting states of the WT, α-$359^{Arg\rightarrow Lys}$, and α-$381^{Phe\rightarrow Leu}$ MoFe proteins, both before and after enzymatic turnover, exhibit the EPR signal of the $S = {}^3/_2$ ground-state FeMo-cofactor.[11,12] The X-band ^{14}N ESEEM spectra of the resting-state WT, α-$359^{Arg\rightarrow Lys}$, and α-$381^{Phe\rightarrow Leu}$ MoFe proteins regenerated by turning over the proteins with $^{14}N_2$ were completely identical to those previously reported, and turning the enzymes over with $^{15}N_2$ caused *no* change (data not shown). Thus, neither the N1 nor the N2 nitrogen is associated with a dinitrogen molecule of catalysis, nor a reduction/catalytic product, including ammonia, that exchanges during turnover. For N1, this is consistent with the previous turnover experiments by Thomann and co-workers[13,14] and with our previous assignments;[10,15] likewise, for N2, this is consistent with our previous assignment.[10] Q-band ^{14}N ESEEM data obtained at $g \sim g_2'$ for the regenerated resting-state MoFe protein turned over with $^{14}N_2$ showed weak modulation that likely can be assigned to ^{14}N1;[16] regardless, the signals from the resting-state MoFe protein regenerated with $^{14}N_2$ and with $^{15}N_2$ were the same (data not shown).

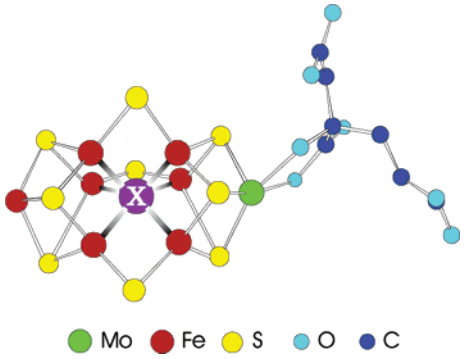

Figure 1. Structure of the FeMo-cofactor showing central ligand "X".[6]

ENDOR and ESEEM can provide complementary information, but $^{14/15}$N ENDOR studies of the resting-state MoFe protein have not been previously reported. We here describe Q-band pulsed ENDOR efforts to search for ^{14}N signals that are associated with the resting-state cofactor, and to test if they change under $^{15}N_2$ turnover.[17] Figure 2A shows Q-band pulsed ENDOR[18] spectra collected at $g \sim g_2'$ of the resting-state MoFe protein regenerated after turnover with $^{14}N_2$. The figure includes spectra taken with both the Davies and the Mims protocols,[18] with the latter employing two values of τ. The different spectra accentuate/suppress different signals, with the result that the multiple spectra together reveal five peaks over the frequency range of 4−9 MHz. A firm assignment of these lines must await the collection and analysis of a full "2D" field-frequency dataset.[7] However, preliminary simulations suggest tentative assignments, as indicated in the figure, to N1, N2, and one or two additional nitrogens (N3 and/or N4 in the figure), all of which have small observed hyperfine couplings.

These pulsed ENDOR experiments were repeated on a sample of the resting-state MoFe protein regenerated after turnover with $^{15}N_2$. As can be seen in Figure 2B, *none* of the peaks are lost, and *no* new features are observed. Thus, *none* of the nitrogenous species that are hyperfine coupled to the electron spin of the cofactor cluster, as detected by a panoply of double-resonance techniques, are altered

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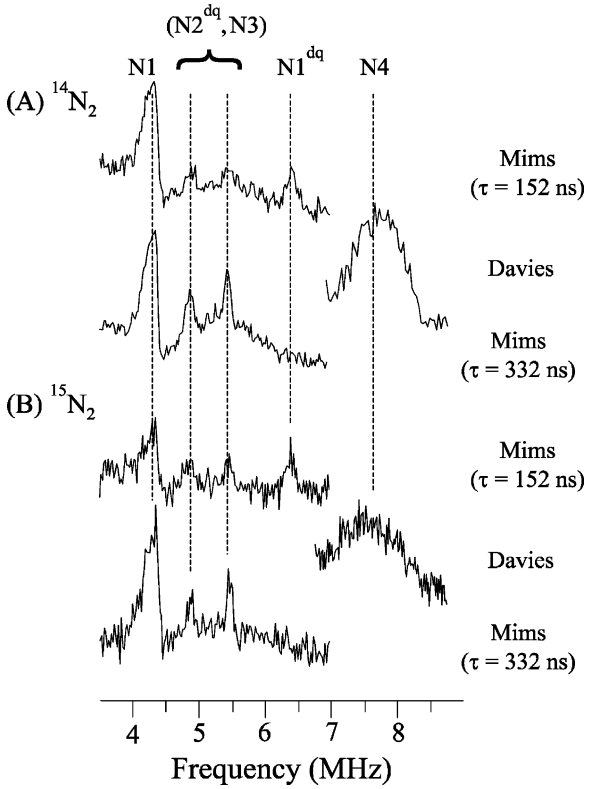

Figure 2. Q-band ^{14}N Mims and Davies ENDOR of the regenerated resting-state WT MoFe protein by turning over with (A) $^{14}N_2$ and (B) $^{15}N_2$. "dq" implies double quantum transition ($\Delta m_I = \pm 2$).[9] Experimental conditions: microwave frequency (GHz), (A) 34.755, (B) 34.822; $\pi/2$ microwave pulse width (ns), (Mims) 56, (Davies) 40; π microwave pulse width (ns), (Davies) 80; radio frequency pulse width (μs), (Mims) 30, (Davies) 60; magnetic field strength (G), (A) 6759, (B) 6809; temperature, 2 K.

by catalytic turnover with $^{15}N_2$, and *no* new ^{15}N signal appears in any of the spectroscopies applied.

Thus, we detect three or four nitrogenous moieties hyperfine-coupled to the cofactor cluster spin, all with small observed couplings (intrinsic $a_{iso} \lesssim 5$ MHz). It seems unlikely to us that a nitrogen incorporated into the cofactor interior could go undetected by the multiple techniques we have employed. On this basis, our experiments indicate that *if* X is a nitrogenous species,[19−22] then it is associated with one of the ^{14}N signals described here and *does not exchange* during catalysis. One may speculate that even a nonexchanging N might participate in catalysis, perhaps through an intermediate(s) in which it forms a bond to the N_2 being reduced.

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(11) EPR, ENDOR, and ESEEM measurements: Q-band continuous-wave (CW) EPR and ENDOR spectra were collected on a modified Q-band Varian E-110 spectrometer equipped with a liquid helium immersion dewar, described elsewhere.[23] Q-band pulsed ENDOR/ESEEM data were obtained on a locally built spectrometer.[24] X-band ESEEM experiments were performed on a locally built X-band pulsed-EPR spectrometer.[25] Mims ($\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau -$ echo) and Davies ($\pi - T - \pi/2 - \tau - \pi - \tau -$ echo) sequences were employed to collect ENDOR spectra.[8,25,26] Electron spin echo intensities were recorded as a function of radio frequency which was applied during the time interval T for ENDOR. Three-pulse echo sequence ($\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau -$ echo)[9] was employed at 2 K to collect ESEEM data.
(12) Sample preparation: Nitrogenase Fe and MoFe proteins were purified as previously described.[27] Samples of wild-type and altered (α-359$^{Arg\rightarrow Lys}$, α-381$^{Phe\rightarrow Leu}$) MoFe proteins were trapped following turnover under 1 atm $^{14}N_2$ or $^{15}N_2$ (Cambridge Isotopes, Inc.). Turnover conditions were initiated by adding the MoFe proteins (100 μM) to a solution containing 50 μM Fe protein, 0.3 mg/mL creatine kinase, 2 mg/mL BSA, 5 mM MgATP, and 16 mM phosphocreatine in 200 mM MOPS buffer (pH 7.0) with 10 mM $Na_2S_2O_4$ in a total reaction volume of 300 μL. The reaction proceeded to completion at 30 °C, corresponding to ca. 10 cycles of dinitrogen reduction per enzyme. Post-turnover samples were trapped in two different ways: (i) the entire reaction solution was concentrated in a Centricon-100 ultrafiltration concentrator to be trapped in liquid N_2; (ii) the MoFe proteins (having a poly-His tag on the C-terminus of the α-subunit) were isolated from the reaction mixture by chromatography on a 3 mL chelating sepharose column charged with Zn and then were trapped.[27]
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