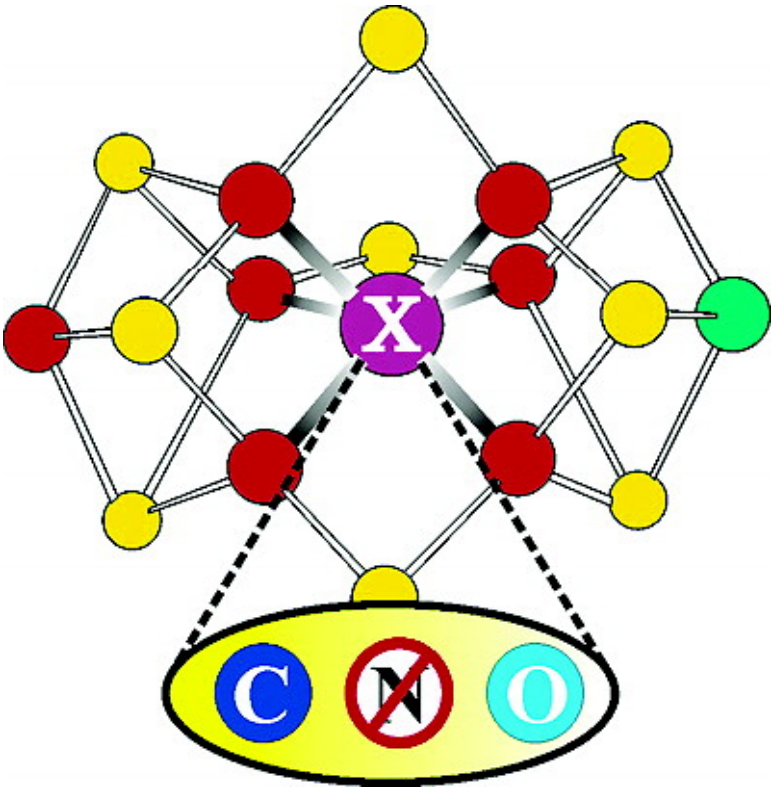
Communication

The Interstitial Atom of the Nitrogenase FeMo-Cofactor: ENDOR and ESEEM Evidence That it is Not a Nitrogen

Tran-Chin Yang, Nathan K. Maeser, Mikhail Laryukhin, Hong-In Lee, Dennis R. Dean, Lance C. Seefeldt, and Brian M. Hoffman

J. Am. Chem. Soc., **2005**, 127 (37), 12804-12805• DOI: 10.1021/ja0552489 • Publication Date (Web): 23 August 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 13 articles that cite this article, as of the time of this article download
- Access to high resolution figures

J|A|C|S
COMMUNICATIONS

Published on Web 08/23/2005

The Interstitial Atom of the Nitrogenase FeMo-Cofactor: ENDOR and ESEEM Evidence That it is Not a Nitrogen

Tran-Chin Yang,[¶] Nathan K. Maeser,[‡] Mikhail Laryukhin,[¶] Hong-In Lee,[†] Dennis R. Dean,*[,§] Lance C. Seefeldt,*[,‡] and Brian M. Hoffman*[,¶]

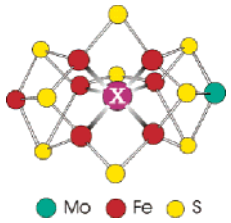
Department of Chemistry, Northwestern University, Evanston, Illinois 60208, Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322, Department of Chemistry Education, Kyungpook National University, Daegu, 702-701, Korea, and Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061

Received August 2, 2005; E-mail: bmh@northwestern.edu; deandr@vt.edu; seefeldt@cc.usu.edu

Nitrogenase, which consists of the electron-transfer Fe protein and active-site-containing MoFe protein, reduces N_2 to two NH_3 under atmospheric pressure and temperature in a reaction coupled to the hydrolysis of ATP.[1,2] Recently, a high-resolution (1.16 Å) X-ray crystallographic study of the MoFe protein revealed electron density from an atom (denoted **X**) inside the active-site metal cluster, the [$MoFe_7S_9$:homocitrate] FeMo-cofactor, at a distance of 2.0 Å from the six "trigonal prismatic" irons and 3.3 D from all the sulfur (Chart 1).[3] The electron density associated with **X** is consistent with a single N, O, or C atom, and it was natural to suggest that **X** is an N atom that derives from N_2 and exchanges during catalysis.[3] We tested the possibility of an exchanging N by using electron−nuclear double resonance (ENDOR)[4,5] and electron spin−echo envelope modulation (ESEEM)[4] spectroscopies to examine the FeMo-co in wild-type (WT) and site-specifically altered MoFe proteins that were turned over with $^{14}N_2$ and $^{15}N_2$. From these measurements, we inferred that *if* **X** is a nitrogenous species,[6−9] then it does not exchange during catalysis. We now have tested whether **X** is a N or not by comparing the ENDOR and ESEEM signals from resting-state ($S = 3/2$) MoFe protein and NMF-extracted FeMo-co from bacteria grown with either ^{14}N or ^{15}N as the exclusive N source.[10−12]

The first evidence as to the identity of **X** was presented long before its presence was even disclosed. In 1987, it was reported that the resting-state MoFe protein's $S = 3/2$ FeMo-co displayed X-band ESEEM from hyperfine-coupled ^{14}N, and that the modulation disappeared when the FeMo-co was extracted into NMF. This indicated that the interacting ^{14}N atoms being observed were associated with protein residues and provided limited evidence against there being a ^{14}N associated with the FeMo-co.[13] Subsequently, we analyzed this modulation in detail and used it to examine the consequences of amino acid substitutions to the MoFe protein in the vicinity of the FeMo-co binding site.[14,15] However, X-band ESEEM might fail to show signals from ^{14}N with hyperfine couplings far from the optimal value near "exact cancellation", where $A/2 \sim \nu_N \sim 0.6$ MHz ($g \sim 3.9$, X-band). We therefore have repeated the ^{14}N comparison between protein-bound and -extracted FeMo-co with ESEEM at Q-band, where ν_N is 4-fold larger. Figure 1A presents the Q-band three-pulse ESEEM patterns for the $S = 3/2$ states of the resting-state MoFe protein and for two preparations of isolated cofactor, as extracted into NMF,[12] in NMF, with the addition of PhSH to coordinate to the terminal Fe and sharpen the $S = 3/2$ signal[16] and also with CN^- to bind to the Mo at the opposite end of the cofactor.[17] The spectrum from the MoFe protein shows ^{14}N modulation as expected from X-band experiments, with both

Chart 1



low (~2.5 MHz) and higher-frequency (~6.1 MHz) components. Simulations following our general ESEEM analysis procedures[18] indicate that this is the same ^{14}N that gives the strong modulation previously characterized in our X-band measurements.[14,15] As can be seen, *no* modulation persists in either of the isolated FeMo-co preparations; the same is true in traces collected at this and other *g*-values over a wide range of values for the critical parameter, τ, the separation between first and second microwave pulses. The absence of ^{14}N modulation in both X- and Q-band ESEEM measurements is powerful evidence against the assignment, X = ^{14}N. However, it is not proof. The depth of the modulation in ^{14}N ($I = 1$) ESEEM is largely controlled by, and increases with, the nuclear quadrupole coupling.[18] However, the interstitial atom X sits on a site of high symmetry (trigonal prismatic) and thus is expected to have a nearly negligible quadrupole coupling and, hence, shallow modulation.

To test the indications from the ESEEM measurements, as before,[6] we performed a parallel Q-band Mims pulsed ENDOR investigation, examining resting-state MoFe protein and extracted FeMo-co derived from *A. vinelandii* grown, but with either ^{14}N or ^{15}N as the exclusive nitrogen source. Figure 1B shows ^{14}N Mims ENDOR spectra collected from the resting-state MoFe protein and NMF-extracted FeMo-cofactor with added PhSH and CN^-, which improves the phase memory as seen in Figure 1A. The resting-state MoFe protein exhibits a natural-abundance ^{13}C signal at ~7.4 MHz and signals over the range of 1−5.5 MHz, arising from ^{14}N nuclei interacting with the FeMo-co, but *none* of the ^{14}N signals of the resting-state MoFe protein is seen in the spectrum of the extracted FeMo-co. As seen in the inset, the spectrum does contain overlapping signals from natural-abundance ^{13}C and distant ^{23}Na ENDOR responses from the buffer solution at $\nu_{Na} \sim 7.8$ MHz. Outside this frequency range, we detect only signals from ^1H (not shown). We have not identified the source of the natural-abundance ^{13}C signal associated with the FeMo-co; the essentially diamagnetic state of Mo in resting-state FeMo-co makes it unlikely that homocitrate is the source, making cluster-bound PhS^- or NMF or **X** itself the likely candidate(s).

We further considered the possibility that the somewhat broadened EPR signal of the extracted FeMo-co, even with PhSH and

[¶] Northwestern University.
[‡] Utah State University.
[†] Kyungpook National University.
[§] Virginia Tech.

10.1021/ja0552489 CCC: $30.25

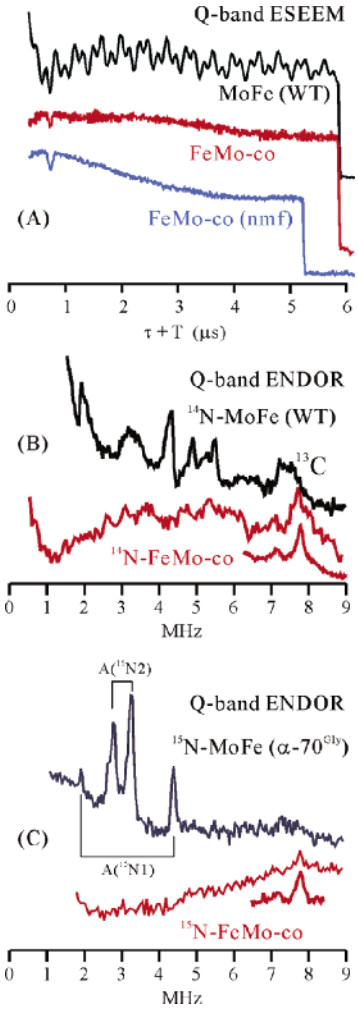


Figure 1. (A) Q-band three-pulse ESEEM spectra of the resting-state WT MoFe protein (black), FeMo-co in NMF with PhSH and CN^- (red trace), and FeMo-co in NMF (blue trace; denoted NMF). (B) Q-band ^{14}N Mims ENDOR spectra of the WT MoFe protein (black trace) and FeMo-co with PhSH/CN^- in NMF (red trace). (C) Q-band Mims ENDOR spectra of ^{15}N-labeled α-70^{Gly} MoFe protein (black trace) and ^{15}N-labeled FeMo-co in NMF/PhSH (red trace). Insets to (B) and (C) are taken at higher rf power. *Conditions*: microwave frequency = 34.80 GHz; repetition rates = 250 Hz; T = 2 K; transients ≈ 200 (ESEEM), 2000 (ENDOR); points/trace = 512 (ESEEM), 256 (ENDOR); $\pi/2$ pulse = 24 ns (ESEEM), 52 ns (ENDOR); τ = 240 ns (ESEEM); τ = 500 ns, RF = 20 μs (ENDOR).

CN^- present, is accompanied by a distributed quadrupole coupling of **X** = ^{14}N, and that this broadens its ENDOR signal;^{15}N (I = 1/2) has no nuclear quadrupole moment and routinely gives much sharper ENDOR signals than ^{14}N.[19] We therefore grew *A. vinelandii* that expresses α-70^{Gly} MoFe protein (unable to reduce N_2 to ammonia) on ^{15}N-urea, purified the MoFe protein, extracted the FeMo-cofactor in NMF/PhSH, and performed ^{15}N ENDOR experiments on both samples (Figure 1C). The ^{15}N ENDOR spectrum of the MoFe protein displays well resolved responses from two kinds of ^{15}N: one with an effective coupling[20] $A'(^{15}N1) \sim 2.5$ MHz, corresponding to a coupling in the $S = 3/2$ manifold of $A^{3/2}(^{15}N1) \sim 1.4$ MHz, $A^{3/2}(^{14}N1) \sim 1.0$ MHz; a second with $A'(^{15}N2) \sim 0.6$ MHz, corresponding to $A^{3/2}(^{15}N2) \sim 0.3$ MHz, $A^{3/2}(^{14}N2) \sim 0.2$ MHz ("goal-post" marks).[20,21] We also looked for smaller hyperfine couplings in experiments with longer τ, but no new signals were observed. Analysis shows that $^{14}N1$ gives rise both to the Q-band ESEEM (Figure 1A) and the deep X-band modulation. The doublet with $A'(^{15}N2) \sim 0.6$ MHz may arise from the ^{14}N nucleus, giving rise to shallow modulation at X-band.[14,22] The extracted FeMo-co shows *none* of the ^{15}N signals seen with the protein, but does show the signals from natural-abundant ^{13}C and ^{23}Na.

The loss of ^{14}N ESEEM (Figure 1A) and ^{14}N ENDOR (Figure 1B) seen in the resting-state MoFe protein upon extraction of the FeMo-co in NMF and loss of the ^{15}N ENDOR signals of the α-70^{Gly} MoFe protein upon extraction of the FeMo-co (Figure 1C) show that these $^{14/15}N$ ENDOR signals from the resting-state MoFe protein all arise from protein-bound N nuclei, and *not from the cofactor itself*. It is hard to imagine that the variety of spectroscopic methods discussed here could have missed a signal from **X** = N unless the nucleus is uncoupled from the electron-spin system, $A^{3/2} \sim 0$ MHz. The Q-band ENDOR of the MoFe protein has detected a ^{15}N signal with $A^{3/2}(^{15}N) \sim 0.3$ MHz, corresponding to a ^{14}N coupling of $A^{3/2}(^{14}N) \sim 0.2$ MHz, and we believe would have detected a coupling of $A^{3/2}(^{14}N_1) \sim 0.1$ MHz or less. Current DFT computations suggest that if **X** = ^{14}N, then a coupling of a MHz or so is expected (similarly for **X** = C or O).[8] Thus, the results presented here strongly indicate that **X** is not an N.

Acknowledgment. This work has been supported by the NSF (MCB-0316038 to B.M.H.) and NIH (GM59087 to L.C.S. and D.R.D.; HL13531 to B.M.H.).

References

(1) Burgess, B. K.; Lowe, D. L. *Chem. Rev.* **1996**, *96*, 2983−3011.
(2) Rees, D. C.; Howard, J. B. *Curr. Opin. Chem. Biol.* **2000**, *4*, 559−566.
(3) Einsle, O.; Tezcan, F. A.; Andrade, S. L. A.; Schmid, B.; Yoshida, M.; Howard, J. B.; Rees, D. C. *Science* **2002**, *297*, 1696−1700.
(4) Schweiger, A.; Jeschke, G. *Principles of Pulse Electron Paramagnetic Resonance*; Oxford University Press: Oxford, UK, 2001.
(5) Hoffman, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3575−3578.
(6) Lee, H.-I.; Benton, P. M. C.; Laryukhin, M.; Igarashi, R. Y.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. *J. Am. Chem. Soc.* **2003**, *125*, 5604−5605.
(7) Hinnemann, B.; Norskov, J. K. *J. Am. Chem. Soc.* **2003**, *125*, 1466−1467.
(8) Lovell, T.; Liu, T.; Case, D. A.; Noodleman, L. *J. Am. Chem. Soc.* **2003**, *125*, 8377−8383.
(9) Dance, I. *Chem. Commun.* **2003**, *3*, 324−325.
(10) MoFe protein was purified essentially as described (Christiansen et al.) from the appropriate *Azotobacter vinelandii* strain (wild-type or DJ1313 for the α-70^{Gly}-substituted MoFe proteins). Strain DJ1313, which is incapable of N_2 fixation, was grown with ^{14}N- or ^{15}N-urea as the sole nitrogen source. FeMo-co was extracted into NMF essentially as described by Shah (Shah and Brill). When added, thiophenol (PhSH) was at a final concentration of approximately 10 mM, and cyanide at a final concentration of approximately 5 mM.
(11) Christiansen, J.; Goodwin, P. J.; Lanzilotta, W. N.; Seefeldt, L. C.; Dean, D. R. *Biochemistry* **1998**, *37*, 12611−12623.
(12) Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3249−3253.
(13) Thomann, H.; Morgan, T. V.; Jin, H.; Burgmayer, S. J. N.; Bare, R. E.; Stiefel, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 7913−7914.
(14) Lee, H.-I.; Thrasher, K. S.; Dean, D. R.; Newton, W. E.; Hoffman, B. M. *Biochemistry* **1998**, *37*, 13370−13378.
(15) DeRose, V. J.; Kim, C.-H.; Newton, W. E.; Dean, D. R.; Hoffman, B. M. *Biochemistry* **1995**, *34*, 2809−2814.
(16) Rawlings, J.; Shah, V. K.; Chisnell, J. R.; Brill, W. J.; Zimmermann, R.; Munck, E.; Orme-Johnson, W. H. *J. Biol. Chem.* **1978**, *253*, 1001−1004.
(17) Cui, Z.; Dunford, A. J.; Durrant, M. C.; Henderson, R. A.; Smith, B. E. *Inorg. Chem.* **2003**, *42*, 6252−6264.
(18) Lee, H.-I.; Doan, P. E.; Hoffman, B. M. *J. Magn. Reson.* **1999**, *140*, 91−107.
(19) Tierney, D. L.; Martásek, P.; Doan, P. E.; Masters, B. S.; Hoffman, B. M. *J. Am. Chem.* Soc. **1998**, *120*, 2983−2984.
(20) Hoffman, B. M.; DeRose, V. J.; Doan, P. E.; Gurbiel, R. J.; Houseman, A. L. P.; Telser, J. *Biol. Magn. Reson.* **1993**, *13* (EMR of Paramagnetic Molecules), 151−218.
(21) The observed $^{15}N_1$ hyperfine coupling constant, $A'(^{15}N)$, is related to the $S = 3/2$ coupling by $A'(^{15}N) = (g'_e/g_e) \times A^{3/2}(^{15}N) = (g'_e/g_e) \times A^{3/2}(^{14}N) \times (g_N{}^{15N}/g_N{}^{14N}) = 3.6/2.0 \times 1.0 \times 1.4 = 2.5$ MHz, where g'_e is the experimental g-value. The center of the $^{15}N1$ doublet is shifted from the ^{15}N Larmor frequency by the pseudo-nuclear Zeeman effect.
(22) $A'(^{15}N2) \sim 0.6$ MHz corresponds to $A^{3/2}(^{14}N2) \sim 0.3$ MHz, which deviates from the value associated with the X-band ESEEM, $A^{3/2}(^{14}N2) \sim 0.5$ MHz. We attribute the difference to perturbation by the mutation at α-70, which modulates the $^{14/15}N_2$ hyperfine coupling constant.

JA0552489