CO Binding to the FeMo Cofactor of CO-Inhibited Nitrogenase: ^{13}CO and ^{1}H Q-Band ENDOR Investigation

Hong-In Lee,[†] Linda M. Cameron,[‡] Brian J. Hales,*[,‡] and Brian M. Hoffman*[,†]

Contribution from the Departments of Chemistry, Northwestern University, Evanston, Illinois 60208, and Louisiana State University, Baton Rouge, Louisiana 70803

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Abstract: The resting state of nitrogenase shows an $S = {}^3/_2$ electron paramagnetic resonance (EPR) signal resulting from the FeMo-cofactor ($MoFe_7S_9$:homocitrate) of the MoFe protein. When the enzyme undergoes turnover under a CO atmosphere, this signal disappears and two new ones appear: one under low pressure of CO (denoted lo-CO; 0.08 atm) and the other under high pressure of CO (denoted hi-CO; 0.5 atm). Our recent Q-band (35 GHz) ^{13}C and ^{57}Fe electron nuclear double resonance (ENDOR) studies demonstrated that one CO is bound to the FeMo-cofactor of lo-CO and two to the cofactor of hi-CO. [Christie, P. D.; Lee, H. I.; Cameron, L. M.; Hales, B. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1996**, *118*, 8707−8709. Pollack, R. C.; Lee, H. I.; Cameron, L. M.; DeRose, V. J.; Hales, B. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1995**, *117*, 8686−8687.] In the present report, we examine the CO-bound FeMo-cofactor in both the lo- and hi-CO forms of the MoFe protein from *Azotobacter vinelandii* by complete orientation-selective ^{13}C and ^{1}H ENDOR measurements. ^{1}H ENDOR studies reveal that well-resolved signals from a solvent-exchangeable proton seen in the resting state FeMo-cofactor are lost in both of the CO-inhibited forms, indicating a loss in hydrogen bonding as compared to the resting state. This supports the hypothesis that CO binds near the "waist" of the cofactor. Determination of ^{13}C hyperfine tensors of bound ^{13}CO to lo-CO and hi-CO leads to the suggestion that the single CO bound to the FeMo-cofactor of lo-CO may bridge or semibridge two iron ions, while each of the two CO bound to hi-CO is a terminal ligand. These ENDOR measurements and recent FTIR results of Thorneley and co-workers [George, S. J.; Ashby, G. A.; Wharton, C. W.; Thorneley, R. N. F. *J. Am. Chem. Soc.* **1997**, *119*, 6450−6451] provide strong mutual support.

Introduction

X-ray diffraction of the MoFe protein (*Av1*)[1] from *Azotobacter vinelandii* (*Av*)[1] and *Clostridium pasteurianum* (*Cp*) disclosed the structures of its two metal-ion clusters, the P-cluster (Fe_8S_7) and the FeMo-cofactor ($MoFe_7S_9$:homocitrate), the site of substrate reduction.[2−6] Since then, there has been much discussion of possible modes of substrate or inhibitor binding the cofactor.[3,7−11] However, these are put forth without any *experimental* evidence as to how substrates and inhibitors interact with either protein-bound cluster.

When nitrogenase turns over under a CO atmosphere, the EPR signal of the $S = {}^3/_2$ resting state cofactor disappears and two new ones appear: one under low pressure of CO (denoted lo-CO; 0.08 atm) with $\mathbf{g} = [2.09, 1.97, 1.93]$ and the other under high pressure of CO (denoted hi-CO; 0.5 atm) with $\mathbf{g} = [2.06, 2.06, 2.17]$.[12−15] Both EPR signals of CO-bound nitrogenase MoFe protein EPR signals arise from the FeMo-cofactor,[16−18] and a Q-band ^{13}C ENDOR study of the two forms gave the first direct observation of a diatomic molecule bound to this substrate binding cluster.[16] It showed that the cofactor of lo-CO has one bound CO; that of hi-CO has two. It further revealed a mechanistic relationship between the CO molecules bound to the two forms, showing that one of the two CO molecules bound to the cofactor in hi-CO is the CO molecule bound in lo-CO, and it gave information regarding the kinetic lability of bound CO.[16]

Enzymatic turnover of nitrogenase is known to involve the reduction of bound substrate with subsequent protonation of the reduced substrate. As such, understanding the mechanism of nitrogen fixation requires a determination of (1) the level of

[†] Northwestern University.
[‡] Louisiana State University.
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(1) Abbreviation used: CW, continuous wave; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; *Av*, *Azotobacter vinelandii*; *Av1*, MoFe protein of nitrogenase from *Azotobacter vinelandii* (*Av*); M center, FeMo cofactor of the MoFe protein of nitrogenase.

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reduction of the cofactor during catalysis, (2) the mode of substrate binding, and (3) the paths of both electron and proton transfer to the cofactor. In another paper,[18] we used orientation-selective ^{57}Fe ENDOR measurements to ascertain the level of reduction of the resting, lo-CO and hi-CO state of the cofactor through a determination of the metal-ion valencies in all three states. In the present paper, we use ^{13}C and ^{1}H ENDOR spectra of lo-CO and hi-CO forms of the MoFe protein from *Av* to address the second and third aspects of the catalytic mechanism, CO binding and proton transfer. Specifically, orientation-selective[19−23] ^{1}H and ^{13}C ENDOR results lead us to propose the binding region and modes of CO binding to the lo- and hi-CO states of the cofactor, while revealing that CO binding influences an important hydrogen bond to the cluster.

Materials and Methods

Sample Preparation. MoFe protein was prepared by standard procedures described elsewhere.[24] The CO-bound forms of the turnover state MoFe protein were prepared by adding Fe protein (in 25 mM Tris, 0.35M NaCl, pH = 7.4) to a solution of MoFe protein that had been equilibrated with CO at a partial pressure of 0.08 atm (lo-CO) or 0.5 atm (hi-CO) in a serum-capped Wheaton vial.[16] The initial concentrations of the proteins and reagents in the turnover mixture were as follows: 0.28 mM MoFe protein, 0.14 mM Fe protein, 50 mM $MgCl_2$, 100 mM Na_2ATP, 300 mM Na(phosphocreatine), 100 mM HEPES, 2 mg/mL creatine kinase, and 100 mM $Na_2S_2O_4$. The reaction was allowed to proceed at 25 °C for 5 min, then rapidly frozen by immersing the sample tube into liquid nitrogen. For H/D exchange, the resting-state MoFe protein was exchanged into D_2O and turned over in D_2O medium.

ENDOR Measurements. Continuous wave (CW) Q-band EPR and ENDOR spectra were recorded as described.[25−27] Q-band pulsed-ENDOR data were collected on a spectrometer described in detail elsewhere.[28] A stimulated-echo microwave pulse sequence, $\pi/2-\tau-\pi/2-T-\pi/2$, and a modified stimulated-echo microwave pulse sequence, $\pi/2-\tau-\pi/2-T-\pi/2-t-\pi$, were employed for Mims ENDOR[29−31] and Refocussed-Mims (ReMims) ENDOR[32] experiments, respectively. The $\pi/2$ and π pulses were 28 and 56 ns. To obtain the principal values and the relative orientations of the hyperfine tensors of the nuclei coupled to the electron spin center in the frozen-solution samples of CO-bound MoFe protein, 2-D datasets comprised of numerous ENDOR spectra collected across the EPR envelopes were analyzed as described elsewhere.[19−23]

Results

^{1}H ENDOR. The "single crystal-like" Q-band ^{1}H CW-ENDOR spectra taken at the high- or low-field edges of the EPR spectra of the resting, lo-CO, and hi-CO states of *Av1* are compared in Figure 1. The ^{1}H ENDOR of the resting state in H_2O buffer shows seven proton doublets with asymmetric intensities, as is often found in Q-band CW-ENDOR, centered at the ^{1}H Larmor frequency and split by the hyperfine couplings of $0.1 \le A \le 3.1$ MHz at g_3 (Figure 1A, H_2O). The spectrum obtained from the sample in D_2O buffer shows six doublets with some showing different phases in their ν_+ and ν_- pairs (Figure 1A, D_2O). As indicated by a "goal-post" mark in the figure, the comparison between the ^{1}H ENDOR spectra of the resting states in H_2O and D_2O buffers reveals a solvent-exchangeable

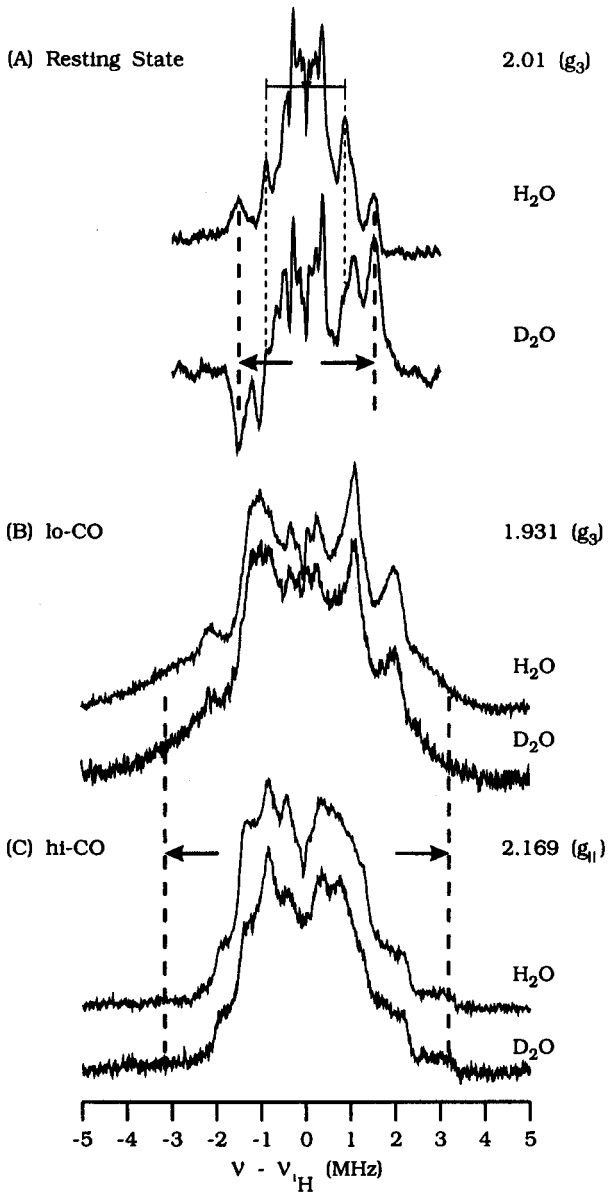

Figure 1. "Single crystal-like" Q-band CW ^{1}H ENDOR spectra of the (A) resting, (B) lo-CO, and (C) hi-CO states of the nitrogenase MoFe protein in H_2O or D_2O buffers. The spectra are centered at the ^{1}H Larmor frequencies. The doublet of the solvent-exchangeable proton in the resting state is indicated by a goal-post mark and thin dash-dot lines. The biggest ^{1}H hyperfine coupling of each state is indicated by arrows and thick dash-dot lines in each spectrum. Experimental conditions: microwave frequency, (A, H_2O) 35.050, (A, D_2O) 35.030, (B, H_2O) 34.977, (B, D_2O) 35.013, (C, H_2O) 34.075, and (C, D_2O) 35.136 GHz; modulation amplitude, 0.67 G; *g*-value, (A) 2.01, (B) 1.931, and (C) 2.169; RF power, (A) 30, (B; C, H_2O) 2.5 (chopped RF with 5% duty-cycle of 50 W), and (C, D_2O) 3.5 W (chopped RF with 5% duty-cycle of 70W); RF sweep speed, 0.2 MHz/s; temperature, 2 K.

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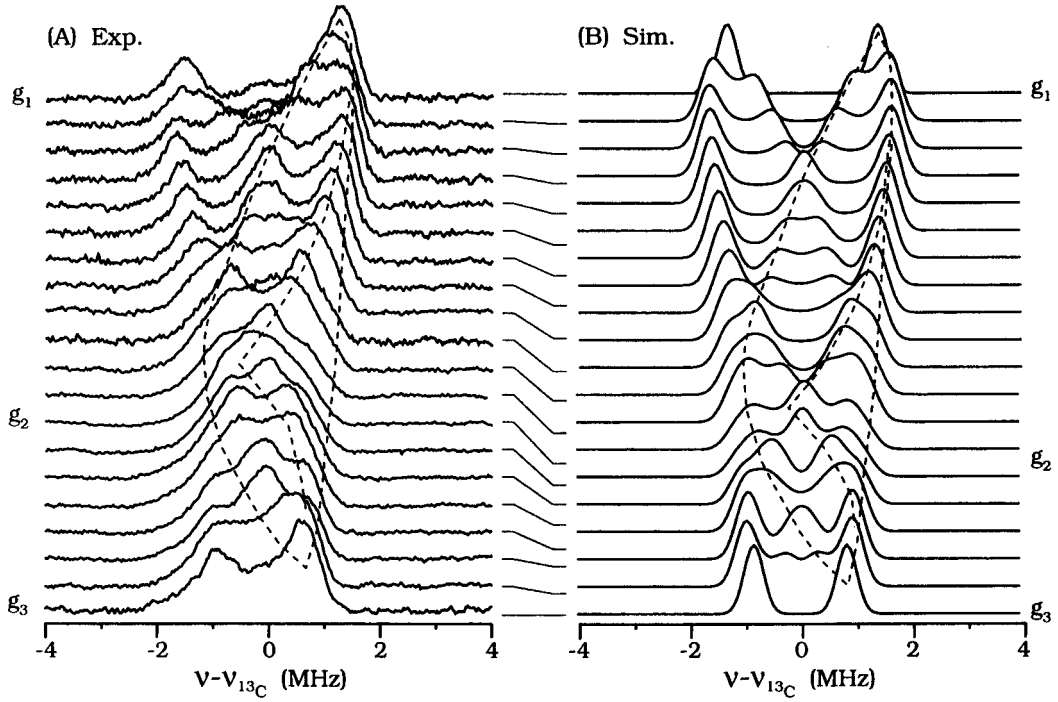

Figure 2. Q-band CW ^{13}C ENDOR spectra taken at several fields across the EPR envelope of (A) the lo-CO state of the MoFe protein under ^{13}CO and (B) their simulations. The spectra are centered at the ^{13}C Larmor frequencies. Dotted lines represent the ^{13}C ENDOR pattern of the one electron spin manifold. Simulation parameters are given in Table 1. Experimental conditions: microwave frequency, 34.992 GHz; modulation amplitude, 0.67 G; RF power, 30 W; RF sweep speed, 0.5 MHz/s; temperature, 2 K. The bandwidth of the RF excitation was broadened to 100 kHz.

proton with $A = 1.75$ MHz.[33−35] The MoFe crystal structure discloses as many as five possible hydrogen bonds to the cofactor. The side chains of Argα96, Hisα195, Argα359, and two backbone amides are hydrogen bond donors to sulfur atoms in the cofactor.[2−4,6] They provide the best candidates for the solvent-exchangeable proton observed in ^{1}H ENDOR. The signals of the lo- and hi-CO MoFe proteins show no loss of a doublet upon H/D exchange (Figure 1B,C), and Q-band CW- and Mims ^{2}H ENDOR spectra of lo-CO and hi-CO prepared in D_2O buffer showed only weak "matrix" ^{2}H signals. This indicates that CO binding abolishes the hydrogen bond to the cluster in both CO-inhibited forms.

It is most probable that in each of the states of the MoFe protein the nonexchangeable proton doublet with the largest hyperfine coupling, indicated by arrows in Figure 1, is from one (or both) of the β-protons of Cysα275.[36] The couplings of $A \sim 6.0$ MHz in lo-CO and $A = 6.4$ MHz in hi-CO are typical of those for a β proton of cysteine bound to an Fe ion of an FeS cluster. They are increased almost 2-fold from $A = 3.1$ MHz in the resting state, with this increase being assigned to the change in the spin state of the cluster. The hyperfine coupling of a nucleus associated with a ligand to a metal ion of a cluster is proportional to the spin-projection coefficient, K, of the bound metal ion.[37] Because this coefficient is inversely related to the total cluster spin, the observed hyperfine couplings should be less for the $S = {}^3/_2$ cluster, even if there is no change in the site hyperfine constants that describe the nucleus in the absence of spin coupling in the cluster. As confirmation, the result for the proton couplings parallels the roughly 2-fold smaller ^{57}Fe hyperfine in the $S = {}^3/_2$ resting state, compared to the $S = {}^1/_2$ CO-bound forms.[18] As each state shows signals from six nonexchangeable protons, no more than two can be associated with the β-protons of the single cysteine ligand, Cysα275. This leaves homocitrate and ring protons of Hisα442 as the most probable origins of the remaining signals.

^{13}C ENDOR. The $S = {}^3/_2$ EPR signal from the FeMo-cofactor of the resting state MoFe protein changes to $S = {}^1/_2$ states of the lo- and hi-CO states formed by binding of one CO (lo-CO) and two CO's (hi-CO) to the cofactor.[16,17] Figure 2A displays a full "2-D" set of Q-band ^{13}C CW-ENDOR spectra taken at fields across the EPR envelope of lo-CO. The hyperfine couplings are highly field dependent, with a maximum value of $A \sim 3.5$ MHz near g_1. This pattern is well simulated (Figure 2B) through calculations that use a hyperfine tensor with comparable isotropic and dipolar components: $\mathbf{A} = [-2.0, 3.5, 2.0] = 1.2 + [-3.2, 2.3, 0.8]$ MHz (Table 1).

Figure 3A shows Q-band ^{13}C CW and ReMims ENDOR spectra collected across the EPR envelope of hi-CO prepared with ^{13}CO. The spectrum taken at $g_{||}$ shows a doublet centered at the ^{13}C Larmor frequency with $A(^{13}\text{C1},g_{||}) = 5.8$ MHz, which represents a single CO [CO(1)] interacting with the cofactor. A second feature at $g_{||}$ that is best visualized in Mims ENDOR with the interval[29−31] $\tau = 400$ ns, (Figure 3B) comes from additional CO [CO(2)] bound to the cofactor. Previous ^{13}C

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(35) The corresponding solvent-exchanged ^{2}H is expected to have a hyperfine coupling of 0.27 MHz. CW ^{2}H ENDOR spectra show deuteron intensity, but the coupling is too small to resolve. In principle this size of coupling would be resolved in a Mims ENDOR when $\tau = \sim 2\ \mu$s. However, the phase relaxation time of the resting-state FeMo-co of *Av1* is less than 1 μs, too short for detection of the ^{2}H signal.

(36) Preliminary ^{1,2}H ENDOR experiments of the MoFe protein with deuterated homocitrate revealed the proton hyperfine couplings of the homocitrate are $\leq$1.5 MHz.

(37) The hyperfine couplings of a ligand to iron ion in FeS clusters are proportional to the spin-projection coefficient, K(Fe), of the coordinated iron ion. The observed hyperfine coupling constant of the ligand is given by $\mathbf{A}_p = K(\text{Fe})a_p$, where a_p is the intrinsic site hyperfine coupling constant.[55] See ref 18.

Table 1. ^{13}C Isotropic Hyperfine Coupling Constants of ^{13}CO Bound to Metal Ion/Clusters in Proteins

protein	^{13}CX	metal−sulfur cluster	^{13}C isotropic hyperfine coupling const (MHz)	ref
lo-CO, *Avl*[a]	CO	$MoFe_7S_9$	1.2[b]	this work
hi-CO, *Avl*[a]	CO(1)	$MoFe_7S_9$	5.37[c]	this work
	CO(2)	$MoFe_7S_9$	0.7[d]	this work
hydrogenase I, *Cp W5*[e]	CO	Fe_yS_z[f]	∼21	40
hydrogenase II, *Cp W5*[e]	CO	Fe_wS_x[f]	35.3	41
CODH, *Ct*[g]	CO	$NiXFe_{3-4}S_4$[h]	∼27	54
Pyrococcus furiosus	CN^-	Fe_4S_4	−3.0	45
Transferrin	CN^-	Fe^{III} [i]	35.50	43

[a] MoFe protein of nitrogenase from *Azotobacter vinelandii* (*Avl*). [b] The principle hyperfine tensor values are $\mathbf{A} = [-2.0, 3.5, 2.0] \pm 0.1$ MHz. The Euler angle of the hyperfine tensor with respect to the **g**-tensor frame is $(\alpha, \beta, \gamma) = (67.5°, 17.5°, 0°)$. The isotropic hyperfine couplings are magnitude values unless indicated. [c] The principal hyperfine tensor values are $\mathbf{A} = [5.8, 5.8, 4.5] \pm 0.05$ MHz, and $A_{||}$ is approximately perpendicular to $g_{||}$. Note, there are no data relating **g** axes of lo- and hi-CO. [d] The principal hyperfine tensor values are not well-defined, but have $\mathbf{A} \approx [0.6, 0.6, 0.9]$ MHz, with $A_{||}$ roughly along $g_{||}$. [e] Anaerobic N_2-fixing bacterium *Clostridium pasteurianum* (*Cp*) *W5*. [f] Cluster conformation is unknown. [g] Carbon monoxide dehydrogenase (CODH) from the acetogenic bacterium (*Clostridium thermoaceticum* (*Ct*). [h] Center C of CODH from *Ct*. Cluster conformation is unknown. [i] Low-spin Fe^{III}.

ENDOR studies of "pulse-chase" experiments discovered that the bound CO of the lo-CO form becomes the CO(1) of hi-CO.[16]

The field dependence of the CW and ReMims ENDOR data (dotted lines) for ^{13}C1 (Figure 3A) is typical for a hyperfine interaction that is dominated by the isotropic component, with a dipolar contribution whose principal axis is perpendicular to $g_{||}$: $\mathbf{A}(C1) = [5.80, 5.80, 4.50] = 5.37 + [0.43, 0.43, -0.87]$ MHz (Table 1). At all fields the weakly coupled ^{13}C (^{13}C2), Figure 3B, shows a sharp doublet with $0.6 \leq \mathbf{A} \leq 0.9$ MHz that rides on a broad background doublet with $\mathbf{A} \sim 1.5$ MHz. The sharp ^{13}C2 ENDOR pattern (dotted lines) in Figure 3B is also dominated by an isotropic contribution, with a small axial hyperfine term parallel to $g_{||}$: $\mathbf{A} = [0.6, 0.6, 0.9] = 0.7 + [-0.1, -0.1, 0.2]$ MHz (Table 1). The broad feature in Figure 3B could be yet a third CO or a distribution of alternate, poorly defined binding geometries of CO(2).

Discussion

Hydrogen Bonding to the Cluster. Catalytic turnover of nitrogenase involves the reduction of substrate with subsequent protonation of the reduced substrate. As such, understanding the mechanism of nitrogen fixation requires a determination of the paths of both electron and proton transfer to the cofactor. Therefore, the key observation in the ^{1}H ENDOR measurements is the loss of the solvent-exchangeable proton signal that is observed in the resting state and that arises from hydrogen bonding to the sulfide ions of the cofactor. The crystal structure revealed five putative hydrogen bond donors as the obvious candidates for the solvent-exchanged proton ENDOR signal: the side chains of Argα96, Hisα195, and Argα359 and the backbone amides of Glyα356 and Glyα357 in the resting state.[2−6] These hydrogen bonds are located in the central "waist" region on the cofactor. Thus we suggest that the CO binds to Fe in the waist area so as to perturb a side chain interaction with the sulfur and hence abolish the exchangeable ^{1}H hyperfine coupling. We speculate that this side chain residue interacts with substrate bound in or near the site of CO binding. ^{1}H ENDOR measurements on resting-state nitrogenase mutants are probing this issue.[38]

^{13}C Hyperfine Couplings. The ^{13}C and ^{57}Fe ENDOR studies of the CO-bound turnover states of nitrogenase clearly identify [FeMo-co][CO]$_n$ as the origin of the EPR signals from both lo-CO ($n = 1$) and hi-CO ($n = 2$) states.[16,17] The characteristics of the ^{13}C hyperfine tensor as determined by the ENDOR measurements provide information about the mode of binding of the bound diatomic inhibitor (CO) in lo-CO and hi-CO. The ^{13}C hyperfine interaction of metal-bound ^{13}CO is the sum of isotropic and anisotropic interactions: $\mathbf{A} = \mathbf{A}_{iso} + \mathbf{A}_{aniso}$. The isotropic interaction originates from direct spin delocalization into the carbon 2s orbital through the M−C σ-bond between metal d-orbital and carbon sp-hybrid as well as from spin polarization of the 2s(C) orbital, either by spin density on the coordinate metal ion acting on the σ-bond or by spin density that is delocalized into carbon p-orbitals. The anisotropic interaction is the sum of contributions from (i) spin density that is in the p_σ-orbital of the carbon directed toward the metal because of the M−C σ-bond or polarization by metal-ion spin, (ii) spin density in the p_π-orbital of C that is generated by the back-donation of the metal d-electrons, and (iii) the direct dipole−dipole interaction between the carbon nuclear spin and the electron spin on the metal.[39] All three of these anisotropic contributions have axial symmetry, but the first and third would be coaxial, with the symmetry axis lying along the M−C bond, while that for a single p_π-orbital on the carbon would have its symmetry axis perpendicular to that bond.

Previous ^{13}C ENDOR studies of ^{13}CO bound to Fe−S clusters of hydrogenase I and II showed $\mathbf{A}_{iso} = 20-35$ MHz (Table 1).[40,41] Hyperfine tensors were essentially isotropic, with a relatively small and axial anisotropic contribution ($\mathbf{A}_{aniso} = [-T/2, -T/2, T]$) where $|T/\mathbf{A}_{iso}| = 0.1-0.2$). This suggests that the CO binds terminally to a single Fe, and that the ^{13}C anisotropic term is a combination of the coaxial contributions from spin density in the σ-bonding p-orbital of the carbon and the through-space dipole coupling to spin on Fe.

The ^{13}C hyperfine interaction tensors of both ^{13}CO molecules bound to hi-CO are similar to those of the ^{13}CO-bound hydrogenases, in that they are dominated by the isotropic component, with relatively small, axially symmetric anisotropic contributions ($|T/\mathbf{A}_{iso}| \sim 0.2$). The magnitude of the couplings to ^{13}CO in hi-CO is much less than that seen with other systems (Table 1). The form of the tensor suggests to us that the CO in hi-CO likewise are terminally bound. The small magnitude could arise in part because the diatomics are bound to metal ions with small spin-projection coefficients, K.[42] However,

(38) Mutation at Argα359 to Lysα359 did show the solvent-exchangeable signal in the resting state. Hence, Argα359 is ruled out as the candidate.

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(42) The small degree of hyperfine anisotropy and poor resolution preclude a good determination of the relative orientations of CO(1) and CO(2). However, taken at face value, the orientations of the hyperfine tensors listed in Table 1 suggest that the two CO molecules bind with the Fe−CO bonds roughly perpendicular.

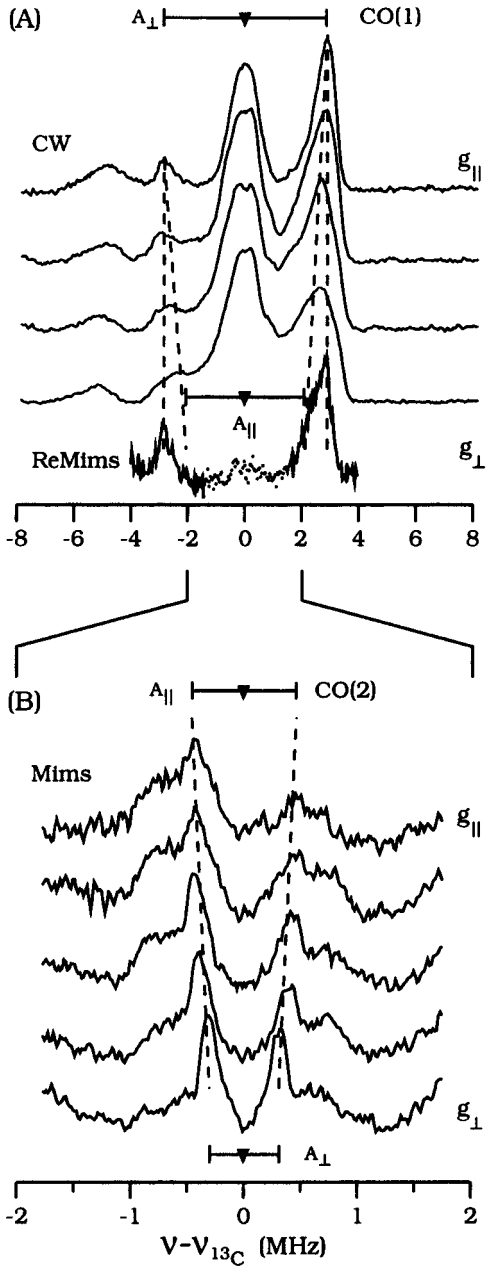

Figure 3. (A) Q-band CW ReMims and (B) Mims ^{13}C ENDOR spectra taken at several fields across the EPR envelope of the hi-CO state of the MoFe protein under ^{13}CO for (A) CO(1) and (B) CO(2), respectively (see text). The spectra are centered at the ^{13}C Larmor frequencies. ^{13}C ENDOR patterns are depicted by dotted lines. Experimental conditions: microwave frequency, (A, CW) 35.073, (A, ReMims) 34.636, and (B) 34.646 GHz; modulation amplitude, (A, CW) 0.67 G; RF power, (A, CW) 20 W; RF sweep speed, (A, CW) 0.5 MHz/s; τ, (A, ReMims) 100 and (B) 400 ns; repetition rate, (A, ReMims; B) 33 Hz; RF pulse width, (A, ReMims; B) 30 μs; number of transients, (A, ReMims) 480 and (B) 200−500; temperature, 2 K. The bandwidth of the RF excitation was broadened to 100 KHz for the CW experiments.

comparison with the results for the isoelectronic $^{13}CN^-$ bound to iron centers shows that this is not necessarily so (Table 1). Cyanide bound to a mononuclear ferric center (which is formally equivalent to a cluster site with $K = 1$) shows large, primarily isotropic, hyperfine couplings, like those of the ^{13}C-bound hydrogenases.[43,44] However, the hyperfine coupling to $^{13}CN^-$ terminally bound to a ferrous site with $K = 0.89$ in the [4Fe-4S]$^+$ cluster of *Pyrococcus furiosus* (*Pf*-Fd) is very similar in magnitude to that for CO(1).[45,46]

The ^{13}C hyperfine tensor of lo-CO shows quite different characteristics. Here, the hyperfine tensor, $\mathbf{A} = \mathbf{A}_{iso} + \mathbf{A}_{aniso} = 1.2 + [-3.2, 2.3, 0.8]$ MHz, is dominated by the anisotropic interaction ($|T/\mathbf{A}_{iso}| = 2.7$), not the isotropic interaction as in hi-CO and hydrogenase-CO. Furthermore, the anisotropic component of the ^{13}CO hyperfine tensor of lo-CO does not have the simple axial form, $[-T/2, -T/2, T]$, as seen for the hi-CO and hydrogenase-CO, but is rhombic. Because each of the observed tensor components is multiplied by the same K,[37] the unusual character of the ^{13}C hyperfine tensor of lo-CO implies to us that the site hyperfine tensor, and thus the binding of CO in lo-CO, is quite different from that in hydrogenase and hi-CO. The rhombicity of $\mathbf{A}_{aniso}$ in lo-CO implies that it is the result of a summation of two or more of the three types of noncoaxial contributions listed above. One might suggest that the observed tensor is associated with a terminal CO where the M−C σ-bond gives a minimal contribution to both isotropic and anisotropic terms, while the anisotropic term is a combination of the noncoaxial M−C point dipole and the local p_π terms, but this does not seem plausible for CO terminally bound to a high-spin ferrous or ferric ion. It might arise from a terminal CO bound to a low-spin Fe, but such an iron would have a strongly anisotropic ^{57}Fe hyperfine interaction, and nothing of this sort has been seen.[18,47] We suggest instead an alternative model where the CO in lo-CO forms some type of bridge between two metal ions in the cluster. In this case the point-dipole interactions and local terms from σ-spin density due to the bond from a single ion would be coaxial, but the interactions with one ion would not be coaxial with those from the other. The result plausibly gives rise to a tensor such as is seen.

In the FeMo-cofactor, there are *a priori* eight possible CO-binding metal sites, including Mo. However, the ^{1,2}H ENDOR results suggest that CO binds near the waist of the cofactor, and thus away from Mo. (Note also, one of the ligands in the saturated ligand-coordination environment of Mo would have to be displaced for the CO binding during the turnover). Moreover, Mo−CO bonding needs d-electron back-donation from Mo to π^*-orbitals of CO, which requires a low oxidation state of Mo, while in another paper it is proposed that Mo in the CO-inhibited FeMo-cofactor is in the same Mo(IV) state as in the resting state,[18] which is in accordance with Mo EXAFS measurements which indicate that Mo does not change valence nor bind CO in CO-inhibited MoFe protein.[48] We thus propose that the Mo site is not involved in either terminal or bridging CO binding.

These considerations lead to a model in which one CO binds to the lo-CO cofactor as a bridge between two of the coordinatively unsaturated waist Fe ions, while two bind there as terminal ligands to different Fe ions in hi-CO, as in Figure 4.[49] The crystal structure and EXAFS studies of the cofactor in the resting state of *Av1* revealed the average distance between two

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(47) However, such a tensor could lead to ^{57}Fe signals that are difficult to detect by ENDOR, and so this decision calls for confirmatory evidence from Mössbauer spectroscopy.

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(49) The distance between two bridged ions (~2.5 Å) is suggestive of Fe−Fe bonding interaction for the bridged ions.[3] This fourth bond might be replaced with the Fe−C−Fe bond.

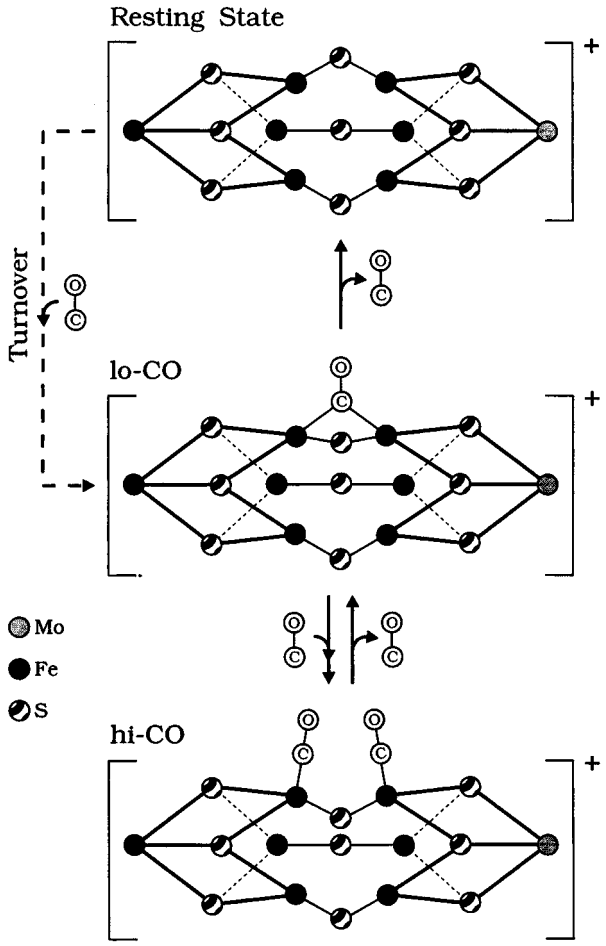

Figure 4. Scheme for CO binding to the FeMo-cofactor as discussed in this study. The cluster charge was determined in another paper.[18]

such iron sites is ~2.5 Å,[2−6,50] the same as the average distance between irons in binuclear iron carbonyl compounds which have bridging carbonyls.[51] The previous ^{13}C ENDOR study of lo-CO and hi-CO[16] showed that lo-CO and hi-CO are interconvertible and the CO(1) of hi-CO originates from the CO of lo-CO. This implies that the CO binding site of lo-CO is not changed when it converts to hi-CO.

These ENDOR experiments are wholly consistent with FTIR measurements of CO-bound nitrogenase from *Klebsiella pneumoniae* (*Kp*) that appeared after submission of the present work.[52] First, experiments under turnover conditions with a high concentration of CO led Thorneley and co-workers[52] to conclude that hi-CO contains two CO that are terminally bound to two different metal sites, with vibrations at 1936 and 1958 cm^{-1}; a third band may be associated with another CO. Under conditions of low CO concentration they observed a single band and concluded that lo-CO contains one bound CO. Although they suggest that it too is terminally bound, in fact its C−O stretching frequency (1906 cm^{-1}) is much lower than those for CO bound to hi-CO, and falls just at the conventionally accepted boundary between terminal and bridging CO.[53] Hence, Figure 4 provides a CO-binding scheme that accommodates both the ENDOR and FTIR data, with the understanding that the bridging CO for lo-CO may well involve an asymmetric or "semi-bridging" CO.

Conclusion

The recent ^{13}C and ^{57}Fe ENDOR studies of the EPR-visible lo-CO and hi-CO states of nitrogenase formed during turnover in the presence of CO identified [FeMo-co][CO]$_n$ as the origin of the EPR signals: lo-CO ($n = 1$) and hi-CO ($n = 2$, and now possibly 3).[16,17] ^{1}H ENDOR studies now reveal that the well-resolved signal from a solvent-exchangeable proton seen in the resting state M center is lost upon CO binding. This is interpreted as a loss of a hydrogen bond to a sulfide of the cluster, indicating the CO binds near the "waist" of the cofactor, likely at or near a site of substrate binding. The complete ^{13}C hyperfine tensors of bound ^{13}CO show substantial differences in the bonding characteristics of the single CO bound to the M center of lo-CO and the two CO molecules bound to hi-CO. We have suggested that the CO of lo-CO may bridge, or semibridge, two iron ions, while each of the two CO bound to hi-CO is a terminal ligand. A scheme linking the three enzyme forms is presented in Figure 4. The ENDOR measurements and recent FTIR results of Thorneley and co-workers[52] provide strong mutual support.

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