# Metal-Ion Valencies of the FeMo Cofactor in CO-Inhibited and Resting State Nitrogenase by $^{57}$Fe Q-Band ENDOR

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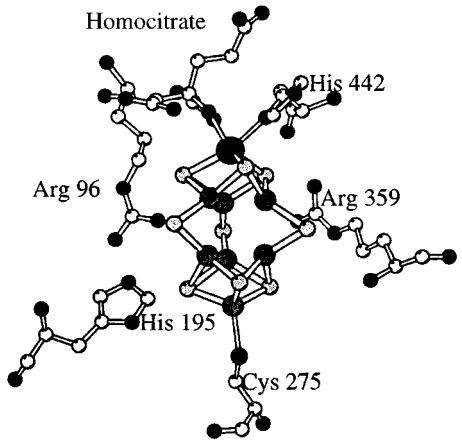
**Abstract:** The resting state of nitrogenase shows an $S = {}^3/_2$ electron paramagnetic resonance (EPR) signal resulting from the FeMo-cofactor (M-center; inorganic portion, [Mo, $Fe_7$, $S_9$]) of the MoFe-protein. When the enzyme undergoes turnover under a CO atmosphere, this signal disappears and two new ones appear: one under low pressure of CO (denoted lo-CO; 0.08 atm) with $g = [2.09, 1.97, 1.93]$ and the other under high pressure of CO (denoted hi-CO; 0.5 atm) with $g = [2.06, 2.06, 2.17]$. Our recent Q-band (35 GHz) $^{57}$Fe and $^{13}$C electron nuclear double resonance (ENDOR) studies clearly identified [FeMo-cofactor][CO]$_n$, as the origin of the EPR signals from both lo-CO ($n = 1$) and hi-CO ($n = 2$) [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 and 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], and a previous paper discusses CO binding in detail [Lee, H. I.; Cameron, L. M.; Hales, B. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1997**, *119*, 10121−10126]. We now present complete orientation-selective $^{57}$Fe ENDOR measurements of the CO-bound FeMo-cofactor in both lo- and hi-CO forms of the MoFe-protein from *Azotobacter vinelandii*. The $^{57}$Fe ENDOR signals associated with the seven Fe ions of the FeMo-cofactor of lo-CO can be completely assigned and interpreted in terms of four magnetically distinct iron signals. Analysis of these signals following the procedures of Mouesca *et al*. [Mouesca, J.-M.; Noodleman, L.; Case, D. A.; Lamotte, B. *Inorg. Chem.* **1995**, *34*, 4347−4359] has led us to propose valence assignments and charges for the cofactor cluster, [Mo, $Fe_7$, $S_9$]$^+$ = [$Mo^{4+}$, $Fe^{3+}{}_1$, $Fe^{2+}{}_6$, $S^{2-}{}_9$]$^+$, organized into one $Fe^{2.5+}$ pair and five $Fe^{2+}$ ions, [$Mo^{4+}$, $(2Fe^{2.5+})_1$, $Fe^{2+}{}_5$, $S^{2-}{}_9$]$^+$. The result is a formal d-electron count of 43. ENDOR and functional studies indicate that the lo-CO, hi-CO, *and* resting states of the M-center are all at the same oxidation level. Hence, the proposed valency assignments apply to all three states.

## Introduction

Nitrogenase, which is comprised of the electron-transfer Fe-protein and the catalytic MoFe-protein, catalyzes the reduction of dinitrogen to ammonia, a key reaction of the biological nitrogen cycle.[1−3] X-ray diffraction of the MoFe-protein from *Azotobacter vinelandii* (*Av*)[4] and *Clostridium pasteurianum* (*Cp*) disclosed the structures of two clusters in the MoFe-protein, P-cluster ($Fe_8S_7$), and FeMo-cofactor ($MoFe_7S_9$:homocitrate), the site of substrate reduction.[5−9] This paper presents a proposal for the valencies of the metal ions and the charge on the cofactor.

The resting state of nitrogenase shows an $S = {}^3/_2$ electron paramagnetic resonance (EPR) signal resulting from the FeMo-cofactor;[10−16] 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) with $g = [2.09, 1.97, 1.93]$ and the other under high pressure of CO




FeMo-cofactor and selected residues

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(4) 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 MoFe-protein of nitrogenase.
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(denoted hi-CO; 0.5 atm) with $g$ = [2.06, 2.06, 2.17].[17−20] Generally, CO noncompetitively inhibits the reduction of all substrates except for protons.[1−3] Early selective $^{57}$Fe labeling of the Fe and MoFe-proteins showed that these signals are associated with the latter but did not reveal whether they arose from the P-cluster or the FeMo-cofactor (M-center).[17−20] Our recent Q-band (35 GHz) electron nuclear double resonance (ENDOR)[21,22] study of a suite of $^{57}$Fe MoFe isotopomers showed that both the lo- and hi-CO EPR signals were from CO-bound M-center.[23,24]

In the present paper, we examine the the CO-bound FeMo-cofactor in both lo- and hi-CO forms of the *Av* MoFe-protein by complete orientation-selective[21,22,25−27] $^{57}$Fe ENDOR measurements. Consideration of the $^{57}$Fe ENDOR data for lo-CO in light of recent discussions of spin coupling in iron clusters by Mouesca *et al.*[28] allows us to propose the valencies and d-electron count of the metal ions in the FeMo-cofactor in the lo-CO form. The lo-CO, hi-CO, and resting states all are interconvertible without redox or catalytic processes, which led us to infer that the cofactor in all three states is at the same redox level, hence the conclusions apply to all three protein forms.[23,24,29] The existence of different spin states at a given oxidation level is well-known for FeS clusters.[30] A previously published paper[31] describes the properties of the bound CO and the influence of CO binding on hydrogen bonding to the cofactor.

## Materials and Methods

**Sample Preparation.** MoFe-protein in which the Fe components of the metal clusters had either naturally isotopic abundance or were uniformly labeled with $^{57}$Fe was prepared by standard procedures described elsewhere.[32] The CO-bound forms of the turnover state MoFe-protein were prepared by adding Fe protein (in 25 mM Tris, 0.35 M 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.[23,24,33] 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 sodium 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 and then rapidly frozen by immersing the sample tube into liquid nitrogen.

**ENDOR Measurements.** Continuous wave (CW) Q-band EPR and ENDOR spectra were recorded at 2 K in dispersion mode under "rapid-passage" conditions, as described elsewhere.[34−36] The band width of the RF excitation was broadened to 100 kHz.[37] The first-order ENDOR spectrum of a nucleus with $I = {}^1/_2$ in a single paramagnetic center is a doublet with frequencies given by[38]

$$\nu_\pm = |\nu_N \pm A^N/2| \quad (1)$$

Here, $\nu_N$ is the nuclear Larmor frequency and $A^N$ is the angle-dependent hyperfine coupling constant, and the doublet is centered at $A^N/2$ when $\nu_N < A^N/2$. The ENDOR pattern is observed as either a hyperfine-split or Larmor-split doublet depending on the relative magnitudes of $\nu_N$ and $A^N$. 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.[21,22,25−27]

## Results

**EPR.** The resting state of the MoFe-protein from *Av* shows a well-defined rhombic EPR signal ($g$ = [4.33, 3.77, 2.01]) arising from the lower Kramers' doublet ($m_S = \pm{}^1/_2$) of the $S = {}^3/_2$ FeMo-cofactor (M-center).[14−16] When the protein turns over under CO, the EPR signal of the resting state disappears and two new signals ($S = {}^1/_2$) associated with the cofactor appear: one is formed under low pressure of CO (lo-CO) by binding of a single CO and has $g$ = [2.09 1.97 1.93]; the other forms under high pressure (hi-CO) by binding of a second CO and has $g$ = [2.06, 2.06, 2.17].[17−20,23] The change from the rhombic $g$-tensor of lo-CO to the axial one of hi-CO upon binding an additional CO is analogous to that observed upon CO binding to the oxidized iron−sulfur center of Hydrogenase I from *C. pasteurianum* (*Cp*) *W5*.[39]

**$^{57}$Fe ENDOR.** Figure 1 shows the "single-crystal-like" $^{57}$Fe ENDOR spectra obtained from the FeMo-cofactor of globally $^{57}$Fe ($I = {}^1/_2$)-enriched lo- and hi-CO samples. Considering the lo-CO spectrum, three $\nu_\pm(^{57}\text{Fe})$ doublets as described by eq 1 are unambiguously identified at the low-field edge ($g_1$) of lo-CO, with hyperfine couplings of $|\boldsymbol{A}| = 15$ ($Fe_a$), 25 ($Fe_b$), and 37 ($Fe_c$) MHz. Careful consideration shows that we must assign a fourth doublet, $Fe_d$, because of the broad feature that overlies what should otherwise be a sharp $\nu_-$ peak of $Fe_a$. Two iron sites were previously identified at the other edge of the EPR envelope ($g_3$), with $|\boldsymbol{A}| = 18$ ($Fe_e$) and 34 ($Fe_f$) MHz;[24] the 2-D, field-dependent plots to be discussed lead to the assignment of a third, with $|\boldsymbol{A}| = 30$ MHz ($Fe_g$) as shown. For hi-CO, two types of iron site are unambiguously identified at $g_{\text{lb}}$, with the hyperfine couplings of $|\boldsymbol{A}| = 22$ ($Fe_h$) and 33 ($Fe_i$) MHz; one additional possible doublet at $g_{\text{ll}}$ (hi-CO) is marked with an open circle (○).

Full 2-D sets of $^{57}$Fe ENDOR spectra were taken at fields across the EPR spectrum of each sample; Figure 2A is such a

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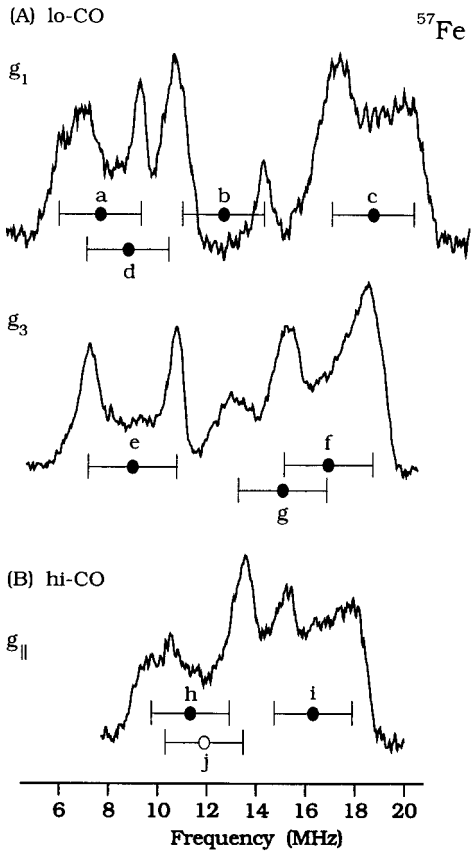


**Figure 1.** "Single-crystal-like" Q-band CW $^{57}$Fe ENDOR spectra of the (A) lo-CO and (B) hi-CO states of the globally $^{57}$Fe-enriched nitrogenase MoFe-protein. The "goal-post" marks indicate $^{57}$Fe doublets centered at $A_{Fe}/2$ (●). *Experimental conditions*: microwave frequency, (a) 35.160 and (b) 34.960 GHz; modulation amplitude, 0.67 G; RF power, 30 W; RF sweep speed, 1 MHz/s; *g*-value, (A) 1.931, 2.086, and (B) 2.169; temperature, 2 K. The band width of the RF excitation was broadened to 100 kHz.

set for lo-CO. The spectra display a rich array of features from ~6 to ~22 MHz, corresponding to $15 \leq |\boldsymbol{A}| \leq 41$ MHz. Although the features are severely overlapped, essentially all of them can be assigned. As indicated, there are clearly three $\nu_{\pm}$ doublets, separated by $\sim 2\nu_{Fe}$, that run across the EPR envelope. These correspond to three iron sites, $Fe_{\beta 1}$, $Fe_{\alpha 1}$, and $Fe_{\alpha 2}$, whose assignment correlates the $Fe_a$, $Fe_b$, and $Fe_c$ doublets at $g_1$ with the $Fe_e$, $Fe_f$, and $Fe_g$ doublets at $g_3$, respectively (Figure 1A). The hyperfine tensors obtained for these three sites by a simulation of the 2-D ENDOR pattern are largely isotropic, as is common for Fe ions in Fe−S clusters; the isotropic components are listed in Table 1, and the full tensors are given in footnotes to Table 1. A single $\nu_-$ feature of a fourth site, $Fe_{\beta 2}$, also can be followed through the envelope (doublet $Fe_d$ in Figure 1A). Its presence among more intense peaks makes it impossible to get complete hyperfine information for this site, but the tensor must also be largely isotropic; an estimate for the isotropic interaction is contained in Table 1. There are no additional $^{57}$Fe signals with higher values of $|\boldsymbol{A}_{iso}|$; if present, these would have been detected easily. Careful examination of the low-frequency region around $\nu_{Fe}$ also failed to disclose any signals from sites with small values of $|\boldsymbol{A}_{iso}|$. In other work on low-spin polynuclear centers, we have detected signals with $|\boldsymbol{A}_{iso}| < 1$ MHz.[40] The $^{57}$Fe ENDOR spectra taken across the EPR envelope of hi-CO are shown in Figure 2B. In this case, the $^{57}$Fe signals span 7−19 MHz, corresponding to

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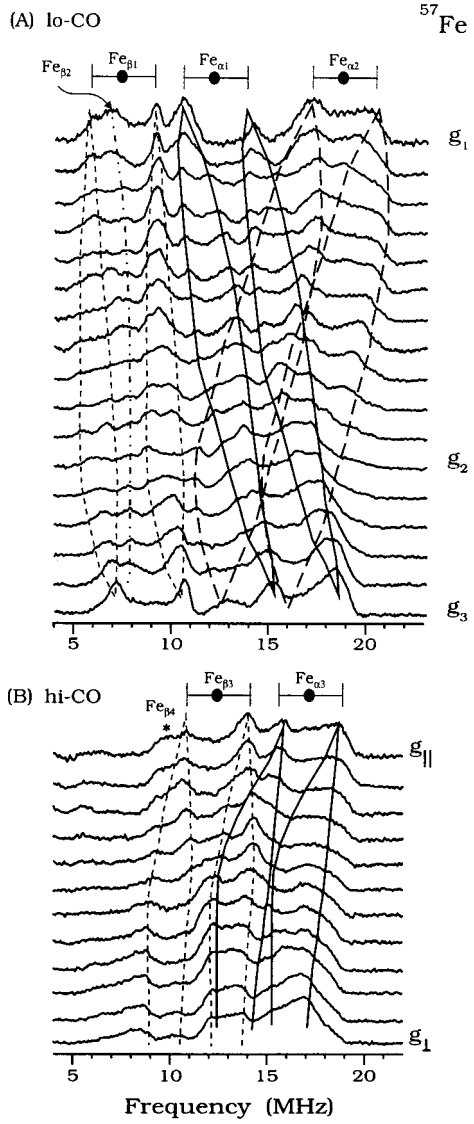


**Figure 2.** Q-Band CW $^{57}$Fe ENDOR spectra taken at fields across the EPR envelopes of (A) lo-CO and (B) hi-CO states of the globally $^{57}$Fe-enriched MoFe-protein. (A) The doublet patterns of the $Fe_{\beta 1}$, $Fe_{\alpha 1}$, and $Fe_{\alpha 2}$ sites in lo-CO are indicated by "goal-post" marks and their experimental variation with magnetic field are indicated by "•••", "—", and "- - -", respectively. $Fe_{\beta 2}$ is indicated by the dash−dot line. (B) Doublet patterns of $Fe_{b3}$ and $Fe_{a3}$ are indicated by "goal-post" marks and "•••" and "—", respectively, in hi-CO. The asterisk (*) represents $Fe_{\beta 4}$ (B). *Experimental conditions* are as in Figure 1.

$17 \leq |\boldsymbol{A}| \leq 35$ MHz, essentially the same range as for lo-CO. Detailed analysis of this complex pattern is not feasible because of the reduced orientation selectivity with an axially symmetric ***g***-tensor. However, from the field-dependent studies, the iron sites of $Fe_j$ and $Fe_i$ in Figure 1B are now clearly identified respectively with $Fe_{\beta 3}$ and $Fe_{\alpha 3}$ in Figure 2B. The spectrum of hi-CO contains signals from at least one more distinct site, marked with an asterisk ($Fe_{\beta 4}$) in Figure 2B (site $Fe_h$ in Figure 1B). Hence, in hi-CO, a minimum of three magnetically distinct iron sites are identified. The estimated hyperfine tensors are in the footnotes to Table 1.

## Discussion

**$^{57}$Fe Hyperfine Couplings.** Spin coupling in multimetallic Fe clusters can be analyzed through examination of the isotropic $^{57}$Fe coupling constants. Table 1 contains the $^{57}$Fe isotropic couplings for the $S = {}^1/_2$ lo-CO and hi-CO forms of the M-center

**Table 1.** $^{57}$Fe Isotropic Hyperfine Coupling Constants of $^{57}$Fe-Enriched Fe−S Clusters

| cluster spin state | enzyme | $^{57}$Fe site | isotropic hyperfine coupling constant (MHz)[a] | ref |
|---|---|---|---|---|
| $^1/_2$ | FeMo−CO *Av1* | $Fe_{\alpha 1}$ | −30[c] | |
| | (lo-CO)[b] | $Fe_{\alpha 2}$ | −31[d] | this work |
| | | $Fe_{\beta 1}$ | 16[e,f] | |
| | | $Fe_{\beta 2}$ | ∼17[f,g] | |
| | FeMo−CO *Av1* | $Fe_{\alpha 3}$ | 31[h] | |
| | (hi-CO)[b] | $Fe_{\beta 3}$ | 24[i] | this work |
| | | $Fe_{\beta 4}$ | ∼22[g] | |
| | aconitase (E)[j] | $Fe^{2.5+}$ | −39, −37 | 28,59 |
| | | $Fe^{2.0+}$ | ∼+16, +33 | |
| | aconitase (ES)[k] | $Fe^{2.5+}$ | −36, −40 | 28,59 |
| | | $Fe^{2.0+}$ | ∼+16, +29 | |
| | *Pf*-Fd-CN[l] | $Fe^{2.5+}$ | −32, −24 | 60 |
| | | $Fe^{2.0+}$ | +17, +15 | |
| | *Pf*-Fd[l] | $Fe^{2.5+}$ | −31, −35 | 60 |
| | | $Fe^{2.0+}$ | +21 (×2) | |
| $^3/_2$ | FeMo−CO *Av1* | $A^1$ | −15 (−18) | 61 |
| | (resting state)[b,m] | $A^2$ | −17 | |
| | | $A^3$ | −12 | |
| | | $B^1$ | +11 | |
| | | $B^2$ | +13 (10) | |
| | *Av2*/urea | $Fe^{2.5+}$ | −7.8 (×2) | 28,62 |
| | | $Fe^{2.0+}$ | −4.1 (×2) | |
| | $[Fe_4S_4(SC_6H_{11})_4]^{3-}$ | $Fe^{2.5+}$ | −8.9 (×2) | 28,63 |
| | | $Fe^{2.0+}$ | −8.9 (×2) | |
| | $2[Fe_4Se_4(SR)_4]^{3-}$ | $Fe^{2.5+}$ | −3.8 (×2) | 28,64 |
| | CpFd | $Fe^{2.0+}$ | −3.8 (×2) | |

[a] Clusters refer to an $[Fe_4S_4]^+$ core except for the $[MoFe_7S_9]$ core of FeMo−CO. Signs are indicated where available. (×2) indicates a spin-delocalized iron pair. [b] *Av*1 = MoFe-protein of nitrogenase from *Azotobacter vinelandii* (*Av*). [c] The principal hyperfine tensor values are $A = -[24, 31, 34] \pm 1$ MHz. Euler angle of the hyperfine tensor with respect to *g*-tensor frame: $(\alpha, \beta, \gamma) = (0°, 15°, 0°) \pm 10°$. [d] The principal hyperfine tensor values are $A = -[39, 26, 27] \pm 1$ MHz. Euler angle of the hyperfine tensor with respect to *g*-tensor frame: $(\alpha, \beta, \gamma) = (15°, 20°, 0°) \pm 10°$. [e] The principal hyperfine tensor values are $|\boldsymbol{A}| = [15, 14, 18] \pm 1$ MHz. Euler angle of the hyperfine tensor with respect to *g*-tensor frame: $(\alpha, \beta, \gamma) = (10°, 0°, 0°) \pm 10°$. [f] The two Fe sites of lo-CO with $A_{iso} \approx 16$ MHz are taken to represent 5 Fe ions; see the text. [g] Estimated magnitude of isotropic hyperfine coupling constant. [h] The principle hyperfine tensor values are $|\boldsymbol{A}| = [27, 31, 34] \pm 1$ MHz. Euler angle of the hyperfine tensor with respect to *g*-tensor frame: $(\alpha, \beta, \gamma) = (0°, 20°, 0°) \pm 10°$. [i] The principle hyperfine tensor values are $|\boldsymbol{A}| = [21, 25, 25] \pm 1$ MHz. Euler angle of the hyperfine tensor with respect to *g*-tensor frame: $(\alpha, \beta, \gamma) = (0°, 10°, 0°) \pm 10°$. [j] Substrate-free reduced aconitase. [k] Substrate-bound aconitase. [l] *Pf*-Fd-CN = Hyperthermophilic archeon *Pyrococcus furiosus* (*Pf*) reduced ferredoxine ($S = {}^1/_2$) with bound $CN^-$. *Pf*-Fd = reduced ferredoxine ($S = {}^1/_2$). [m] The values in parentheses are alternative assignments.

of the MoFe-protein, for the $S = {}^3/_2$ resting state M-center, as well as for selected 4Fe−4S clusters with both values of the total spin. The seven iron ions of the lo-CO M-center give rise to four distinct types of $^{57}$Fe signals, two with an isotropic coupling of $|\boldsymbol{A}_{iso}| \approx 31$ MHz ($Fe_{\alpha 1}$, $Fe_{\alpha 2}$) and two with $|\boldsymbol{A}_{iso}| \approx 16$ MHz ($Fe_{\beta 1}$, $Fe_{\beta 2}$). The hi-CO M-center exhibits ENDOR signal from three distinct types of $^{57}$Fe sites, with roughly comparable couplings, $22 \leq |\boldsymbol{A}_{iso}| \leq 31$ MHz; because of the reduced hyperfine selectivity available for this state as a result of its axial ***g***-tensor, we do not discuss it in detail. The resting state M-center exhibits five distinct types of $^{57}$Fe signals, with isotropic couplings of $10 \leq |\boldsymbol{A}_{iso}| \leq 18$ MHz.

The observed hyperfine interaction, $\boldsymbol{A}_{exp}(Fe_x)$, for a given iron ion in a spin-coupled cluster is proportional to the projection of the ion's local spin onto the total spin $K(Fe_x)$[28,30]

$$\boldsymbol{A}_{exp}(Fe_x) = K(Fe_x) a_{site}(Fe_x) \quad (2)$$

where $a_{site}(Fe_x)$ is the isotropic hyperfine constant for the *x*th site when not spin-coupled and $K(Fe_x)$ is the spin-projection coefficient of the site given by[28,30]

$$K(Fe_x) = \langle \boldsymbol{S}(Fe_x) \cdot \boldsymbol{S}_t \rangle / \langle \boldsymbol{S}_t \cdot \boldsymbol{S}_t \rangle \quad (3)$$

Here, $\boldsymbol{S}(Fe_x)$ is the electron spin of the site, $\boldsymbol{S}_t$ is the total cluster spin of the spin-coupled cluster, and $\langle \boldsymbol{S}_t \cdot \boldsymbol{S}_t \rangle = S_t(S_t + 1)$ when total spin is conserved. Undoubtedly, the hyperfine couplings for the $S = {}^3/_2$ resting state are smaller than those of the $S = {}^1/_2$ lo-CO (and hi-CO) state, but comparable to those of other 4Fe−4S clusters with $S = {}^3/_2$ (Table 1) because of this spin dependence of the denominator of *K*.

We now show how the valencies of the Fe ions of the lo-CO M-center, and hence the d-electron count of the cofactor, can be deduced from the $^{57}$Fe ENDOR data. We begin with the assumption that the Mo ion in CO-inhibited MoFe is in a spinless Mo(IV; $S = 0$) state, as we inferred for the resting state enzyme.[41] Given that $^{95,97}$Mo ENDOR signals can be seen in both isotopically enriched and natural-abundance ($^{95,97}$Mo, total abundance 25.5%) resting state enzymes, where the hyperfine coupling to Mo is small,[41] if Mo had been reduced under turnover to a paramagnetic Mo(III; $S = {}^1/_2$) state where the coupling to $^{95,97}$Mo would likely be larger, then Mo ENDOR signals should be even easier to detect in CO-inhibited samples. However, no such signals were detected in either of the natural-abundance CO-inhibited enzyme forms,[42] and the same is true for preliminary measurements on $^{95}$Mo-enriched CO-inhibited samples.[42b] The assumed valence state of Mo is in accordance with Mo EXAFS measurements which indicate that in CO-inhibited MoFe-protein the Mo neither changes valence from the resting state nor binds CO.[43] Given that a ferrous ion has an even-electron count ($d^6$; $S = 2$) and a ferric ion has an odd ($d^5$; $S = {}^5/_2$), the obvious first consequence of Mo being in an even-spin state is that the seven iron ions must include an odd number of $Fe^{3+}$ ions in order to generate a total cluster spin of $S = {}^1/_2$ for lo-CO (and hi-CO) MoFe-protein. In the highly reducing nitrogenase environment, it is inconceivable that the M-center can be in a "HiPIP-like" state, with more than half the Fe ions in the oxidized, ferric form. Thus, contrary to other proposals,[44] we conclude that an even-spin Mo in the FeMo-cofactor can be accompanied only by one or three ferric ions. In such reduced clusters, the ferric ion generally occurs as a mixed-valence ($2Fe^{2.5+}$) pair in which one $Fe^{3+}$ and one $Fe^{2+}$ ion have one electron (hole) delocalized between them.[45−48] As a result, we infer that the FeMo-cofactor of lo-CO has $p = 1$ or 3 such pairs, and that the metal ions in the cofactor are arranged as $[(2Fe^{2.5+})_{1\text{ or }3}, Fe^{2+}{}_{5\text{ or }1}, Mo^{4+}]$.

To proceed, we consider the ENDOR-determined hyperfine coupling constants (Table 1), which involve the unknown spin-projection coefficients (eq 2). At the most primitive level, one can make direct preliminary comparisons between the $^{57}$Fe

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(42) (a) In ref 24, the subject of the paper is $^{57}$Fe ENDOR studies, but the control measurements on natural-abundance enzyme provide a careful examination of the frequency range where $^{95,97}$Mo signals with hyperfine couplings that are unresolved by EPR must appear ($\nu \leq 30$ MHz), and they showed no such signals. (b) Unpublished results.

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hyperfine couplings for different Fe−S centers that have the same total spin. The experimental isotropic coupling, $|\boldsymbol{A}_{iso}| \approx$ 31 MHz, seen for two sites in lo-CO is common for the mixed-valence pairs of $S = {}^1/_2$ [4Fe−4S] clusters, which contain only a single mixed-valence pair and ferrous ions (Table 1).[28] The occurrence of similar values in different clusters is not unexpected because such pairs have a high spin $({}^9/_2 - {}^7/_2 - {}^5/_2)$[30] and in general would have a relatively large spin-coupling coefficient, $K$, in any reduced cluster. In contrast, ferrous ($^{57}Fe^{2+}$) ions typically show smaller $K$ and $|\boldsymbol{A}_{iso}|$, comparable to those seen for the other two distinct sites of lo-CO (Table 1).[28] This suggests that $Fe_{\alpha 1}$, $Fe_{\alpha 2}$ of lo-CO (and $Fe_{\alpha 3}$ of hi-CO) represent one or more such $Fe^{2.5+}$ pairs, while $Fe_{\beta 1}$ and $Fe_{\beta 2}$ of lo-CO (and $Fe_{\beta 3}$, $Fe_{\beta 4}$ of hi-CO) are $Fe^{2+}$ ions. In this case, the presence of p = 3 pairs is ruled out because only 1 of the seven Fe ions would exist as an $^{57}Fe^{2+}$ ion, while we observe resonances from two ($Fe_{\beta 1}$, $Fe_{\beta 2}$). Thus, the two sites of lo-CO, $Fe_{\alpha 1}$ and $Fe_{\alpha 2}$ most likely correspond to the two members of a single $^{57}Fe^{2.5+}$ pair; by difference, $Fe_{\beta 1}$ and $Fe_{\beta 2}$ would represent five $^{57}Fe^{2+}$ ions, conceivably existing as two $^{57}Fe^{2+}$ pairs and an isolated $^{57}Fe^{2+}$.[49,50]

To test this suggestion we consider the parameter $a_{test}$ introduced by Mouesca *et al.*[28]

$$a_{test} = \sum \boldsymbol{A}_{exp}(Fe_x) = \sum K(Fe_x) a_{site}(Fe_x) \qquad (4)$$

where the sum runs over all seven Fe ions.[51] If the Mo has $S = 0$, and thus does not participate in the spin coupling scheme, then $\sum K(Fe_x) = 1$ and $a_{test}$ is the weighted average of the intrinsic isotropic constants for the Fe sites. Values of $a_{test}$ of −16 to −25 MHz have been calculated by Mouesca *et al.*[28] for a number of smaller clusters. If the $S = {}^1/_2$ state of lo-CO arises from spin coupling among one ($p = 1$) $Fe^{2.5+}$ pair with $|\boldsymbol{A}_{iso}| \approx$ 31 MHz for each Fe, plus five $Fe^{2+}$ ions, each with $|\boldsymbol{A}_{iso}| \approx 16$ MHz, then the only way to achieve such a value of $a_{test}$ is to have a large positive value of $K$ for the pair, a positive $K$ for one of the $Fe^{2+}$ ions, and negative values of $K$ for four of the $Fe^{2+}$ ions. This yields, $\boldsymbol{A}_{iso}(Fe^{2.5+}) \approx -31$ MHz (×2), $\boldsymbol{A}_{iso}(Fe^{2+}) \approx 16$ MHz (×4), and $\boldsymbol{A}_{iso}(Fe^{2+}) \approx -16$ MHz (×1), with $a_{test} \approx -14$ MHz for lo-CO. While this value is slightly outside the range of the reported values, the discussions of Mouesca *et al.*[28] show that a decrease in the magnitude of $a_{test}$ as seen for lo-CO is consistent with a highly reduced cofactor cluster having a preponderance of $Fe^{2+}$ ions, which are expected to have a lower value of intrinsic isotropic coupling constant and hence of $a_{test}$. Thus, the value of $a_{test}$ for lo-CO supports the suggestion that the FeMo-cofactor contains only $p = 1$ mixed-valence pair.[52] Taking into account the proposed $Mo^{4+}$ state molybdenum, this leads to a formulation of the inorganic portion of the cluster as $[Mo, Fe_7, S_9]^+ = [Mo^{4+}, {Fe^{3+}}_1, {Fe^{2+}}_6, {S^{2-}}_9]^+ = [Mo^{4+}, (2Fe^{2.5+}), {Fe^{2+}}_5, {S^{2-}}_9]^+$, with a formal d-electron count of 43.[53]

A number of independent observations support the conclusion that the FeMo-cofactor is at the same redox level in resting state, lo-CO, and hi-CO proteins and that the proposed valency assignments thus apply to all three states. The most direct evidence is that the hi-CO state quenched with ethylene glycol (EG at 40%) can be converted into lo-CO in the *absence* of turnover by simply pumping off atmospheric CO. Subsequent back addition of CO to the atmosphere regenerates hi-CO while extensive pumping on quenched lo-CO converts it to the resting state, all in the absence of electron transfer, meaning that lo-CO, hi-CO, and resting state all correspond to the same oxidation state of the cofactor.[29] In addition, past spectroscopic,[10−13] chemical,[11,54] and electrochemical[55] studies of both the protein-bound cofactor and the extracted cofactor found only three oxidation state, $M^+$, $M$, and $M^-$ (sometimes termed oxidized, semi-reduced, and reduced, respectively), where $M$ represents the oxidation state of the resting enzyme with $S = {}^3/_2$. No further reduction could be effected with potentials even lower than ∼−1 V (NHE). Since the $M$ state is the only half-integer spin state of the three, the $S = {}^1/_2$ lo- and hi-CO signals must correspond to CO bound to this valency state, or to a hitherto unobserved $M^{2-}$ level. Finally, our oxidation assignments of Fe are the only acceptable ones that yield a cluster with half-integer spin state and a net negative charge. Thus, when homocitrate is included in the cofactor structure, the overall charge on the cluster [$MoFe_7S_9$:homocitrate] is ($2^-$), assuming a ($3^-$) charge on the homocitrate. In contrast, a paramagnetic state with $p = 3$ mixed-valence pairs would likely be neutral. Previous interpretations[2,56] of the negative charge of the extracted cofactor (as demonstrated by DEAE-binding studies,[57] counterion chromatography,[56] and electrophoretic[58] measurements) have been to assign the association of anionic solvent and exogenous ligands to a positively charged cluster. Our valency assignments automatically make the cluster negatively charged and thus eliminate the necessity of including such ligands in the structure.

## Conclusion

The recent $^{13}C$ and $^{57}Fe$ ENDOR studies of CO-bound turnover state of nitrogenase clearly identified the binding cluster in MoFe-protein and the properties of the bound diatomic inhibitor (CO): $[FeMo\text{-}co][CO]_n$ is the origin of the EPR signals from both lo-CO ($n = 1$) and hi-CO ($n = 2$).[23,24] The $^{57}Fe$ ENDOR signals presented here for the seven Fe ions of the FeMo-cofactor of lo-CO can be completely assigned and

(49) This argument would preclude the possibility that a $^{57}Fe^{2.5+}$ pair has gone undetected because the pair's vector-coupling coefficient is small; the assignment into types would not be altered if from 1 to 3 of the $^{57}Fe^{2+}$ ions had undetectably small values of $|A_{iso}|$ because of small vector coupling coefficients.

(50) Such a highly reduced cofactor, with a single mixed-valence pair and five ferrous ions is consistent with the absence of an appreciable resonance Raman signal from the MoFe-protein and, as pointed out by a helpful reviewer, with low-temperature MCD measurements, while a cofactor with $p = 3$ mixed-valence pairs is not.

(51) This procedure has been used successfully to discuss spin coupling in the $[F_4S_4]^+$ ($S = {}^1/_2$) cluster of *Pyrococcus furiosus* ferredoxin, see ref 60.

(52) As the final step in a spin-coupling analysis one can sometimes derive spin-coupling coefficients, the $K$ of eq 3, provided that the $^{57}Fe$ site hyperfine coupling constants, $a_{site}$ in eq 2, can be treated as transferrable. Mouesca *et al.*[28] have given consensus values for a mixed-valence pair and for a ferrous ion of conventional Fe−S clusters. However, the six tricoordinate Fe of the FeMo-cofactor are sufficiently different from the tetrahedral iron of $Fe_2S_2$ and $Fe_4S_4$ clusters that it is unlikely that $a_{site}$ is transferable.

(53) For completeness, we have used eq 4 to explore the possible spin-coupling scheme for spinless Mo with $p = 1$ or 3 mixed-valence pairs. We have also considered spin coupling for a cluster with Mo(III, $S = {}^1/_2$). In this latter case, the arguments presented in the text permit only $p = 2$ mixed-valence pairs and indicate that the net d-electron count in the cluster would be the same (43) as with Mo(IV): the change from Mo(IV) to Mo(III) must be accompanied by the change of one Fe from Fe(II) to Fe(III). We have calculated $a_{test}$ for all acceptable assignments of the two observed values, $A_{iso} \approx \pm 31$ and ±16, to mixed-valence pairs and to $Fe^{2+}$ ion(s) for both even- and odd-spin Mo. The results are listed in the Supporting Information, Table SI.

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interpreted in terms of four magnetically distinct $^{57}$Fe signals. These signals have been analyzed within a conceptual framework provided by the deep understanding of FeS clusters generated through the joint efforts of Mössbauer[48] (and ENDOR)[59] spectroscopy and theory.[28,30] The interpretation of the ENDOR data for the iron−molybdenum-cofactor of the lo-CO nitrogenase MoFe-protein has led us to propose valence assignments for the inorganic part of the cofactor cluster: [Mo, $Fe_7$, $S_9]^+$ = [$Mo^{4+}$, $Fe^{3+}{}_1$, $Fe^{2+}{}_6$, $S^{2-}{}_9]^+$, with a formal d-electron count of 43.[53] It is further proposed that these ions are organized into one $Fe^{2.5+}$ pair and five $Fe^{2+}$ ions: [$Mo^{4+}$, $(2Fe^{2.5+})_1$, $Fe^{2+}{}_5$, $S^{2-}{}_9]^+$. A variety of arguments indicate that the lo-CO, hi-CO, *and* resting states of the M-center all are at the same oxidation level (see above). Hence, the proposed valency assignments apply to all three states.

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**Supporting Information Available:** Supplementary discussion of alternative valency assignments and corresponding spin-projection coefficients (2 pages). See any current masthead page for ordering and Internet access instructions.

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