# Identification of the CO-Binding Cluster in Nitrogenase MoFe Protein by ENDOR of $^{57}$Fe Isotopomers

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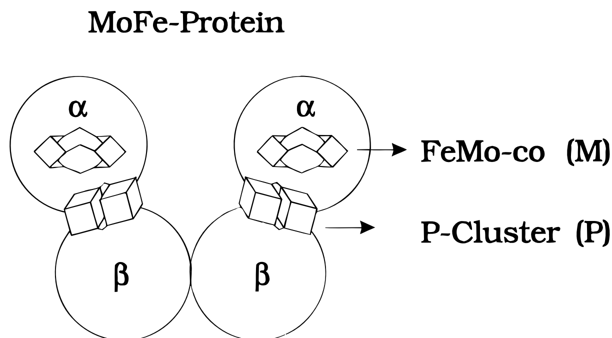
**Abstract:** The X-ray structure of the nitrogenase MoFe protein has established the organization and architecture of its multimetallic cofactors, the P-cluster ($Fe_8S_{7-8}$) and the FeMo-cofactor ($MoFe_7S_9$:homocitrate). Nonetheless, until recently it has not been possible to detect or characterize a substrate or inhibitor interacting with the functioning enzyme. In the present study we have used $^{57}$Fe ENDOR to study the CO-inhibited turnover states of a novel suite of $^{56,57}$Fe isotopomers of the MoFe protein, including those in which these two clusters are *selectively,* as well as uniformly, labeled. CO-inhibited MoFe protein exhibits two distinct EPR signals, one under low and another under high CO pressure. The $^{57}$Fe measurements, along with an earlier $^{13}$C ENDOR study of bound $^{13}$CO (Pollock, 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), show that the two EPR signals arise from CO-bound FeMo-cofactor, in one case with one bound CO and in the other with two bound CO, and they further provide initial insights into the properties of the inhibitor-bound cluster.

## Introduction

Nitrogenase catalyzes the reduction of dinitrogen to ammonia at ambient temperatures and pressure, a reaction of critical environmental importance that defies biomimesis. The nitrogenase MoFe protein incorporates the sites of substrate reduction,[1−5] and its beautiful crystal structure has revealed the organization and architecture of its multimetallic clusters:




the P-cluster ($Fe_8S_{7-8}$) and the FeMo-cofactor ($MoFe_7S_9$: homocitrate).[6−8] Nonetheless, the structural bases of enzymatic function remain a profound mystery. In particular, while enzymatic studies on selected mutants strongly indicated the FeMo-cofactor as the active site,[9−12] no small-molecule substrate or inhibitor had *ever* been shown to directly interact with either protein-bound cluster.

As the initial step in characterizing the nitrogenase active site(s), Q-band $^{13}$C electron nuclear double resonance (ENDOR)[13,14] spectroscopy of CO-inhibited nitrogenase gave the first direct observation of a diatomic molecule bound to a metal center of the MoFe protein.[15] Prior to this it was known that the $S = {}^3/_2$ electron paramagnetic resonance (EPR) signal of the resting state enzyme's FeMo-co cluster (called the M-center) disappears under turnover conditions in the presence of CO, and that two new $S = {}^1/_2$ EPR signals appear: one is formed under low pressure of CO (denoted, lo-CO) and has $g$ values of $(g_1, g_2, g_3) = (2.09, 1.97, 1.93)$; the other is formed under high pressure (denoted, hi-CO) and has $g$ values of $(g_{\|}, g_{\perp}) = (2.17, 2.06)$.[16−19] Both signals had been shown to be associated

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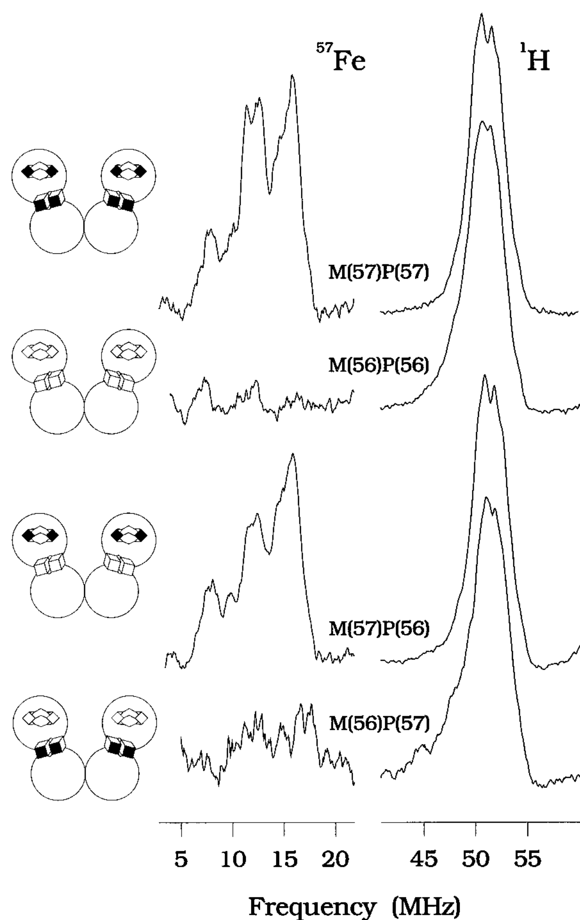


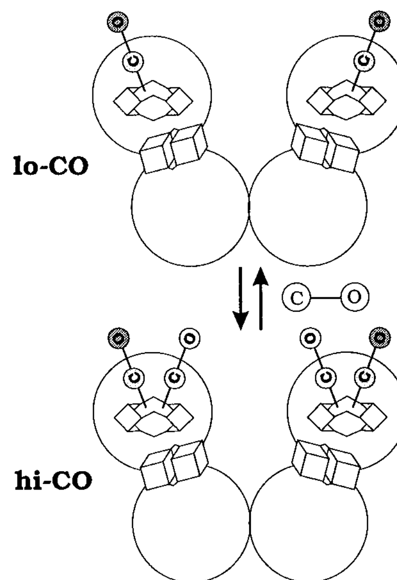

**Figure 1.** ENDOR spectra obtained at $g = 2.06$ ($g_\perp$) of the hi-CO EPR signal from MoFe protein of nitrogenase turnover samples that are globally $^{57}$Fe enriched [M(57)P(57)], natural abundance [M(56)-P(56)], selectively $^{57}$Fe labeled on the FeMo-cofactor [M(57)P(56)], and the P-cluster [M(56)P(57)]. Spectra are normalized to $^{1}$H ENDOR peaks as internal standards. No features were observed at 22−40 MHz in any spectra. $^{57}$Fe signal regions are enlarged three fold for display purposes. *Conditions:* microwave frequency, 34.95−35.24 GHz; modulation amplitude, 0.67 G; modulation frequency, 100 kHz; sweep speed, 3 MHz/s.

with turnover states of the MoFe protein by EPR studies in which first the MoFe protein and then its partner, the Fe protein were selectively labeled with $^{57}$Fe.[16−19] The combination of CW and pulsed Q-band $^{13}$C ENDOR measurements disclosed that lo-CO has one bound CO molecule, but hi-CO has two.[15] In addition, these measurements (i) demonstrated that the lo- and hi-CO EPR signals are associated with the same MoFe metal cluster; (ii) gave mechanistic relationships between the CO molecules bound to the two forms; and (iii) gave information regarding the kinetic lability of bound CO. However, the experiments did not identify the cluster responsible for the EPR signals of CO-inhibited protein, namely, M-center or P-cluster. The present report now makes this identification through a Q-band ENDOR study not only of uniformly $^{57}$Fe labeled MoFe protein, denoted [M(57)P(57)], but also of a novel pair of $^{57}$Fe MoFe isotopomers, denoted [M(57)P(56)] and [M(56)P(57)], in which the M-center and P-cluster are *selectively* labeled as well.

## Experimental Section

**Sample Preparation.** MoFe protein in which the metal clusters had either natural isotopic abundance ([M(56)P(56)]) or were uniformly labeled with $^{57}$Fe were prepared from *Azotobacter vinelandii (Av)* by standard procedures.[20] The mixed-isotope proteins were prepared

**Scheme 1**




through the additional use of protein isolated from the UW 45 strain, which is completely unable to make the M cluster. To make the MoFe protein selectively labeled with $^{57}$Fe in the M-center, *Av* UW45 was grown on natural-abundance Fe and then reconstituted with $^{57}$Fe-enriched M-center extracted from protein isolated from *Av* grown on $^{57}$Fe. The MoFe protein selectively labeled in the P-cluster was prepared by inverting the procedure. This protocol eliminates all possibilities of label-scrambling.[21] The four MoFe isotopomers were used to prepare protein in the hi-CO state[15] for ENDOR measurements.

**Endor Measurements.** Q-Band EPR and ENDOR spectra were recorded on a modified Varian E-109 spectrometer equipped with a liquid helium immersion dewar, described elsewhere,[22] using rf excitation whose bandwidth had been broadened to 100 kHz.[23] All spectra were collected at 2 K and in dispersion mode, under "rapid-passage" conditions.[24,25] Q-Band pulsed ENDOR spectra were collected on a spectrometer described in detail elsewhere.[26]

## Results and Discussion

Figure 1 shows $g = 2.06$ ($g_\perp$) ENDOR spectra of the four hi-CO isotopomers. This field was chosen because it gives the strongest signals, but equivalent results are obtained at any field. All samples show intense $^{1}$H resonances centered at the $^{1}$H Larmor frequency ($\nu_H \sim 52$ MHz), and the ENDOR intensities at other frequencies are normalized to the proton signals as internal standards. The spectrum of the globally $^{57}$Fe enriched hi-CO (Figure 1, [M(57)P(57)]) clearly shows $^{57}$Fe ENDOR signals in the 6−20 MHz range where the spectrum of natural-abundance hi-CO (Figure 1, [M(56)P(56)]) is essentially featureless. The ENDOR spectrum obtained from the hi-CO sample selectively labeled on FeMo-cofactor (Figure 1, [M(57)-P(56)]) shows the same $^{57}$Fe ENDOR pattern given by [M(57)-P(57)], and with the same intensity relative to its $^{1}$H signal. This $^{57}$Fe signal is *not* seen for the sample where only the P-cluster is $^{57}$Fe labeled (Figure 1, [M(56)P(57)]). These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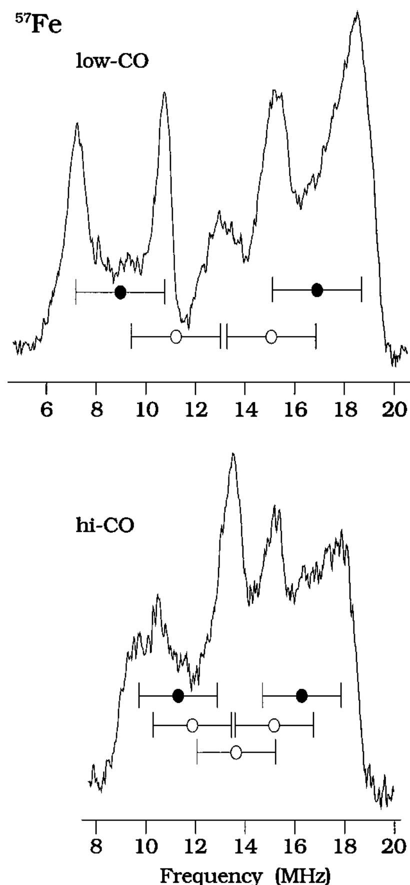


**Figure 2.** Single-crystal-like $^{57}$Fe ENDOR spectra of globally $^{57}$Fe enriched lo-CO ($g = 1.93$ ($g_3$)) and hi-CO ($g = 2.17$ ($g_{\|}$)) nitrogenase turnover samples. $^{57}$Fe doublets of $\nu_-$ and $\nu_+$ are connected by (|−|). The center frequencies ($A/2$) of the $^{57}$Fe doublets are denoted by closed circles (●) or open circles (○) when the doublets are unambiguously or tentatively identified, respectively. The lo-CO form was prepared only with the natural abundance and globally enriched protein. *Conditions*: as in Figure 1 except for sweep speed, 1 MHz/s.

observations clearly identify the FeMo-cofactor as the metal cluster that gives rise to the hi-CO EPR signal. The previous $^{13}$C ENDOR investigation showed that the lo-CO and hi-CO EPR signals are associated with the same metal cluster.[15] Thus, the resting state, lo-CO, and hi-CO EPR signals *all* arise from odd-electron states of the FeMo-cofactor. In the resting state it has no bound diatomic molecule; in lo-CO it has one bound CO, and in hi-CO it has two, as depicted in Scheme 1.[27]

Figure 2 shows optimized single-crystal-like $^{57}$Fe ENDOR spectra of globally $^{57}$Fe enriched lo-CO and hi-CO samples. In such spectra, each class of magnetically distinct $^{57}$Fe gives one doublet with frequencies of $\nu_{\pm} = |A/2| \pm \nu_{Fe}$. Thus, a minimum value for the number of $^{57}$Fe classes can be obtained by analyzing such spectra, and the corresponding hyperfine couplings can be measured. The $^{57}$Fe features span the range of approximately 6−20 MHz for lo-CO and ~9−19 MHz for the hi-CO form. As indicated, in each spectrum, $^{57}$Fe doublets from two types of iron site can be assigned unambiguously. For the lo- and hi-CO states respectively one may tentatively assign 2 and 3 additional doublets from other distinct iron sites. $^{57}$Fe ENDOR measurements across the EPR envelopes of lo- and hi-CO are under way and in fact suggest that for lo-CO there may be as many as 7 distinguishable Fe sites. The $^{57}$Fe signals from lo-CO show isotropic hyperfine couplings in the range 9 MHz $\le |A_{iso}| \le$ 30 MHz, as well as substantial hyperfine anisotropy. Such detailed information is more difficult to obtain for the hi-CO state because of the reduced orientation selectivity with its axially-symmetric $g$ tensor. The $^{57}$Fe results for FeMo-co in the lo-CO state are quite different from those of FeMo-co in the resting-state enzyme, where ENDOR identified five magnetically distinct Fe sites with $11 < |A_{iso}| < 17$ MHz.[28] These preliminary comparisons suggest that the effective symmetry of the cofactor is reduced in the lo- and hi-CO states, relative to the resting state, but more detailed analysis of the $^{57}$Fe data for CO-inhibited MoFe protein is needed.

In summary, the recent $^{13}$C ENDOR study of the MoFe protein of nitrogenase[15] gave the first glimpses into the properties of the diatomic inhibitor (CO) bound to turnover states of an *unidentified* cluster of the MoFe protein. $^{57}$Fe ENDOR measurements collected on $^{57}$Fe isotopomers of the MoFe protein now clearly identify CO-bound FeMo-cofactor, [FeMo-co][CO]$_n$, as the origin of the EPR signals from both lo- ($n = 1$) and hi-CO ($n = 2$) forms, and also provide the first evidence about the properties of the Fe sites in active cluster states. A question that remains to be answered is the exact reduction state of the cofactor that gives the lo- and hi-CO signals. The hi-CO signal is observable in experiments with very low electron flux,[29] and thus the FeMo-co is most likely at the (odd-electron) reduction level of the resting-state cluster or is doubly reduced. We favor the former even though no new EPR signals arise when CO is added to the MoFe protein in the absence of turnover. Instead we speculate that the hi-CO signal arises when a FeMo-co cluster cycles through a reduced state that binds the two CO molecules then returns to the resting-state reduction level in an alternate conformation that retains the bound CO and gives the observed EPR signal. Because lo- and hi-CO forms can be interconverted in the absence of catalytic turnover following ethylene glycol quenching,[29] then the lo-CO signal must arise from the same reduction state of the cluster. Hence, the alternate cluster conformation must be relatively stable with either one- or two-bound CO, and convert to the resting-state conformation only after the loss of both.

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(27) The small $^{13}$C hyperfine coupling constants for CO bound to FeMo−CO of nitrogenase,[15] $A(^{13}C) \lesssim 6$ MHz, are in contrast to the large couplings of $^{13}$CO bound to the metal centers in Fe hydrogenase I and II, $A(^{13}C) \sim$ 21 and 34 MHz, respectively.[31,32] However, a comparably small $^{13}$C interaction has been found recently for the isoelectronic diatomic, $^{13}CN^-$, bound to the $[4Fe-4S]^+$ cluster of Pyrococcus furiosus (Pf−Fd).[30]

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