Investigation of CO Bound to Inhibited Forms of Nitrogenase MoFe Protein by ^{13}C ENDOR

Robert C. Pollock,[†] Hong-In Lee,[‡] Linda M. Cameron,[§] Victoria J. DeRose,[‡] Brian J. Hales,*[,§] W. H. Orme-Johnson,*[,†] and Brian M. Hoffman*[,‡]

Departments of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
Northwestern University, Evanston, Illinois 60208
Louisiana State University, Baton Rouge, Louisiana 70803

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Nitrogenase catalyzes one of the most remarkable biological reactions, the reduction of dinitrogen to ammonia at ambient temperature and pressure.[1−3]

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16\,MgADP + 16P_i \quad (1)$$

The MoFe protein of nitrogenase contains two metal centers, the P-cluster (Fe_8S_{7-8}) and the FeMo-cofactor (Fe_7S_9Mo: homocitrate), which is believed to be the substrate binding site.[1−5] This protein has been the subject of numerous biophysical studies,[4,5] culminating in the disclosure of its beautiful and unexpected structure.[6,7] Current X-ray crystallographic modeling shows that the P-cluster is composed of two ferredoxin-like Fe_4S_4 cubane clusters linked by two cysteine thiols and one disulfide; the cofactor can be viewed as Fe_4S_3 and $MoFe_3S_3$ clusters linked by three sulfides. Surprisingly, knowledge of the structure gives no obvious insights into the nature of substrate or inhibitor binding. We report here a first step in addressing this problem: Q-band CW and pulsed electron-nuclear double resonance (ENDOR)[8,9] spectroscopy gives the first direct observations of a diatomic molecule, CO, bound to a metal center of the MoFe protein.

The $S = {}^3/_2$ electron paramagnetic resonance (EPR) signal from the resting state of the FeMo-co cluster of nitrogenase disappears under turnover conditions. When turnover occurs in the presence of CO, two new signals are observed, one at low CO concentration (denoted lo-CO) with $g_{av} < 2$ (2.09, 1.97, 1.93) and one at high CO (denoted, hi-CO) with $g_{av} > 2$ (2.17, 2.06, 2.06).[10] The observation of EPR line broadening upon ^{57}Fe labeling of the MoFe protein but not the Fe protein showed that both signals are associated with the former.[10] However, neither EPR signal shows broadening in the presence of ^{13}CO.[10] Thus, the EPR signals from CO-inhibited nitrogenase might arise from a cluster of the MoFe protein that actually contains bound CO or from a cluster that is "upstream" of the site of inhibition. The presence, number, and mechanistic relationships of CO bound to CO-inhibited nitrogenase are addressed here by using ENDOR to study samples prepared through turnover in the presence of ^{13}CO.[11,12]

Figure 1 displays single-crystal-like ENDOR[8,9] spectra taken[13] from the EPR signals elicited under turnover with low (A1: ^{13}CO, $P = 0.08$ atm) and high (B1, B2: ^{13}CO, $P = 0.5$ atm) pressure of ^{13}CO. The observed peaks from both lo- and hi-CO are absent in samples prepared with ^{12}CO (A2, B6: ^{12}CO) and thus must arise from ^{13}C of the labeled inhibitor; careful examination showed no additional ^{13}C signals at higher frequency. In the ^{13}C spectrum of lo-CO taken at the low-field edge of the EPR envelope, $g_1 = 2.09$, the appearance of one doublet centered at $\nu(^{13}C)$ and split by the hyperfine coupling, $A(^{13}C) \sim 3$ MHz, indicates that the signal is associated with a single ^{13}CO coupled to the spin system.[18] Examination of spectra at numerous fields across the EPR envelope confirms this and shows that this small coupling is mostly dipolar. In contrast, ^{13}CO bound to the metal centers in Fe hydrogenase I and II yields large and roughly isotropic couplings, $A(^{13}C) \sim 21$ and ~ 34 MHz, respectively.[20,21] However, a comparably small, mainly dipolar, ^{13}C interaction has been found recently for the isoelectronic diatomic, $^{13}CN^-$, bound to the $[4Fe-4S]^+$ cluster of *Pyrococcus furiosus* (*Pf*-Fd).[22]

The spectrum taken at $g_{||} = 2.17$ for nitrogenase inhibited with high pressures of ^{13}CO (Figure 1, B2: ^{13}CO) shows a doublet centered at $\nu(^{13}C)$ with $A(^{13}C1) \sim 6$ MHz, which again represents a single ^{13}CO interacting with the paramagnetic center; the unequal intensities of the two peaks, also evident but less pronounced in Figure 1, A1, is common in Q-band ENDOR. In addition, there is a large central peak for hi-CO (B2) that is not seen for lo-CO (A1). Q-band[23] pulsed ENDOR[24] measurements resolve this peak into a well-defined doublet pattern with $A(^{13}C2) \sim 1$ MHz, Figure 1, B1, thereby establishing that it represents a second, more weakly coupled $^{13}CO(2)$ bound to hi-CO. Measurements taken across the hi-CO EPR envelope suggest that the $^{13}C1$ coupling is largely isotropic, in contrast to the lo-CO ^{13}C signal, whereas $^{13}C2$ of hi-CO has a dipolar coupling whose maximum value of $A(^{13}C2) \sim 1$ MHz is seen near $g_\perp$. Neither CO(1) nor CO(2) of the hi-CO form has characteristics with a clear precedent; the small coupling to C2 almost certainly indicates that it is

[†] Massachusetts Institute of Technology.
[‡] Northwestern University.
[§] Louisiana State University.

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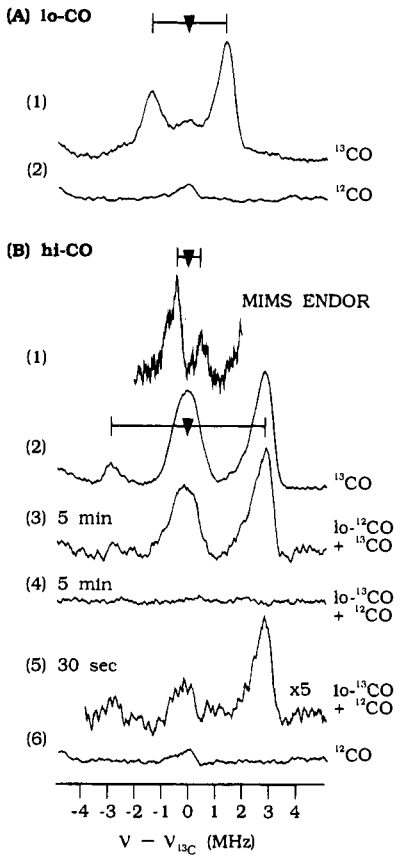

Figure 1. Single-crystal-like ^{13}C ENDOR spectra from lo-CO (A) and hi-CO (B) EPR signals from nitrogenase turnover samples. Spectra A, B1, B2, and B6 were from normal lo-CO and hi-CO samples. The hi-CO "pulse-chase" samples (B3–B5) were prepared as described in the text and footnote 25. All spectra are centered at the ^{13}C Larmor frequency (▼). ^{13}C hyperfine splittings are connected by |—|. Conditions: temperature, 2 K; microwave frequencies, 34.9–35.2 GHz (cw) or 34.646 GHz (pulsed); lo-CO spectra, $g = 2.09$; hi-CO spectra, $g = 2.17$. For Q-band MIMS ENDOR of hi-^{13}CO (B1): microwave pulse widths, 60–60–60 ns; interval between first and second microwave pulses, 400 ns; rf pulse width, 30 μs; 300 transients. All other traces are CW-ENDOR data obtained by using 100 kHz field modulation.[13]

bound to a metal ion that is diamagnetic (e.g., low-spin, $S = 0$, ferrous ion) or has *ca.* zero spin projection.

The simplest interpretation of the observation that lo-CO exhibits a signal from one bound inhibitor while hi-CO has two is that the two EPR signals arise as two CO bind sequentially to a single metal center of the MoFe protein, but it is equally possible that the two signals come from the two different centers. In the course of our efforts to probe such mechanistic relationships between the two inhibited forms, we have discovered that it is possible to interconvert between them[25] and have used this ability to distinguish between alternatives by carrying out three "pulse-chase" experiments according to eq 2, where n and m

$$\text{native MoFe} \xrightarrow[\text{(turnover)}]{^{n}\text{CO (0.5 atm)}} \text{hi-}^{n}\text{CO} \xrightarrow{\text{glycol}} \xrightarrow{\text{pump}}$$
$$\text{lo-}^{n}\text{CO} \xrightarrow{^{m}\text{CO (0.5 atm)}} \xrightarrow{\text{incubate } (t)} [\text{lo-}^{n}\text{CO} + {}^{m}\text{CO}]{:}t \quad (2)$$

refer to isotope. The experiments are as follows: (1) the hi-^{12}CO form was prepared, the reaction was quenched with ethylene glycol, ^{12}CO was pumped off (to yield the lo-^{12}CO state), and ^{13}CO was added back and incubated for 5 min before freezing to give a hi-CO state (denoted [lo-^{12}CO + ^{13}CO]:5 min); (2) the hi-^{13}CO form was prepared, the reaction was quenched with ethylene glycol, ^{13}CO was pumped off, and ^{12}CO was added back and incubated for 5 min to give a hi-CO state (denoted [lo-^{13}CO + ^{12}CO]:5 min); (3) the second experiment was repeated with only ~30 s exposure to ^{12}CO before freezing (denoted [lo-^{13}CO + ^{12}CO]:30 s).

The first of these hi-CO samples gave a ^{13}C ENDOR signal indistinguishable from that of hi-^{13}CO prepared directly (Figure 1, B3; [lo-^{12}CO + ^{13}CO]:5 min); the second gave no ^{13}C ENDOR signal (Figure 1, B4; [lo-^{13}CO + ^{12}CO]:5 min). These two experiments can be explained by either of the following: (a) the hi-CO signal represents CO bound to one metal cluster while the lo-CO represents CO bound to the other; or (b) both signals arise from the same cluster, but the inhibitor molecule bound to the lo-CO form is kinetically labile in the hi-CO form.

The ENDOR spectrum from the hi-CO form present in the third sample, which was frozen ~30 s after a high pressure of ^{12}CO was added to lo-^{13}CO generated by pumping, not only confirms the kinetic lability postulated in alternative b, but also gives the mechanistic relationship of hi- and lo-CO. The spectrum shows the ^{13}C1 signal of hi-CO, Figure 1, B5, but the intense ^{13}C2 signal of the normal hi-^{13}CO sample (Figure 1, B2) is essentially absent; the ENDOR intensity at $\nu(^{13}C)$ is attributable to natural-abundance ^{13}C. As the only ^{13}CO in the sample had been bound to the lo-^{13}CO protein form, the observation of the hi-^{13}CO(1) signal requires that the CO molecule of lo-CO becomes CO(1) of the hi-CO form and that the lo- and hi-CO EPR signals thus arise from the same cluster. The inhibitor molecule that becomes CO(2) must bind to lo-CO directly from the bulk when the CO partial pressure is increased. This suggests that the lo-CO form is generated when a single CO binds and that the conversion from lo- to hi-CO is caused by the binding of the second CO. Finally, the ^{13}C1 ENDOR signal from a [lo-^{13}CO + ^{12}CO] sample is present in reduced intensity after 30 s incubation, but absent after 5 min incubation, and this sets the time scale for exchange of CO(1) from hi-CO. Data not shown set a similar time scale for exchange of CO on lo-CO.

These first direct observations of small-molecule binding to the MoFe protein thus (i) demonstrate that the lo- and hi-CO EPR signals seen under turnover are associated with the same metal cluster;[26] (ii) disclose that lo-CO has one bound CO molecule, but hi-CO has two; (iii) give mechanistic relationships between the CO bound to the two forms; and (iv) give information regarding the kinetic lability of bound CO. Further interpretations will be permitted when the identity of this cluster, FeMo-cofactor or P-cluster, is established by ^{57}Fe ENDOR studies of enzyme in which an individual cluster type is selectively ^{57}Fe enriched. Additional experiments such as the pulse-chase ones reported here will further contribute to our understanding of the mechanism of N_2 fixation.

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(26) It is intriguing to note that we find that both lo- and hi-CO forms to show proton-ENDOR patterns that contain signals from at least five nonexchangeable protons, with the largest coupling being 5–6 MHz for the former and ~6 MHz for the latter. Such results are more in line with expectations for a cluster having multiple bound cysteines, as is true for the P-clusters, than for one having only a single bound cysteine, as with the FeMo-cofactor.