Time-Resolved Binding of Carbon Monoxide to Nitrogenase Monitored by Stopped-Flow Infrared Spectroscopy

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Received April 7, 1997

In this communication we report the use of stopped-flow fourier transform infrared spectroscopy (SF-FTIR) to monitor the binding of carbon monoxide to nitrogenase from *Klebsiella pneumoniae*. Nitrogenase is a bacterial metalloenzyme whose physiological function is to catalyze the reduction of dinitrogen to ammonia[1−4] with a concomitant reduction of $2H^+$ to H_2 and hydrolysis of MgATP to MgADP and P_i.[5] Nitrogenase comprises two proteins: the MoFe protein and the Fe protein. The MoFe protein contains a structurally unique metal−sulfur cluster; the FeMo cofactor ($MoFe_7S_9$:homocitrate), which is thought to be the substrate binding site.[1−4] Despite extensive biochemical, structural, spectroscopic, and kinetic characterization[1−4] as well as biomimetic chemical studies[6,7] the site and nature of dinitrogen binding to the FeMo cofactor and the chemistry of its subsequent reduction remain unresolved. In part, this can be ascribed to the novel and complex nature of the enzyme chemistry. One technical problem is that many reduced enzyme intermediates exist, and in general these can only be generated as mixtures of transient species.[3] Another problem is the lack of an effective spectroscopic probe of the dinitrogen *substrate* and its chemical changes on binding to the MoFe active site. To address these issues we have been developing SF-FTIR as a probe of the dynamics of metalloenzyme−substrate interactions. This communication addresses the application of SF-FTIR to the binding of CO to molybdenum nitrogenase. We show that under high-CO concentrations several transient metal center bound CO species exist, each with a unique kinetic profile. This suggests that the chemistry of CO inhibition of nitrogenase may be significantly more complex than has been believed.

CO is a potent noncompetitive inhibitor of the reduction of dinitrogen by nitrogenase.[8−10] It also inhibits the enzyme's general catalytic ability to reduce acetylene and other multiply bonded molecules.[8−10] CO does not, however, inhibit enzyme turnover. MgATP hydrolysis is sustained under CO, and the electron flux through the enzyme is directed toward increasing the rate of reduction of protons to dihydrogen.[11] During enzyme turnover, CO inhibited nitrogenase exhibits one of two transient electron paramagnetic resonance (EPR) signals.[13−15] These are denoted high-CO (**g** = 2.17, 2.06, 2.06) and low-CO (**g** = 2.09, 1.97, 1.93) because they occur under high and low-CO partial pressures, respectively. Electron-nuclear double resonance (ENDOR) studies have shown that one CO binding environment on the FeMo cofactor is apparent under low-CO, while two are observed under high-CO.[15,16]

The SF-FTIR experiments presented here employed a two syringes drive system.[17] One syringe contained 1:3.4 molar ratio of *K. pneumoniae* MoFe protein (Kp1): Fe protein (Kp2) giving ∼52 μM FeMo-cofactor centers after mixing.[19] The other syringe contained a buffered solution of MgATP and/or CO.[21] Infrared data were collected between 2200−1750 cm^{-1}. Figure 1 comprises illustrative spectra. In the absence of CO (Figure 1d) or MgATP (not shown) no time-dependent infrared bands were observed.[22] However, reacting the enzyme solution with a "high-CO" mixture yielding 10 mM MgATP and 0.5 mM CO after mixing[23] produced a complex transient infrared spectrum containing one relatively intense band at 1935.6 cm^{-1} and two smaller bands at 1958 cm^{-1} and 1906 cm^{-1} (Figure 1a).[24] All these bands shifted ∼44 cm^{-1} when ^{13}CO was substituted for the natural abundance ^{12}CO confirming that they arise from bound CO. The time dependence[25] of these bands are very different (Figure 2). The 1906 cm^{-1} band grows and decays relatively rapidly, peaking at about 7−8 s. By contrast the intense 1935.6 cm^{-1} and smaller 1958 cm^{-1} bands grow more slowly, peaking at 55 and 100 s, respectively, before slowly decaying. Both bands still have small but observable intensity after 600 s.

Low-CO conditions, using approximately a stoichiometric equivalent of CO to MoFe centers, produced a transient infrared spectrum with a single band at 1904 cm^{-1} (Figure 1c). This

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(21) All experiments used 25 mM HEPES pH 7.3 buffer containing 10 mM $MgCl_2$ and 10 mM sodium dithionite. Background spectra were generated by shooting, in turn, Kp1:Kp2 and MgATP/CO against buffer and averaging the result. Signal to noise for the time-dependent experimental spectra was enhanced by performing many shots and averaging. All results were reproduced.
(22) The ν(N≡N) stretch of terminally bound metal-dinitrogen complexes can also absorb in this region. No conclusion can be drawn from the apparent absence of such bands in Figure 1d as the Kp2:Kp1 stoichiometry is significantly lower than the optimum for dinitrogen turnover.
(23) EPR spectrum of this mixture frozen 1 min after mixing contained an intense high-CO signal (not shown).
(24) There is also a feature at 1880 cm^{-1} which is apparently time dependent, but our current data cannot confirm its nature.
(25) Peak intensity as a function of time was determined by fitting an averaged spectrum similar to Figure 1a to sum of Gaussian lineshapes. The peak widths and positions were then constrained and, for each spectrum of the time course, peak intensities and a baseline function were allowed to float. This approach significantly reduced the "noise" in the time course and corrected out a small time dependent baseline instability in these samples which probably arises from the evolution of very small bubbles of hydrogen.

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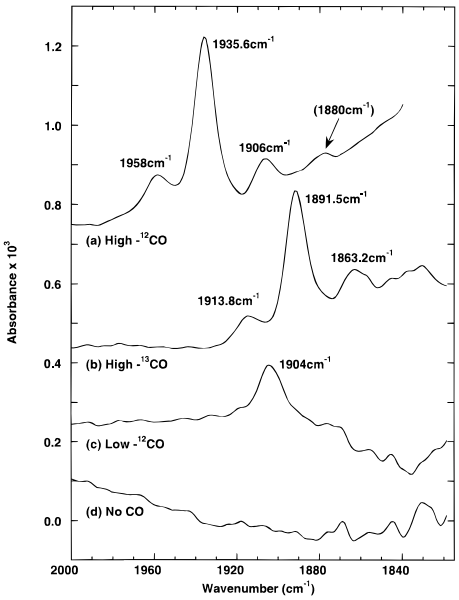

Figure 1. Transient infrared spectra of *Klebsiella pneumoniae* nitrogenase−CO complex. These are illustrative spectra obtained by averaging the data sets over 4−54 s after mixing. All samples contained 42 μM Kp1, 142 μM Kp2, 25 mM HEPES pH 7.3, 10 mM $MgCl_2$, 10 mM sodium dithionite, and 10 mM ATP. No creatine kinase regenerating system was used. CO concentrations assume buffer saturated with CO is 1 mM: (a) ∼0.5 mM CO (high CO) (average of nine experiments) and (b) ∼0.5 mM ^{13}CO (high ^{13}CO) (average of three experiments). The reduced intensity compared to (a) is due to the presence of 15% $^{13}C^{18}O$: (c) ∼60 μM CO (low CO) (average of two experiments) and (d) no CO (average of two experiments).

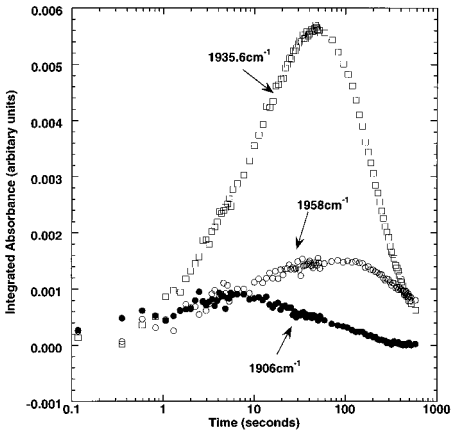

Figure 2. Time dependence of the principal bands observed in the *Klebsiella pneumoniae* nitrogenase high−CO complex infrared spectrum in Figure 1a: ● 1906 cm^{-1}; □ 1935.6 cm^{-1}; and ○1958 cm^{-1}. The time course is an average of nine stopped-flow experiments. Scanning times: 0−6 s in 0.2 s steps; 6−53 s in 1.2 s steps; and 53−515 s in 11.8 s steps.

band's time course was very similar to that observed for the 1906 cm^{-1} high-CO band, and at this point it is reasonable to conclude both these bands arise from the same species.

A number of broad conclusions can be drawn from these data. First, the energies of all the bands presented here are typical of ν(CO) stretching modes arising from CO terminally bound to metal sites (M-C≡O) such as heme−CO complexes.[26] We cannot eliminate the possibility of additional species with CO bound between two metal sites with a bridging carbon as these may produce ν(CO) stretches below the 1800 cm^{-1} limit of our current data set.[27] It is unlikely that the bands in Figure 1 represent minority species as their maximum extinction coefficients (1935.5 cm^{-1} ∼1930 M^{-1} cm^{-1}, 1906 cm^{-1} ∼400 M^{-1} cm^{-1}, 1958 cm^{-1} ∼350 M^{-1} cm^{-1}) and linewidths (∼10 cm^{-1}) are comparable with those of heme−CO complexes such as myoglobin−CO which have intensities of ∼1000 M^{-1} cm^{-1}.[28]

Second, each of the three ν(CO) peaks identified in the high-CO spectrum (Figure 1a) corresponds to a chemically distinct species. This is because all three peaks have disparate time courses, and only a single peak is observed at low-CO concentrations. This excludes the possibility that the different peaks arise from conformational differences within an otherwise identical binding site, as observed for, for example, heme−peroxidase CO complexes.[29,30]

The most straightforward interpretation of our data is that there is more than one metal site binding CO in the protein.[31] One site binds CO relatively rapidly under both high- and low-CO concentrations to give the 1906 cm^{-1} band, while the 1935.6 and 1958 cm^{-1} sites bind CO much more slowly and only under high-CO concentrations. It is not possible to say from the current data whether the high-CO 1935.6 and 1958 cm^{-1} bands represent two separate binding sites on the FeMo cofactor or the same binding site but with different cofactor redox states. It is unlikely that two CO molecules bind to the same metal within the cluster as this should lead to significant time dependent frequency shifts of the ν(CO) stretches arising from combination effects, and these are not observed.

Our data also allows speculation that the bound CO giving the rapidly formed "low-CO" 1906 cm^{-1} species is primarily responsible for CO inhibition and that the additional slow binding under high-CO conditions is secondary. This is supported by a study of enzyme activity under similar experimental conditions[32] which shows that the enzyme is fully inhibited by CO within 15 s.[33] These activity measurements also indicate that turnover suddenly slows after about 60 s whether CO is present or not; presumably because of ADP product inhibition. This time scale is similar to that for the maximum intensity for the high-CO signals (1935.6 cm^{-1}: 55 s, 1958 cm^{-1}: 100 s), and it suggests that the slow decay of the "high-CO" 1935.6 and 1958 cm^{-1} peaks is a consequence of loss of enzyme turnover.

There are unresolved problems. Why, for example does the low-CO 1906 cm^{-1} ν(CO) band start to decay after only 7 s? Nevertheless it is evident that the binding chemistry of CO to nitrogenase is significantly more complex than previously thought. It is also clear that SF-FTIR using relatively dilute (50 μM) enzyme is an effective probe of this chemistry. While the data presented here are limited to the "water-window", with appropriate adjustments to pathlength and the use of deuterated buffers much of the infrared spectrum is accessible. Also time resolutions of ∼15 ms should be possible. Hence this technique may well find application to a number of problems in bioinorganic chemistry.

Acknowledgment. R.N.F.T. and S.J.G. thank the UK BBSRC for a ROPA award. C.W.W. thanks the UK BBSRC and EC for support. The authors would like to thank Ms. Tess Regan for her assistance.

JA971088S

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