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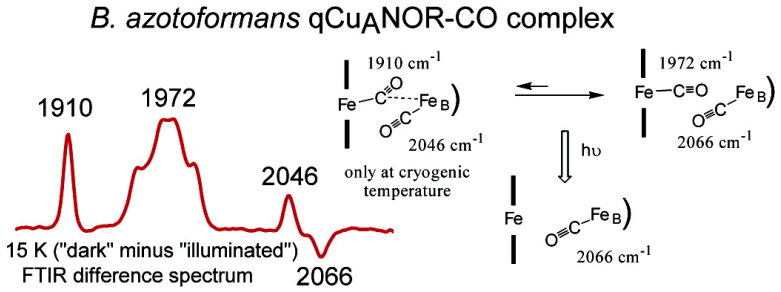

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Two CO Molecules Can Bind Concomitantly at the Diiron Site of NO Reductase from *Bacillus azotoformans*

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Nitric oxide reductase (NOR) catalyzes the two-electron reduction of two NO molecules to N_2O. The enzyme is encountered in denitrifying organisms as well as in some pathogenic bacteria, where it plays a protective role against NO toxicity. All NORs isolated so far are integral membrane proteins distantly related to the cytochrome *c* oxidase (CcO) superfamily.[1] Most studies have focused on NORs from *Paracoccus denitrificans*, where the electron acceptor chain includes a cytochrome *c* (cNOR enzymes), and from *Ralstonia eutropha*, where the electron entry to the active site corresponds to a quinol binding site (qNOR enzymes). Recently, a bifunctional NOR was isolated from *B. azotoformans* and was shown to utilize a dinuclear copper site analogue to CcO's Cu_A site as well as a quinol binding site (qCu_ANOR).[2] Despite the variability in electron acceptors in bacterial NORs, these enzymes share a common active site composed of a high-spin heme coupled to a non-heme iron (Fe_B in analogy with Cu_B in CcO). The close vicinity of the two iron centers in cNOR was confirmed by resonance Raman (RR) experiments, which characterized a μ-oxo diferric bridge and an Fe−Fe distance <3.5 Å.[3] Earlier, we proposed a reaction mechanism based on catalysis by proximity (also known as catalysis by approximation), where both irons bind one NO to promote the formation of the N−N bond.[4] In this report, we describe direct spectroscopic evidence for the binding of two diatomic molecules within the active site of qCu_ANOR.

Exposure of dithionite-reduced qCu_ANOR to CO results in UV−vis absorption changes consistent with the binding of CO to a high-spin heme.[2] Accordingly, the RR spectra obtained with Soret excitation show heme skeletal modes characteristic of a heme-CO complex (Figure S1, Supporting Information). The room-temperature (^{12}CO minus ^{13}CO) FTIR difference spectrum identifies two ν(CO) bands (Figure 1A). The band at 1972 cm^{-1} downshifts by 44 cm^{-1} with ^{13}CO gas and is readily assigned to the ν(C−O) from the heme-CO complex. Virtually identical frequencies were observed in the RR and FTIR spectra of the heme-CO complex formed in cNOR.[4] The second band at 2068 cm^{-1} — not found in cNOR — downshifts 47 cm^{-1} to 2021 cm^{-1} with ^{13}CO gas (Figure 1A). This ν(C−O) at 2068 cm^{-1} is consistent with a non-heme iron carbonyl (Fe_BCO) where back-bonding donation from the iron d_π orbitals to the carbonyl π^* orbitals is weakened compared to that observed in heme-CO complexes. The integrated areas of the two ν(CO) bands are equivalent, indicating that these two species are present in comparable concentrations.[5]

Interestingly, if qCu_ANOR is exposed to high chloride concentration, the ν(CO) from Fe_BCO at 2068 cm^{-1} is lost and only the heme-CO is detected as a single ν(C−O) band at 1970 cm^{-1} (Figure 1B). This form of qCu_ANOR is equivalent to that of cNOR, where FTIR detects CO binding exclusively at the heme, regardless of the chloride content.[6]

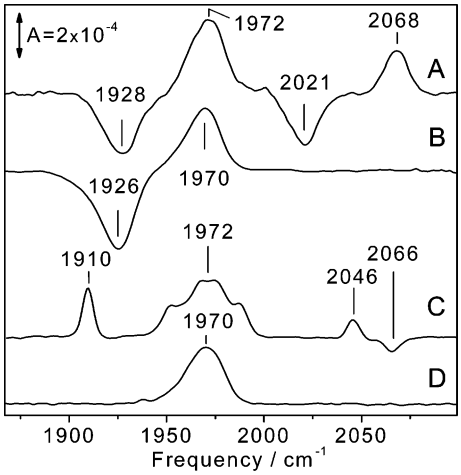

Figure 1. FTIR difference spectra of qCu_ANOR−CO at 293 and 15 K. Room-temperature traces are (^{12}CO minus ^{13}CO) in a chloride-free sample (A) and in the presence of 3 M Cl^- (B). The low-temperature traces are ("dark" minus "illuminated") in Cl^--free sample (C) and with 3 M Cl^- (D).

At 15 K and in the presence of 3 M chloride, the ("dark" minus "illuminated") FTIR difference spectrum displays a single ν(C−O) at 1970 cm^{-1} (Figure 1D) that is nearly identical to the signal observed in the room-temperature data (Figure 1B). This signal is consistent with the photolabile character of heme-CO complexes. Moreover, the intensity of the 1970 cm^{-1} band in the light-induced FTIR difference spectrum obtained at 15 K indicates that complete photolysis of the heme-CO complex is achieved and CO rebinding is fully inhibited. Increasing the temperature to 80 K allows total rebinding of the photolyzed CO to the heme iron (data not shown). This temperature dependence is consistent with an efficient geminate recombination process as observed in myoglobin[7] rather than a nongeminate recombination process as found in CcO.[5] Indeed, in CcO, the photolyzed CO binds to the CuB and is released only when the temperature reaches ∼200 K.

In the absence of excess chloride, the ("dark" minus "illuminated") FTIR difference spectrum obtained at 15 K is much more complex. A group of partially resolved components form a cluster of bands between 1950 and 1990 cm^{-1} that can be assigned to distinct but comparable heme-CO conformers within the substrate pocket. Such distributions of heme ν(C−O) have been observed in many hemoproteins, including globins and terminal oxidases.[8,9] In addition to this cluster of bands, we observe a positive band at 1910 cm^{-1} and an S-signal with a positive band at 2046 cm^{-1} and a negative band at 2066 cm^{-1} (Figure 1C). The isotopic sensitivity of these signals when the samples are prepared with ^{13}CO demonstrates that they all correspond to ν(CO) modes (Figure S2, Supporting Information). Comparison of different samples of qCu_A-

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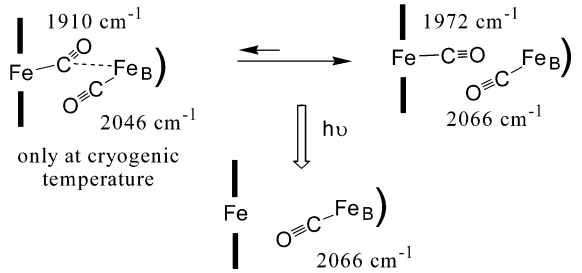
Scheme 1. Different Configurations Adopted by the CO Ligands in qCu_ANOR[a]



[a] At 15 K and in the absence of excess Cl^-, the heme CO adopts a semi-bridging configuration which perturbs the stretching frequency of Fe_BCO.

NOR show some variation in the relative intensity of the ~1972 cm^{-1} cluster of bands compared to that of the 1910 and 2046/2066 cm^{-1} signals, but the intensity ratio of the latter two signals remains constant. The interdependence of these two photoinduced features is also confirmed by the influence of temperature on the FTIR difference spectra. Specifically, when the temperature is raised to ~100 K, the 1910 cm^{-1} band and the 2046/2066 cm^{-1} S-signal are unaffected, but the intensity at ~1972 cm^{-1} decreases, presumably because of geminate rebinding of the photolyzed CO to the heme iron, as is seen at high chloride concentrations (data not shown).

The results lead us to the following interpretation (Scheme 1). As stated above, the ~1972 cm^{-1} cluster of bands in qCu_ANOR is assigned to ν(CO) bands from the heme iron carbonyl in distinct configurations within the active site. Accordingly, discreet configurations are observed at 15 K, while at room temperature, rapid sampling of these configurations leads to a Gaussian distribution centered at 1972 cm^{-1}. In addition to these discreet but comparable heme-CO configurations, a ν(CO) at 1910 cm^{-1} corresponds to a semi-bridging configuration of the heme-CO with Fe_B that is stabilized at low temperature. Indeed, carbonyls bridging two metals display ν(CO) bands downshifted by as much as 200 cm^{-1} compared to terminal CO groups, and semi-bridging configurations with intermediate ν(CO)s have also been reported in inorganic compounds.[10] As the heme-CO adopts a bridging configuration between the two irons, the electron density on Fe_B increases and results in a ν(CO) shift in Fe_BCO from 2066 to 2046 cm^{-1}. Thus, the photolysis of the bridging CO is accompanied by an S-signal originating from Fe_BCO that occurs at 2046 cm^{-1} in the dark spectrum and at 2066 cm^{-1} after illumination. This latter frequency matches the 2068 cm^{-1} stretch observed for Fe_BCO at room temperature, where the bridging CO is not observed. Concomitant detection, by room-temperature FTIR, of the ν(CO) bands from heme a_3 and Cu_B carbonyl complexes has been reported previously in cytochrome ba_3 from *Thermus thermophilus*.[11] However, these data were interpreted in terms of a binding equilibrium involving a single CO ligand bound to either heme a_3 or Cu_B within the active site. Upon photodissociation from the heme moiety, the CO bound to heme a_3 readily binds to the Cu_B site.[9] In contrast, photodissociation of the heme-CO complex in qCu_ANOR results in a perturbation of the ν(C−O) associated with Fe_BCO, indicating that *two* CO molecules bind concomitantly at the diiron site.

Chloride can affect the structure and function of metalloproteins via direct binding to the metal center or by inducing conformational changes through interactions with protein side chains. For example, chloride acts as a heterotropic ligand in hemoglobin, where it binds at the 2,3-diphosphoglycerate site.[12] In CcO, XAS studies have shown direct chloride binding to Cu_B.[13] In preliminary experiments with *B. azotoformans* membrane fragments, rather than isolated qCu_ANOR, we have confirmed that a chloride effect is also observed. However, more experiments are required to determine whether the decreased CO affinity at the non-heme iron site is due to direct coordination to Fe_B(II) or if chloride acts indirectly by perturbing the substrate pocket structure. For example, chloride could mimic a native effector, e.g., a specific anionic molecule, a small regulatory protein, or a specific lipid interacting with the protein matrix. This study, including the detection of *two interacting iron−carbonyls* at the diiron site of NOR, clearly strengthens mechanistic models of bacterial NO reductase catalysis where the formation of an iron-nitrosyl dimer, i.e., a $[\{FeNO\}^7]_2$ unit, promotes N−N bond formation. In fact, we recently characterized such a $[\{FeNO\}^7]_2$ unit at the non-heme diiron center of protein R2 of *Escherichia coli* ribonucleotide reductase that slowly produces N_2O.[14]

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Supporting Information Available: High-frequency RR spectrum of qCu_ANOR before and after addition of CO; low-temperature FTIR difference spectrum of the ^{13}CO complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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