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Model Studies of Azide Binding to Functional Analogues of CcO

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N_3^- binding to a functional model of CcO is investigated in its Fe^{3+}, $Fe^{3+}Cu^+$, and $Fe^{3+}Cu^{2+}$ forms. A combination of EPR and FTIR indicates that N_3^- binds in a bridging mode in the bimetallic sites and signature N_3^- bands are identified for several forms of N_3^- binding to the site. The presence of the distal metal increases the binding affinity of N_3^-. This bridging enables antiferromagnetic interaction between the two metal centers in the $Fe^{3+}Cu^{2+}$ state, which results in an EPR-silent ground state.

Cytochrome c oxidase (CcO) is the terminal electron donor to oxygen in mitochondria, reducing it to H_2O. This generates a proton gradient that drives ATP synthesis.[1] The O_2 reduction occurs at the heterobimetallic active site of CcO consisting of a heme a_3 and a Cu_B center within 5 Å.[2a–c] The reaction mechanism of this fundamentally important enzyme and its interactions with small molecules (e.g., N_3^-, CO, and NO) have been a focus of major research for several decades.[1,3] In particular, the nature of its interaction with its inhibitor N_3^- has been investigated using Fourier transform (FTIR), electron paramagnetic resonance (EPR), and resonance Raman techniques.[4a–c] It is generally accepted that one or two N_3^-'s can bind either in a bridging or in a terminal manner to the resting oxidized state of the active site ($Fe^{3+}Cu^{2+}$). However, unambiguous assignment of the spectroscopic data is generally complicated by spectroscopic features from heme *a* and Cu_A centers (also present in the enzyme). Furthermore, the role of the Cu_B center in N_3^- binding to the CcO active site and the possibility of N_3^- binding to a possible mixed-valent form (i.e., $Fe^{3+}Cu^+$ or $Fe^{2+}Cu^{2+}$ forms of these complexes, not $Fe^{3+}Cu^{2+}$ with heme *a* and CuA reduced) are yet unexplored. Synthetic biomimetic model complexes provide a controlled environment for studying these key interactions that take place in a protein active site. Thus, there is a need for a systematic study of N_3^- binding to the CcO active site model that will serve as a reference for analyzing spectroscopic data obtained in protein active sites.

Several synthetic CcO models have been reported in the past decade,[5] and one of them has been used to model N_3^- binding.[6] Unfortunately, in the absence of FTIR data, the presence of multiple N_3^--bound forms, and the lack of O_2 reduction activity of this complex, it is hard to correlate those results to the ones obtained in CcO. Recently, one synthetic model complex has been shown to have O_2-reducing activity, under physiological conditions, comparable to the parent enzyme (Scheme 1).[7a–c] In this study, we show that this functional model can be used to investigate N_3^- binding to the CcO active site. We use EPR and FTIR techniques to characterize different modes of N_3^- binding that helps gain insight into the origin of noncompetitive inhibition of CcO by these anionic ligands.[8]

The monometallic Fe^{3+} complex can be synthesized by oxidizing the Fe^{2+} complex with ferrocinum tetrafluoroborate (Fc^+) in dichloromethane (CH_2Cl_2). The mixed-valent (in the active site) $Fe^{3+}Cu^+$ state has not been well characterized in CcO[9] or in any other model systems.[7b] It can be obtained by adding 1 equiv of Cu^{2+} to a Fe^{2+} complex in a CH_2Cl_2 solution, whereupon Fe^{II} gets oxidized to Fe^{3+} and the resulting Cu^+ binds to the distal pocket of the model (Figure S1 in the Supporting Information). It can also be synthesized by adding 1 equiv of Cu^+ to a Fe^{3+} complex. The addition

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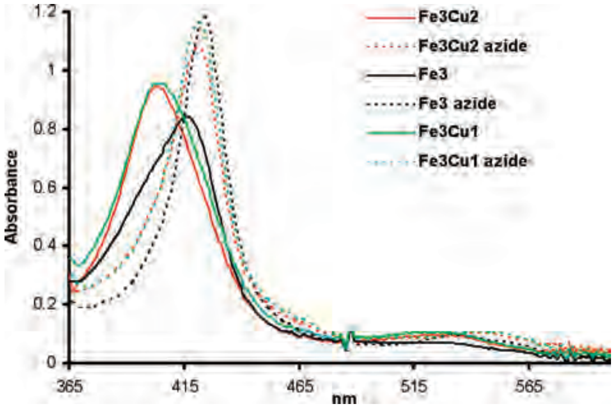
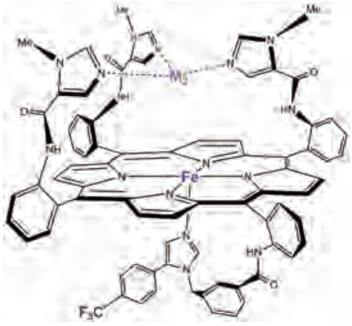


Figure 1. Absorption spectra of Fe^{3+}, $Fe^{3+}Cu^{+}$, and $Fe^{3+}Cu^{2+}$ states and their N_3^--bound forms.

Scheme 1. Representation of the Functional Model Complex in its Fe^{3+} (No M2), $Fe^{3+}Cu^{+}$ (M2 = Cu^{+}), and $Fe^{3+}Cu^{2+}$ (M2 = Cu^{2+}) States (Metals Have Triflate Counterions)



of 2 equiv of Cu^{2+} to the Fe^{2+} complex or 1 equiv of Fc^{+} to the $Fe^{3+}Cu^{+}$ complex generates the $Fe^{3+}Cu^{2+}$ complex.

Binding N_3^- to the monometallic Fe^{3+} porphyrin complex in CH_2Cl_2 shifts the Soret band from 415 to 424 nm, and the Q band shifts from 527 to 534 nm (Figure 1, solid black and dashed black lines). For the $Fe^{3+}Cu^{+}$ complex (Figure 1, solid green and dashed green lines), the Soret shifts from 406 to 423 nm upon N_3^- binding, while in the $Fe^{3+}Cu^{2+}$ state (Figure 1, solid blue and dashed blue lines), the Soret shifts from 404 to 422 nm. These changes in the absorption features upon N_3^- binding to Fe^{3+}, $Fe^{3+}Cu^{+}$, and $Fe^{3+}Cu^{2+}$ states indicate that N_3^- ligates to the Fe^{3+} center in all three cases. In all of the above cases, the red shift in the Soret band is indicative of a change of the spin state of Fe^{3+} from high spin to low spin upon N_3^- binding.[6,10]

Quantitative addition of N_3^- to these complexes indicates that the monometallic Fe^{3+}, the mixed-valent $Fe^{3+}Cu^{+}$, and the fully oxidized $Fe^{3+}Cu^{2+}$ require 4, 2, and 1 equiv of N_3^- for complete binding, respectively. This is consistent with cooperativity between the two metals in this bimetallic active site model, where the presence of the distal metal and a subsequent increase in its charge enhance anionic ligand binding to the heme Fe.[6] This could be due to the bridging nature of the N_3^- ligand and is evaluated below.

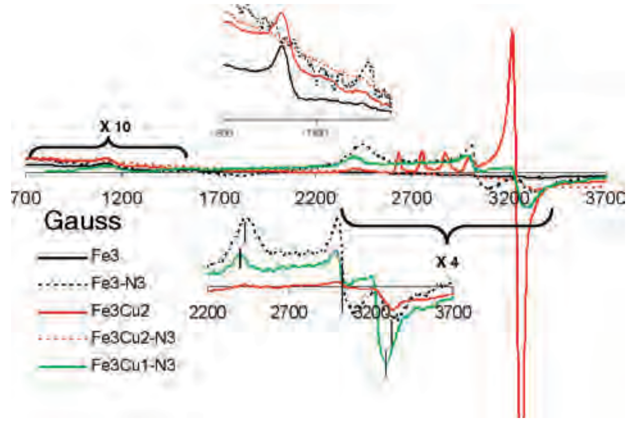


Figure 2. EPR spectra of the Fe^{3+}, $Fe^{3+}Cu^{+}$, and $Fe^{3+}Cu^{2+}$ states and their N_3^--bound forms collected at 77 K in frozen CH_2Cl_2. The upper inset shows the low-field region, and the lower inset shows the low-spin Fe^{3+} signals in the high-field region (three *g*'s are indicated by vertical lines). The small EPR signal in the $Fe^{3+}Cu^{2+}N_3^-$ complex is due to some unconverted $Fe^{3+}N_3^-$.

EPR data indicate that the azide binding to the monometallic high-spin Fe^{3+} complex (Figure 2, solid black line and upper inset) leads to a low-spin Fe^{3+} center (Figure 2, dashed black line and lower inset) as suggested above by the blue shift of the Soret band. Binding of N_3^- to the mixed-valent $Fe^{3+}Cu^{+}$ complex, which has the same EPR signal as the monometallic Fe^{3+} complex, also leads to the growth of a new low-spin Fe^{3+} signal (Figure 2, green line and lower inset). The *g* values of this signal are different from those obtained from the $Fe^{3+}N_3^-$ complex. This implies that the azide binding to the low-spin Fe^{3+} centers in these are different. The fully oxidized $Fe^{3+}Cu^{2+}$ state of the complex has a high-spin Fe^{3+} signal at 1150 G (Figure 2, red line; 4.5 K EPR data in Figure S2 in the Supporting Information) and a Cu^{2+} signal at 2600−3400 G. Spin quantification of this Cu^{2+} signal against a standard at 77 K accounts for >95% of the sample, indicating that the Fe^{3+} and Cu^{2+} centers are magnetically uncoupled. This indicates that in the fully oxidized $Fe^{3+}Cu^{2+}$ state there is no bridging ligand between the two paramagnetic centers.[11] On the other hand, binding of N_3^- leads to disappearance of the Cu^{2+} and Fe^{3+} EPR signals (Figure 2, dashed red). Thus, N_3^- binds as a bridging ligand between the $S = {}^1/_2$ Fe^{3+} (indicated by the absorption and EPR of the $Fe^{3+}N_3^-$ complex) and $S = {}^1/_2$ Cu^{2+} centers. This enables antiferromagnetic coupling between these sites, leading to an EPR-silent ground state. Thus, this model successfully reproduces the bridging interactions observed in most CcO's; i.e., while in its resting $Fe^{3+}Cu^{2+}$ state, there is no bridging ligand and the heme a_3 and Cu_B can be bridged by an N_3^- ligand.[2a,b] The bridging of the two metals with a single N_3^- implies that, even with the lack of crystallographic data, it is safe to conclude that the distances between the two metals in this model must be ~6 Å as observed in the CcO active site.

FTIR has been used extensively to study the interaction of N_3^- with ferric porphyrin models.[10] The FTIR of the N_3^- bound Fe^{3+} complex shows a N_3^- asymmetric stretch at 2010 cm^{-1} (Figure 3, blue line), which corresponds to a low-spin

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(11) EPR on the fully oxidized $Fe^{3+}Cu^{2+}$ species obtained in wet CH_2Cl_2 also shows the same EPR, indicating the lack of a bridging ligand.

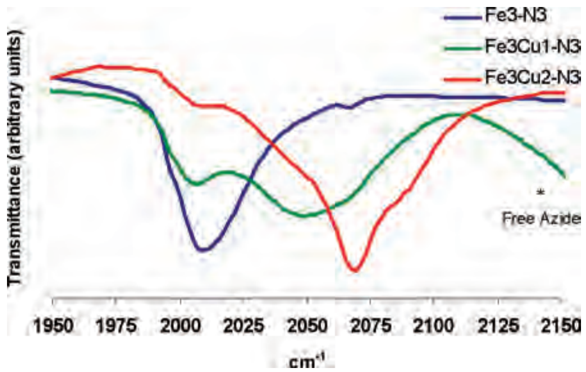

Figure 3. FTIR spectrum of the azide-bound complexes of the Fe^{3+}, $Fe^{3+}Cu^{+}$, and $Fe^{3+}Cu^{2+}$ complexes (*Y* axis arbitrarily scaled). The shoulder around 2010 cm^{-1} in both the $Fe^{3+}Cu^{+}$ and $Fe^{3+}Cu^{2+}N_3^{-}$ complexes is due to some unconverted $Fe^{3+}N_3^{-}$ (Figure S4 in the Supporting Information).

$Fe^{3+}N_3^{-}$ species, as indicated by EPR data. Using a single N^{15}-substituted N_3^{-} (i.e., $^{15}NNN^{-}$; Figure S3 in the Supporting Information), two bands are observed at 1990 and 1998 cm^{-1}.[4a,c,12] N_3^{-} binding to the mixed-valent $Fe^{3+}Cu^{+}$ complex shows a major IR band at 2048 cm^{-1} (Figure 3, green line). This band is 38 cm^{-1} shifted relative to the N_3^{-} band in the $Fe^{3+}N_3^{-}$ complex. This is consistent with a bridging N_3^{-} between the Fe^{3+} and Cu^{+} centers where more electron density is shifted from its N−N π*-type highest occupied molecular orbitals. This also parallels the small differences in the *g* values in the EPR spectra of the $Fe^{3+}N_3^{-}$ complex and the mixed-valent $Fe^{3+}Cu^{+}N_3^{-}$ complex. With N^{15}-substituted azide, this 2048 cm^{-1} shifts to 2033 cm^{-1} (Figure S3 in the Supporting Information). The ν_{assym} are further blue-shifted to 2068 cm^{-1} with a shoulder at 2090 cm^{-1} in the fully oxidized $Fe^{3+}Cu^{2+}N_3^{-}$ complex (Figure 3, red line), where, from EPR, N_3^{-} bridges the Fe^{3+} and Cu^{2+} centers. With $^{15}N_3^{-}$, the 2068 cm^{-1} band shifts to 2054 and 2063 cm^{-1} and the 2090 cm^{-1} shoulder shifts to 2083 cm^{-1} (Figure S3 in the Supporting Information).

In summary, the biomimetic models studied here indicate that N_3^{-} can bind in the $Fe^{3+}Cu^{+}$ (mixed-valent) as well as $Fe^{3+}Cu^{2+}$ (resting oxidized) states of the CcO active site. The presence of the distal metal increases the binding affinity for N_3^{-} because of its bridging interaction. This enhances N_3^{-} interaction with the $Fe^{3+}Cu^{+}$ and $Fe^{3+}Cu^{2+}$ states, making it an efficient inhibitor of CcO. This bridging between the metals results in an antiferromagnetically coupled ground state in the fully oxidized state of the active site. The terminal N_3^{-} binding is characterized by an IR vibration at 2010 cm^{-1}, while the bridging N_3^{-} is characterized by vibrations at 2048 and 2068 cm^{-1} for the mixed-valent and fully oxidized states, respectively. These results should provide benchmark features for the analysis of FTIR data obtained in a CcO active site.

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Supporting Information Available: The 4.5 K EPR spectra of the Fe^{3+} complexes, the kinetic traces of the distal metal binding, and the FTIR data in solution and with ^{15}NNN azide. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) A single ^{15}N-substituted azide should give rise to two ν_{assym} modes of N_3^{-} because it could bind with either the ^{15}N or ^{14}N atom of the azide.