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Catalytic Reduction of NO to N_2O by a Designed Heme Copper Center in Myoglobin: Implications for the Role of Metal Ions

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One of the most fascinating subjects in chemistry and biology is structural and functional comparison between heme copper centers in heme copper oxidases (HCOs) and heme−non-heme iron centers in bacterial NO reductases (NORs). Both contain a heterobinuclear center with a heme in close proximity to either a copper (in HCOs) or a non-heme iron (in NORs).[1,2] While the heme copper center catalyzes four-electron reduction of O_2 to H_2O,[3−5] the heme−non-heme iron center promotes two-electron reduction of NO to N_2O ($2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$).[6,7] The structural similarities between HCOs and NORs suggest that they might have evolved from a common phylogeny.[8] Therefore, while copper was selected by nature for O_2 reduction, iron is preferred for NO reduction, indicating that the presence of a Cu_B or an Fe_B site is a prerequisite for enzyme catalysis. Therefore, an interesting issue is why a protein is efficient at O−O bond cleavage when using a copper ion and proficient at N−N bond formation when using an iron ion.

An entry point to addressing the above issue is cross-reactivity between the two enzymes. Previous studies have shown that a bacterial NOR from *Paracoccus denitrificans* has HCO activity.[9] Several families of HCOs, such as the ba_3 and caa_3 oxidases from *Thermus thermophilus* and cytochrome cbb_3 oxidase from *Pseudomonas stutzeri*, have displayed NOR reactivity, while other families, such as cytochrome oxidase from bovine heart, show no NOR activity.[10−13] The reasons for the different reactivities between HCOs and NORs and among HCOs from different species still remain to be clarified. A contributing factor is difficulty in replacing one metal ion with another in native HCOs or NORs. Synthetic models that mimic the native enzymes both structurally and functionally are rare in literature.[14−16]

To provide insights into the reduction of O_2 by HCO, a Cu_B center has been designed into wild-type Mb (called Cu_BMb, Figure 1A) to create a binuclear heme copper center as a small model protein.[17] Studies of Cu_BMb have shown that the Cu_B center plays a critical role in O_2 binding and reduction[17] and in modulating the redox potential of the heme when the heme and copper are coupled.[20] In addition, proton delivery, perhaps through a hydrogen-bonding network, is important in heterolytic O−O bond cleavage.[18,19] Finally, both the heme type[19] and the presence of chloride[20,21] also play a role in its O_2 reduction. Here, we report the effects of metal ions in the Cu_B center on the reaction of NO with Cu_BMb. This study shows that the redox property and the oxidation state of metal ions in the Cu_B center can exert significant structural and reactivity changes for NO reduction, and the implications of such interactions in HCOs are discussed.

The absorption spectrum of ferrous-Cu_BMb-NO, prepared from the reaction of met-Cu_BMb with excess dithionite and $NaNO_2$ under Ar, displays a Soret band at 420 nm and visible absorption bands at 548 and 583 nm (Figure 1B), similar to the spectrum of ferrous-WTswMb-NO.[22,23] The EPR spectra of ferrous Cu_BMb-NO and

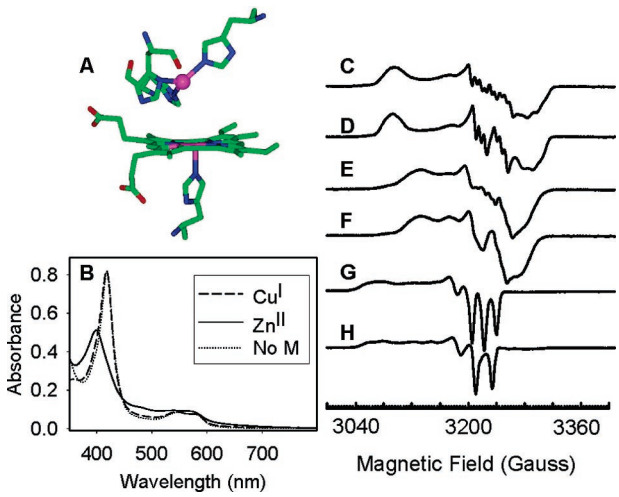

Figure 1. (A) Active site of a computer model of Cu_BMb. (B) UV−vis spectra of ferrous Cu_BMb-NO in the absence of metal ions (dotted line), in the presence of Cu(I) (dashed line), and in the presence of Zn(II) (solid line). EPR spectra of (C) ferrous-Cu_BMb-NO and (D) ferrous-Cu_BMb-^{15}NO in the absence of metal ions; (E) ferrous-Cu_BMb-NO and (F) ferrous-Cu_BMb-^{15}NO in the presence of copper; and (G) ferrous-Cu_BMb-NO and (H) ferrous-Cu_BMb-^{15}NO in the presence of Zn(II). Samples were recorded in 20 mM Tris, pH 8, at 45 K and 0.2 mW power; microwave frequency, 9.050 GHz.

Cu_BMb-^{15}NO, prepared using $NaNO_2$ and $Na^{15}NO_2$, respectively, displayed *g* values at 2.090, 2.003, and 1.972 (Figure 1C,D). The hyperfine splitting from both bound NO and the proximal histidine nitrogen can be clearly observed, indicating the formation of a six-coordinate ferrous heme-nitrosyl species.[24]

The effect of copper on the binding of NO to ferrous Cu_BMb was studied under the same conditions. As shown in Figure 1E,F, the EPR spectra of ferrous-Cu_BMb-NO in the presence of copper showed shifted *g* values at 2.067, 2.006, and 1.97. Although the hyperfine splitting from NO is still clearly observed, the hyperfine splitting from the proximal histidine becomes less resolved in comparison to the Cu-free species, probably due to a weakening of the proximal heme Fe−His bond after the binding of Cu. In the presence of Zn(II), the UV−vis spectrum of ferrous Cu_BMb-NO showed a Soret band at 399 nm, a charge-transfer peak at 484 nm (as a shoulder), and visible bands at 543 and 568 nm, which are characteristic of the formation of a five-coordinate ferrous heme-NO species (Figure 1B).[25,26] This conclusion is further supported by the characteristic *g* values (2.107, 2.032, and 2.009) and the hyperfine splitting pattern of the EPR spectra of the ferrous Cu_BMb-NO and Cu_BMb-^{15}NO species in the presence of Zn(II) (Figure 1G,H), which is similar to the EPR spectrum of five-coordinate ferrous hemoglobin-NO.[24] Thus, although the binding of Cu(I) in the Cu_B center can only weaken the proximal heme Fe-His bond, the bound Zn(II) caused the complete cleavage of

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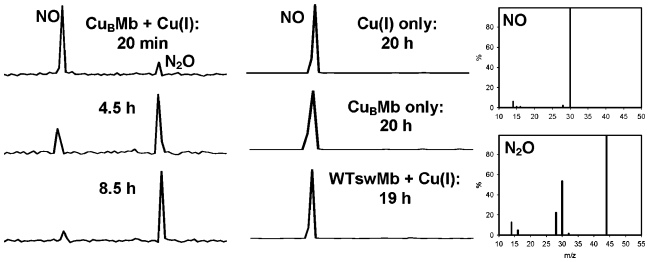

Figure 2. GC/MS chromatogram of NO reduction by Cu_BMb and Cu(I). The GC peaks have been normalized.

the Fe-His bond and formation of a five-coordinate heme-NO complex. As a control experiment, the EPR spectra of WTswMb-NO showed little change in the presence of Cu(I) or Zn(II) (see Supporting Information). These results demonstrated that the bound Cu(I) and Zn(II) in Cu_BMb play different roles in the binding of NO to heme.

To examine the reactivity of Cu_BMb toward NO reduction, purified NO was added to a solution containing Cu_BMb under He in the presence of metal ions and ascorbate/tetramethyl-*p*-phenylenediamine (TMPD). In the presence of Cu(I), Cu_BMb catalyzes the reduction of NO to N_2O, as evidenced by the appearance of a second peak at a longer retention time in the GC, which corresponds to a 44 MW peak (N_2O) in the MS (Figure 2). In addition, the relative GC peak intensity of N_2O:NO increases as a function of time, indicating further reduction of NO to N_2O as the reaction proceeds. The turnover number for NO reduction was calculated to be ~2 mol NO·mol Cu_BMb^{-1}·min^{-1}, close to the 3 mol NO·mol $enzyme^{-1}$·min^{-1} reported for the ba_3 oxidases from *T. thermophilus*.[10−13] Under identical conditions, the catalytic reduction of NO was not observed with Cu_BMb alone, with Cu(I) alone, or with WTswMb in the presence of Cu(I) (Figure 2). These results demonstrated that Cu(I) plays a critical role in the reduction of NO to N_2O in Cu_BMb.

It has been shown that HCOs from different species show either substantial NO reduction activity or no activity. The results presented here strongly suggest that the designed heme copper center in Cu_BMb is a close structural and functional model of HCOs with NO reduction activity. Our small and well-characterized protein model, which possesses no other chromophores and an easily substitutable Cu_B metal binding site, allowed us to gain new insights. First, no NO reduction was observed in the absence of metal ions or in the presence of redox-inactive Zn(II) in the designed Cu_B center, even with a large excess of reductant (see Supporting Information). Catalytic reduction of NO by Cu_BMb occurred only in the presence of Cu(I). These results demonstrate that electron transfer from the Cu_B center is essential for NO reduction. Second, it has been shown that NO can labilize the heme Fe-His bond in both heme proteins and model compounds.[27,28] Binding of NO to the reduced binuclear center in NOR results in formation of a five-coordinate ferrous heme-NO complex,[29,30] and a five-coordinate heme-NO species has been detected and proposed as a key intermediate in the reduction of NO by cytochrome cbb_3 oxidase.[13] In contrast to these observations, it has been suggested that five-coordinate ferrous heme-NO complexes could represent dead-end products incompetent in N_2O production.[6] Our UV−vis and EPR studies indicated that Cu(I) binding to Cu_BMb further weakens the heme Fe-His bond, in addition to the NO trans effect, suggesting that bond weakening, but not necessarily bond cleavage into a five-coordinate species, is a contributing factor in NO reduction. Finally, the binding of Zn(II) to the Cu_B center of the same Cu_BMb protein produced a five-coordinate heme-NO species, resulting from the cleavage of the proximal heme Fe-His bond (Figure 1). Although the binding of metal ions in the designed Cu_B center can cause conformational changes, the different effects of Cu(I) and Zn(II) on the structure of ferrous-Cu_BMb-NO probably result from their different oxidation states, with higher metal ion oxidation states facilitating greater weakening of the proximal heme Fe-His bond via increasing interactions with the Fe-bound NO. Therefore, reduced Fe(II) in NOR has the desirable features of both Zn(II), which is capable of further weakening of the heme Fe-His bond necessary for NO reduction, and Cu(I), which possesses redox activity. These reasons may be why iron has been chosen by nature for the reduction of NO to N_2O.

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Supporting Information Available: Experimental details for EPR and GC/MS measurements, as well as the EPR spectra of WTswMb-NO in the presence of Cu(I) and Zn(II). This material is available free of charge via the Internet at http://pubs.acs.org.

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