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A Purple Cupredoxin from *Nitrosopumilus maritimus* Containing a Mononuclear Type 1 Copper Center with an Open Binding Site

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Supporting Information

ABSTRACT: Mononuclear cupredoxin proteins usually contain a coordinately saturated type 1 copper (T1Cu) center and function exclusively as electron carriers. Here we report a cupredoxin isolated from the nitrifying archaeon *Nitrosopumilus maritimus* SCM1, called Nmar1307, that contains a T1Cu center with an open binding site containing water. It displays a deep purple color due to strong absorptions around 413 nm (1880 M^{-1} cm^{-1}) and 558 nm (2290 M^{-1} cm^{-1}) in the UV−vis electronic spectrum. EPR studies suggest the protein contains two Cu(II) species of nearly equal population, one nearly axial, with hyperfine constant $A_{\parallel} = 98 \times 10^{-4}$ cm^{-1}, and another more rhombic, with a smaller $A_{\parallel}$ value of 69×10^{-4} cm^{-1}. The X-ray crystal structure at 1.6 Å resolution confirms that it contains a Cu atom coordinated by two His and one Cys in a trigonal plane, with an axial H_2O at 2.25 Å. Both UV−vis absorption and EPR spectroscopic studies suggest that the Nmar1307 can oxidize NO to nitrite, an activity that is attributable to the high reduction potential (354 mV vs SHE) of the copper site. These results suggest that mononuclear cupredoxins can have a wide range of structural features, including an open binding site containing water, making this class of proteins even more versatile.

Cupredoxins are copper-containing proteins that exhibit redox properties important for electron-transfer (ET) function in many key biological processes, from photosynthesis to respiration.[1−10] The most prominent members of this class of proteins are the type 1 copper (T1Cu) proteins, which contain a copper center coordinated by one S_{Cys} and two N_{His} in a trigonal plane, with axial interactions above and sometimes below the plane.[11−15] Mononuclear cupredoxins have been studied for decades, and almost all of them natively contain a coordinately saturated Cu site and perform ET function.[1−10,13,16−30] The only exception so far is nitrosocyanin, which is in a cupredoxin scaffold but contains an additional water ligand.[31,32] Spectroscopic and X-ray crystallographic studies suggested that nitrosocyanin contains a type 2 copper (T2Cu) center, similar to many copper enzymes.[31,32]

Advances in genomics and culturing techniques have led to the discovery of previously unknown microbes that play fundamental roles in Earth's biogeochemical cycles. An important example is the nitrifying archaeon *Nitrosopumilus maritimus* (*N. mar*).[33] *N. mar* and closely related strains are globally abundant in marine ecosystems,[34−50] where they catalyze the oxidation of ammonia to nitrite, a critical step in the global nitrogen cycle.[36,37,47,49] Interestingly, *N. mar* does not contain any cytochrome *c* proteins, the most ubiquitous class of ET proteins, and appears to have replaced them with cupredoxin-like proteins.[51]

Given the important role that *N. mar* plays in the global nitrogen cycle, and its unusual replacement of heme-based ET proteins with copper-based counterparts, characterizing some of the cupredoxins from *N. mar* would expand our understanding of the interplay between heme-based and cupredoxin-based biochemical processes. Here we report recombinant expression, purification, and characterization of a small cupredoxin protein from *N. mar* called Nmar1307. Spectroscopic and X-ray crystallographic studies show that Nmar1307 contains a mononuclear T1Cu center with a water as ligand. This open binding site prompted us to investigate its reactivity toward small substrates like NO, which led to the discovery of reversible NO oxidation/NO_2^- reduction activity. This suggests a possible role for Nmar1307 in the ammonia oxidation pathway or in detoxification of nitrification byproducts.

Sequence analysis suggests that Nmar1307 contains an N-terminal transmembrane helix (Figure S1). In order to obtain a water-soluble protein for biochemical and biophysical studies, we cloned the *Nmar1307* gene without this transmembrane helix. This soluble copper-containing domain was expressed in *Escherichia coli* and purified using two consecutive ion-exchange chromatographic steps and one size exclusion chromatography step (see Supporting Information and Figure S2). Electrospray ionization mass spectrometry of the purified protein showed a single peak with a mass of 10 746 ± 1 Da (see Figure S3), which is within experimental error of the calculated mass of copper-free apo-Nmar1307 (10 747 Da), indicating successful expression and purification of the copper-free apo protein.

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Addition of Cu(II) to apo-Nmar1307 in 50 mM Tris buffer at pH 8.0, close to the pH of the ocean where this protein resides, converted the colorless protein into one with a strong purple color, showing absorption peaks at 413 and 558 nm and extinction coefficients of 1880 and 2290 M^{-1} cm^{-1}, respectively, determined by spin-counting electron paramagnetic resonance (EPR) (Figure 1a and Figure S4). Its

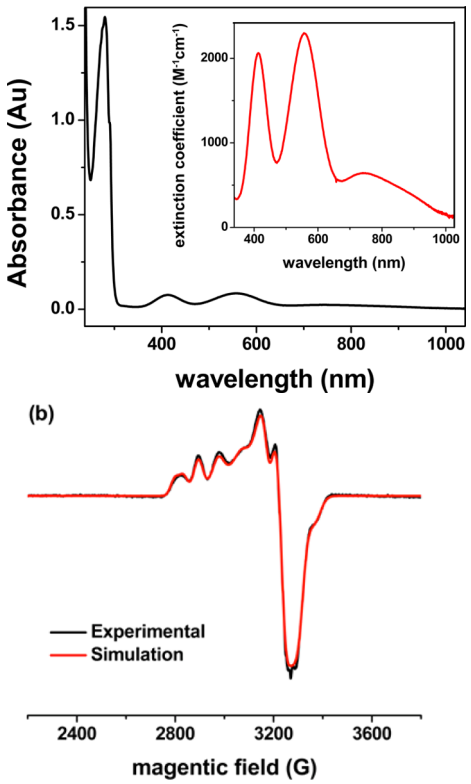

Figure 1. (a) UV−vis and (b) EPR spectra of Cu(II)-Nmar1307 in 50 mM Tris buffer, pH 8.0.

UV−vis spectrum is very similar to those of distorted T1Cu proteins.[8] To corroborate this conclusion, we collected EPR spectra of the protein at both X-band (Figures 1b and S5a) and Q-band (Figure S5b). The best fit was obtained when the X- and Q-band spectra were simulated with two species (1:1 ratio), one nearly axial and the other rhombic. The *g* values and hyperfine constants are listed in Table 1. Copper species I

Table 1. EPR Simulation Parameters of Nmar1307

species	g_x, g_y, g_z	A_x, A_y, A_z (10^{-4} cm^{-1})
50% species I	2.056, 2.042, 2.269	15, 9, 98
50% species II	2.034, 2.070, 2.272	59, 28, 69

[a]Line width values for *x*, *y*, *z* are 48, 49, 30 and 30, 57, 35 gauss for species I and II, respectively. Euler angles for species I are 118, 13, −118. For species II, *g* and *A* are found to have coincident axes.

exhibits a nearly axial EPR spectrum, with $A_{\parallel} = 98 \times 10^{-4}$ cm^{-1}, similar to that of M121H azurin.[52] On the other hand, Copper species II displays a more rhombic EPR spectrum, with a smaller $A_{\parallel}$ value of 69×10^{-4} cm^{-1}, similar to plantacyanin from cucumber peel.[53] Both species have a distorted T1Cu center with either tetrahedral or tetragonal distortions.[54]

The spectroscopic features of the protein remained largely unchanged when the pH was changed (see Figure S6). Similar pH-independent behavior has been observed in nitrosocyanin, which also has a water ligand in the Cu binding site.[55] The ratio of the two species is ∼50:50 at pH 8, but at high glycerol concentrations (see Figure S7), the rhombic component is slightly favored (40:60 for 60% glycerol). Therefore, we attribute the observation of two species in the EPR spectra to different conformations of the water ligand in the Cu center, one with higher rhombicity.

The molecular structure of Cu(II)-Nmar1307 (PDB ID: 5FC9) was determined by X-ray crystallography at 1.6 Å resolution (see Figure 2 and Tables S1 and S2). (Note: The

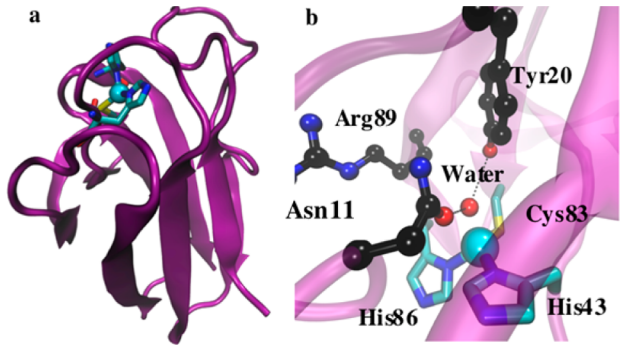

Figure 2. (a) Crystal structure of Cu(II)-Nmar1307 (PDB ID: 5FC9). (b) Zoom-in of the copper binding site with nearby amino acids shown in black ball-and-stick representation. The coordinating water is shown as a red sphere, the primary ligands as cyan sticks, and the Cu ion as cyan sphere.

residue numbering does not include the N-terminal transmembrane residues and begins with the first residue in the crystal structure.) The protein crystallized with four molecules in the asymmetric unit, with each of the four molecules being the same except for different conformations of a few surface residues that display high degrees of freedom. As shown in Figure 2a, Nmar1307 displays an overall structure typical of the cupredoxin fold observed in T1Cu proteins.[1,2,10] Figure 2b indicates that the copper is coordinated to one Cys (S_{γ}Cys83-Cu = 2.25 Å) and two His (N_{δ}His43-Cu = 2.01 Å and N_{δ}His86-Cu = 2.07 Å) in a trigonal plane. The Met/Leu/Gln axial ligand above the trigonal plane commonly found in other cupredoxins is replaced by Arg89 in Nmar1307. However, the structure of Nmar1307 clearly shows that Arg89 is rotated away from the copper, creating an open coordination site (Figure 2b). While we cannot rule out the possibility of the Arg being the axial ligand, the observed rotamer in the crystal structure and the steric clash between this Arg and Tyr20 deem it unlikely (see Figure S8). The next closest possible axial ligands (O_{δ} from Asn11 or O_{η} from Tyr20) are both >4 Å from the copper center, too far away to be considered as axial ligands. Instead, the extra electron density found 2.25 Å from the T1Cu in the Cu coordination sphere can be assigned to a water molecule. This water is 2.54 and 2.97 Å away from potential hydrogen-bonding groups of Asn11 and Tyr20, respectively. Therefore, unlike most mononuclear cupredoxins that contain a Met, Leu, or Gln residue in the axial position,[3,10] the copper site in Nmar1307 is occupied by a water ligand, which could potentially be displaced by an exogenous ligand. The possible entry path for water is shown in Figure S9.

The open coordination site and binding of a solvent molecule directly to the copper suggest that this protein could display catalytic activity toward small molecules. Since *N.*

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mar is known to play a role in the nitrogen cycle, and because an enzyme containing only a single copper atom is likely to only participate in one-electron transformations, the ability of Cu(II)-Nmar1307 to oxidize NO to NO_2^- was tested. Upon reaction of Cu(II)-Nmar1307 with NO, a slow decrease of the visible absorption bands (Figure 3a) and a decrease of Cu(II)

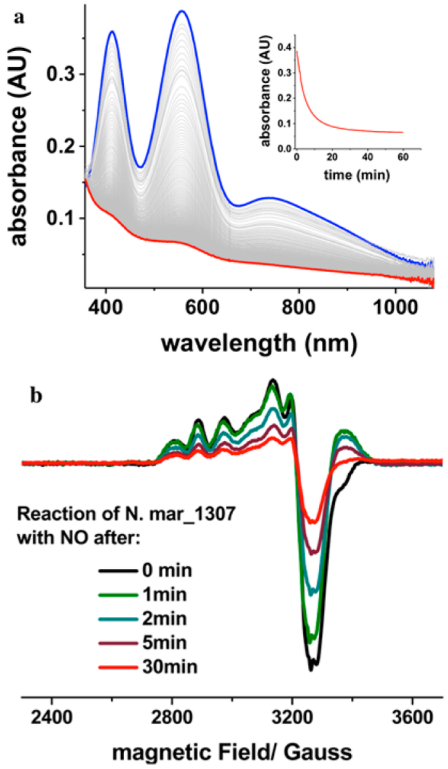

Figure 3. (a) Time course UV−vis spectra showing bleaching of the ligand-to-metal charge-transfer bands of 193 μM Cu(II)-Nmar1307 upon reaction with 13 equiv of NO in 50 mM Tris buffer, pH 8.0, under anaerobic conditions. (b) X-band EPR spectra of 0.3 mM Cu(II)-Nmar1307 reacting with 13 equiv of NO in 50 mM Tris, pH 8.0, collected at 30 K at different time points after reaction with NO. The small peak at 3300 G is due to unreacted NO.

EPR signals (Figure 3b) was observed, suggestive of reduction of the Cu(II) to Cu(I). In contrast, no reaction was observed between NO and wild-type azurin (Figure S10).[56] The reduced Cu(I)-Nmar1307 can be fully recovered upon reoxidation with ferricyanide (Figure S11), indicating that the copper center remains intact. The product of NO oxidation, NO_2^-, was confirmed using the Griess assay (Figure S12). Since the reaction was performed under single-turnover conditions with no external co-substrate added, we believe that the reaction goes through a previously proposed mechanism[57,58] in which NO is first oxidized to NO^+ by the protein. This species then immediately reacts with water to form NO_2^-.

The reaction with NO is highly pH dependent (Figure S13). At pH 8.0, the midpoint potential of the Cu(II)/Cu(I)-Nmar1307 (E_m = 354 mV, see Figure S14) is higher than that of NO_2^-/NO_{aq} (E_m = 200 mV vs SHE), enabling Cu(II)-Nmar1307 to oxidize NO. It is known that decreasing pH will increase the E_m of the NO_2^-/NO(aq) redox couple by ∼120 mV per pH unit. Therefore, at lower pH, such as pH 6.0, incomplete reduction of Cu(II)-Nmar1307 by NO (Figure S14) and, in the opposite direction, Cu(I) being oxidized by NO_2^- were observed (Figure S15).

In conclusion, we have discovered a T1Cu cupredoxin with an open binding site containing water, suggesting the likelihood that this class of proteins can function as a enzyme in addition to the well-known electron-transfer functions. A recent study has suggested that NO might be an obligatory intermediate in ammonia oxidation among the ammonia-oxidizing archaea, delivering electrons to the ammonia monooxygenase.[59] The finding of NO oxidation to NO_2^- by Nmar1307 isolated from *N. mar* reported here provides support for this proposal. However, the rate of the reaction is slow (second-order rate constant of 6.11 M^{-1} s^{-1}, Figure S16) and may not be physiologically relevant. The current study of Nmar1307 in isolation is only the first step in elucidating the biological function of this T1Cu protein with an open binding site, which will require future efforts in identifying, isolating, and reconstituting some of its redox partners. Nonetheless, the results demonstrate a T1Cu cupredoxin with an open binding site containing water and with catalytic activity, showing the versatility of this class of protein.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b13128.

Detailed experimental procedures; Figures S1−S15 showing protein expression, assays, and pH-dependent EPR; and Tables S1 and S2 giving crystallographic parameters and EPR simulated bond lengths (PDF)

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Notes

The authors declare no competing financial interest.

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