

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

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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 ($1450 \text{ M}^{-1}\text{cm}^{-1}$) and 558 nm ($1770 \text{ M}^{-1}\text{cm}^{-1}$) in the UV-vis electronic spectrum. EPR studies suggest the protein contain two Cu(II) species of nearly equal population, one nearly axial, with a $98 \times 10^{-4} \text{ cm}^{-1}$, and another more rhombic with a smaller $A//$ value of $69 \times 10^{-4} \text{ cm}^{-1}$. 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. Both UV-vis absorption and EPR spectroscopic studies suggest that the Nmar1307 can oxidize NO to nitrite, an activity that is attributable to its high reduction potential (361 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 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* which, along with 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 nitro-

gen 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 the Nmar1307 contains a mononuclear T1Cu center with a water as ligand. This open binding site prompted us to investigate its reactivity towards small substrates like NO, which led to the discovery of reversible NO oxidation/NO₂⁻ 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 supplementary information and Figure S2). Electrospray ionization mass spectrometry of the purified protein showed a single peak with a mass of $10,746 \pm 1$ Da (see Figure S3), which is within the experimental error of the calculated mass of copper-free apo-Nmar1307 (10,747 Da), indicating the successful expression and purification of copper-free apo protein.

Addition of Cu(II) to the apo-Nmar1307 in 50 mM Tris buffer 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 with peaks at 413 nm and 558 nm and extinction coefficients of 1450 and 1770 $\text{M}^{-1}\text{cm}^{-1}$, respectively, determined by ICP-MS (Figure 1a). This 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 (Figure 1b, S4a) and Q-band (Figure S4b). 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 exhibits a nearly axial EPR spectrum, with $A// = 98 \times 10^{-4} \text{ cm}^{-1}$, 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 $69 \times 10^{-4} \text{ cm}^{-1}$, similar to that of cucumber basic protein.⁵³ Both species belong to distorted T1Cu center with either tetrahedral or tetragonal distortions.⁵⁴

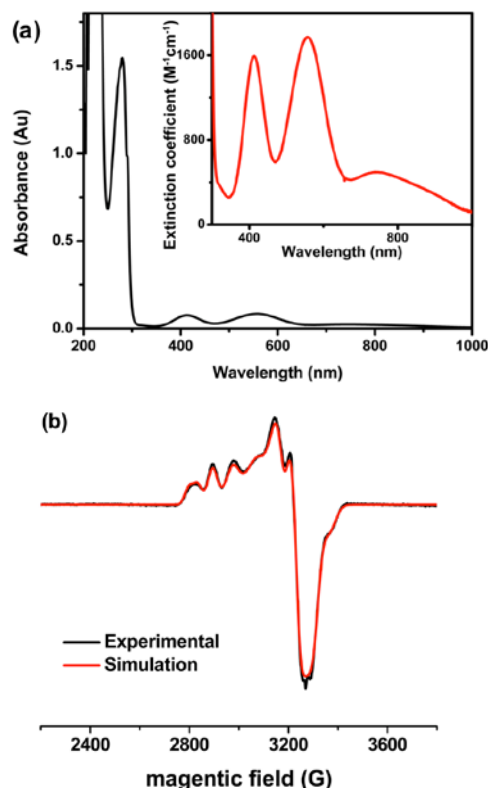


Figure 1. (a) UV-vis and (b) EPR spectra of Cu(II)-Nmar1307 in 50 mM Tris buffer, pH 8.0.

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

* 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

The spectroscopic features of the protein remained largely unchanged upon changes in pH (see Figure S5). 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 S6), the rhombic component is slightly favored (40:60 for 60% glycerol). Therefore, we attribute the observation of two species in the EPR 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, Tables S1 and S2). (Note: the residue numbering does not include the N-terminal transmembrane residues and begins with the first residue in the crystal structure.) The protein crystallized with 4 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 (S₇Cys83-Cu = 2.25 Å) and two His (N₈His43-Cu = 2.01 Å and N₈His86-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 deems it unlikely (see Figure S7). The next closest possible axial ligands (O₈ from Asn11 or O₁ 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 Å 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 S8.

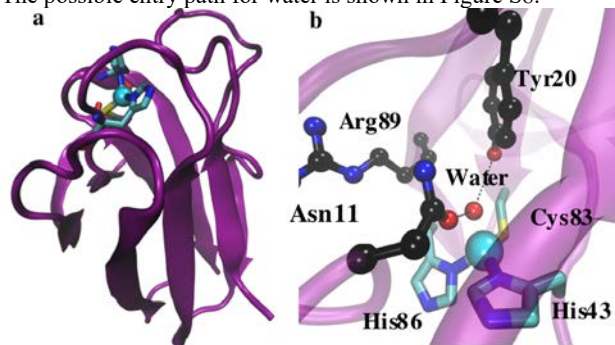


Figure 2. (a) Crystal structure of Cu(II)-Nmar1307 (PDB ID: 5FC9). (b) Zoom-in of the copper-binding site with nearby amino acids shown in black ball-and-stick representation. The coordinating water is shown as a red sphere, the primary ligands as cyan stick and the Cu ion as cyan sphere.

The open coordination site and binding of a solvent molecule directly to the copper suggests that this protein could display catalytic activity toward small molecules. Since *N. 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 1-electron transformations, the ability of Cu(II)-Nmar1307 to oxidize NO to NO₂⁻ was tested. Upon reaction with NO, a slow decrease of the visible absorption bands (Figure 3a) and a decrease of Cu(II) 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 S9).⁵⁶ The reduced Cu(I)-Nmar1307 can be fully recovered upon reoxidation with ferricyanide (Figure S10), indicating that the copper center remains intact. The product of NO oxidation, NO₂⁻, was confirmed using the Griess assay (Figure S11). Since the reaction was performed in 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 is then immediately reacts with water to form NO₂⁻.

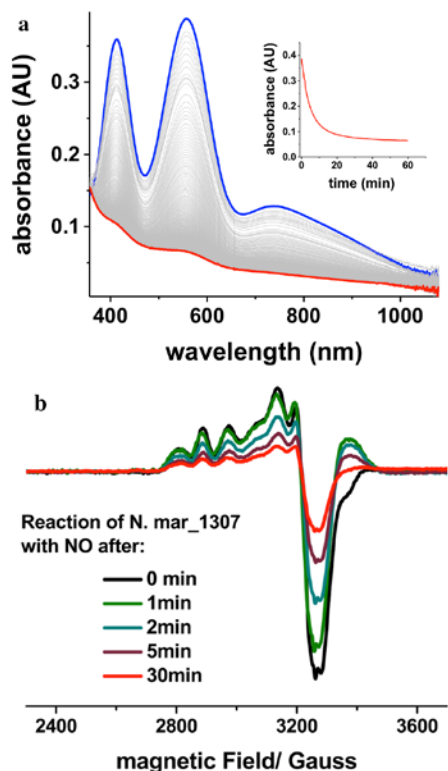


Figure 3. (a) Time course UV-vis spectra showing bleaching of the LMCT bands of 250 μ M Cu(II)-Nmar1307 upon reaction with 10 equivalents of NO in 50 mM Tris buffer pH8.0 under anaerobic condition. (b) X-band EPR spectra of 0.4 mM Cu(II)-Nmar1307 reacting with 10 equivalents of NO in 50 mM Tris pH 8.0 collected at 30 K at different time points after reacting with NO. The small peak at 3300 G is due to unreacted NO.

The reaction with NO is highly pH dependent (Figure S12). At pH 8.0, the midpoint potentials of the Cu(II)/Cu(I) (E_m =361 mV, see figure S13) is higher than that of $\text{NO}_2^-/\text{NO}_{\text{aq}}$ (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 $\text{NO}_2^-/\text{NO}_{\text{aq}}$ redox couple by ~ 120 mV per pH unit. Therefore, at lower pH, such as pH 6.0, the incomplete reduction of Cu(II)-Nmar1307 by NO (Figure S13) and the opposite direction of Cu(I) being oxidized by NO_2^- were observed (Figure S14).

In conclusion, we discovered a T1Cu cupredoxin with an open-binding site containing water, suggesting the likelihood that this class of proteins can function as a catalyst 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 $6.11 \text{ M}^{-1}\text{s}^{-1}$, Figure S15) 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 re-constituting 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 via the Internet at <http://pubs.acs.org>. Detailed experimental procedure, tables associated with crystallographic parameters and EPR simulation, and figures of protein expression, assays, and pH-dependent EPR.

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Notes

The authors declare no competing financial interests. These authors contributed equally to the current work.

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