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

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Supplemental information:

Materials and methods

Cell culture and expression of N. mar proteins

The protein coding DNA sequence of *Nmar1307* was cloned into the pET-22b periplasmic expression vector (Novagen) from genomic DNA using the NcoI and XhoI restriction sites. The His-tag was removed by incorporating a stop codon at the XhoI site before the tag. Nmar1307 natively has an N-terminal helix that anchors the protein to the cell membrane. This membrane-associated helix was omitted from the cloned sequence here in order to result in solubly expressed protein. The pET-22b leader sequence gets cleaved after expression at the Met residue.

Rosetta strain *Escherichia coli* (Novagen) was then transformed with the cloned sequences and cell stocks were made. This strain of *E. coli* was used because the codon usage of *N. mar* is very different from *E. coli* and Rosetta cells have extra translational machinery and tRNAs to compensate for this. Transformed cells were then used to streak LB plates with 100 mg/L ampicillin. These plates were incubated at 37 °C overnight to obtain single colonies. Single colonies were then used to inoculate 5 mL LB cultures with 100 μg/mL of ampicillin, which were then incubated at 37 °C until an OD_{600} of 0.6-0.8 was achieved. A 2 L portion of 2xYT media was then inoculated with 1 mL of the 5 mL culture and 100 mg/L of ampicillin. The 2 L cultures were then incubated at 37 °C overnight, typically reaching an OD_{600} of around 1.5 after 18 hours. In the morning, cells were induced with 100 mg/L of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and allowed to express protein at 37 °C for an additional 4 hours. After expressing for 4 hours, proteins were extracted *via* the osmotic shock procedure outlined below.

Osmotic shock procedure for periplasmically expressed N. mar proteins

The volumes in the procedure outlined below were scaled based on the volume of the original culture. After harvesting the *E. coli* cells by centrifugation, the cells were re-suspended in 1/8 the volume of the original culture of a solution containing 20 % w/v sucrose, 50 mM tris HCl buffer at pH 8.0, and 5 mM ethylenediaminetetraacetate (EDTA). The cells were incubated in this solution at room temperature for 1 hour and re-harvested. The supernatant was discarded and the cells were re-suspended in 1/8 the volume of the original culture of a solution with 4 mM NaCl and 1 mM dithiothreitol (DTT) for 10 minutes at 4 °C to effectively lyse the periplamsic membrane. Precipitants were then removed by centrifuging and the supernatant was collected. The pH of the supernatant was then lowered to 4.0 by slowly adding a solution of 500 mM sodium acetate at pH 4.0 (1/10 the volume of the supernatant). Lowering the pH in this way caused more precipitation, which were again removed by centrifuging and the supernatant was collected.

Column purification of Nmar1307 and re-constitution with copper

After extracting the protein from the cells by the osmotic shock procedure above, the pH of the Nmar1307 supernatant solution was raised to 6.0 by slow addition of aqueous NaOH. The supernatant was then applied to a FF Q-Sepharose column (50 mL, GE Healthcare) equilibrated in 50 mM sodium acetate buffer at pH 6.0 by adding the beads directly to the supernatant and letting it shake on an orbital shaker at 4 °C overnight. The protein was eluted from the column by a NaCl gradient on an Akta basic FPLC (GE Healthcare). The desired protein typically eluted at around 300 mM NaCl.

Nmar1307 was then reconstituted with copper by adding a slight excess of $CuSO_4$ to the partially purified protein solution. Full incorporation took several hours to complete. It was believed that the slow incorporation of copper compared to some other cupredoxin proteins may have been due to binding of copper ion to other sites. To remove this adventitious metal sites, we added excess EDTA to the protein solution and incubated at 4 °C with stirring for 1 hour to overnight. The excess EDTA was then removed by a desalting column (for small protein preps) or by the next Q-column (for larger preps). Copper incorporated protein was then separated from non-copper bound protein and other contaminants by re-applying the protein to a Q-Sepharose column and re-eluting it with a NaCl gradient. The purification finished by applying the protein to a size exclusion column (GE Healthcare). The purity of the final protein solution was assessed with SDS-Page and electrospray ionization mass spectrometry (ESI-MS).

Redox titration of Cu(II)-Nmar1307 with ferrocyanide at pH 8

The redox titration method was adopted from a previously reported procedure.[1] Cu(II)-Nmar1307 solution in 100 mM Tris pH 8.0 was degassed and equilibrated with Ar on a Schlenk line and was then transferred into an anaerobic chamber (Coy Laboratories). To 1.0 ml of 165 μM Cu(II)-Nmar1307 solution, different amounts of ferrocyanide solution (from 500 mM stock solution in water) were added at room temperature under stirring. The final concentration of ferrocyanide was at 0.5, 1.0, 2.0, 3.0, 4.0 and 6.0 mM. The UV-vis spectra were monitored on an Agilent 8453 photodiode array spectrometer, located inside the anaerobic chamber, after each addition of ferrocyanide. The reduction of Cu(II)-Nmar1307 was

monitored by decreasing LMCT band at 558 nm where the ferricyanide and ferrocyanide do not contribute to absorption. Corrections are made for dilution. The extinction coefficient at 558 nm (ε_{558nm} = 2290 M^{-1} cm^{-1}) was used to calculate the concentration of Cu(II)-Nmar1307 solution. The concentration of Cu(I)-Nmar1307 was calculated by subtracting the concentration of Cu(II)-Nmar1307 from the total concentration of Cu-Nmar1307 (165 μM). The reduction potential of Cu(II)-Nmar1307 was calculated based on Nernst equation by linear plotting the potentials of ferri/ferrocyanin redox pair versus log([Cu(II)/Cu(I)]). The intercept (354 mV) represents the reduction potential of Cu(II)-Nmar1307 at pH 8.0. The slope of the fitting was 65 mV, close to the theoretic value 59 mV.

Redox titration of Cu(I)-Nmar1307 with ferricyanide at pH 8

Cu(II)-Nmar1307 solution in 100 mM Tris pH 8.0 was degassed and equilibrated with Ar on a Schlenk line and was then transferred into an anaerobic chamber (Coy Laboratories). The Cu(II)-Nmar1307 was reduced by adding ascorbate/TMPD (10:1 mixture) solid till the solution turned colorless. The excess ascorbate/TMPD was removed by running the solution through a PD-10 desalting column (GE Healthcare Life Sciences). To 1.0 ml of 570 μM Cu(I)-Nmar1307 solution, different amounts of ferricyanide solution (from either 100 mM or 500 mM stock solution in water) were added at room temperature under stirring. The final concentration of ferricyanide was at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.5, 2.0, 5.0 and 10 mM respectively. The UV-vis spectra were monitored on an Agilent 8453 photodiode array spectrometer, located inside the anaerobic chamber, after each addition of ferricyanide. The oxidaiton of Cu(I)-Nmar1307 was monitored by increasing LMCT band at 558 nm where the ferricyanide and ferrocyanide do not contribute to absorption. Corrections are made for dilution. The extinction coefficient at 558 nm (ε_{558nm} = 2290 M^{-1} cm^{-1}) was used to calculate the concentration of Cu(II)-Nmar1307 solution. The concentration of Cu(I)-Nmar1307 was calculated by subtracting the concentration of Cu(II)-Nmar1307 from the total concentration of Cu-Nmar1307 (570 μM). The reduction potential of Cu(II)-Nmar1307 was calculated based on Nernst equation by linear plotting the potentials of ferri/ferrocyanin redox pair versus log([Cu(II)/Cu(I)]). The intercept (354 mV) represents the reduction potential of Cu(II)-Nmar1307 at pH 8.0. The slope of the fitting was 54 mV, close to the theoretic value 59 mV.

Spectroscopic characterization of Nmar1307

Electrospray ionization mass spectroscopy (ESI-MS) was collected on a Waters Quattro II Tandem Quadrupole/Hexapole/Quadrupole instrument, maintained by University of Illinois Mass Spectroscopy Facility, by adding formic acid to samples to ionize. UV-visible spectra of Nmar1307 were collected on a Carey 3E (Varian) in 50 mM or 100 mM Tris buffer pH 8.0. X-band EPR spectra were taken using X-band Varian E-122 spectrometer at the Illinois EPR Research Center (IERC) at 77K in the same buffer as a glass with 0, 20, or 60 % glycerol at 1 mW power and modulation of 4 gauss. For pH-dependent studies the protein was exchanged into universal buffer (UB) (containing 50 mM NH_4OAc, 40 mM MOPS, 40 mM MES and 40 mM Tris) which spans a wide range of pH with the same ionic composition. Q-band EPR

spectra were collected on an E580 Elexsys spectrometer with a superQ-FT attachment and an ER5106QT (CW) probe. The samples were collected at a temperature of 77 K, power of 0.047 mW and modulation of 8 gauss. EPR simulation used the program SIMPOW.[2] First and second derivative X-band and Q-band spectra were simultaneously fitted to give the lowest sum of the rms residuals for the four spectra.

Cu(II)-Nmar1307 extinction coefficient determination by spin-counting EPR

The extinction coefficient of absorption bands were calculated based on spin-counting EPR, using CuSO4 solutions with known concentration as calibration. The total spins in samples were quantified by double integration of the spectrum. A standard curve was obtained by linear fitting of double integrated EPR spectra versus the $CuSO_4$ concentrations. The concentration of Cu(II)-Nmar1307 was calculated by dividing the double integrated EPR spectrum of Cu(II)-Nmar1307 by the slope of the linear fitting. UV-vis spectrum of Cu(II)-Nmar1307 was taken and its extinction coefficient was calculated based on the absorption intensity and concentration of Cu(II)-Nmar1307.

Crystallization of Nmar1307, data analysis, and molecular visualization

Crystals of Nmar1307 were grown by the hanging drop vapor diffusion method. Protein solution was prepared in 50 mM sodium acetate buffer at pH 6.0 to a concentration of about 1.5 mM. A 2 µL portion of this protein solution was then mixed with 2 µL of a well buffer solution consisting of 100 mM TrisHCl buffer at pH 8.0, 20 mM $CuSO_4$, 100 mM $LiNO_3$ and varying amounts of polyethylene glycol (PEG). The highest quality crystals formed from wells with 35 % w/v PEG 4000 after about 2 months at 4 °C. We should note that the reported crystal structure is not at pH 6 but higher because the protein drop was mixed 1:1 with a buffer with high capacity at pH 8.

Data was collected at Brookhaven National Laboratory, beamline X-29A. The data was then indexed using HKL2000 software and phased and refined using Shelx97 and PHENIX[3] software. Azurin backbone (PDB entry 4AZU) was used as the initial model for refinement. Coot software[4] was used for analysis of the refined data. Data visualization was performed using VMD[5] and pymol visualization packages. The entry channel of waters was predicted using HOLLOW package.[6]

NO oxidation activity of Cu(II)-Nmar1307 at pH8.0

Cu(II) loaded Nmar1307 protein solution in 50 mM Tris pH 8.0 buffer was degassed in a Schlenk line and transferred to an anaerobic chamber (Coy Laboratories). 5 molar equivalents of Proli NONOate solution (Cayman Chemicals, from a 50 mM stock solution in 10 mM NaOH) was added to 2000 µl of 193 µM Cu(II)-Nmar1307 solution in 50 mM Tris pH8.0 buffer. The UV-vis spectra were monitored on an Agilent 8453 photodiode array spectrometer located inside the anaerobic chamber. The extinction coefficient at 558 nm (ε_{558nm} = 2290 M^{-1} cm^{-1}) was used to calculate the concentration of Cu(II)-Nmar1307 solution. The concentration of Proli NONOate (Cayman Chemical, MI) was determined using ε_{252nm} = 8400 M^{-1} cm^{-1}.[7]

EPR spectra were collected at 30 K on an X-band Varian E-122 spectrometer at the Illinois EPR Research Center equipped with an Air Products Helitran Cryostat and EIP frequency counter. The EPR samples were prepared as described above except higher protein concentration was used for better resolution. Glycerol was added to a final concentration of 20% v/v and the solution was quickly frozen in liquid nitrogen.

pH-dependent activity of Cu(II)-Nmar1307 reacting with NO

Cu(II)-Nmar1307 solution in 50 mM MES at pH6.0, 50 mM BisTris at pH7.0 or 50 mM Tris at pH8.0 was degassed and equilibrated with Ar on a Schlenk line and was then transferred into an anaerobic chamber (Coy Laboratories). To 1.0 ml of 116 μM Cu(II)-Nmar1307 solution, 13 molar equivalents of Proli NONOate solution (Cayman Chemicals, from 60 mM Proli NONOate stock solution in 10 mM NaOH) was added at room temperature under stirring. The color of the solution changed from purple to colorless. The UV-vis spectra were monitored on an Agilent 8453 photodiode array spectrometer located inside the anaerobic chamber. The extinction coefficient at 558 nm (ε_{558nm} = 2290 M^{-1} cm^{-1}) was used to calculate the concentration of Cu(II)-Nmar1307 solution. The concentration of Proli NONOate (Cayman Chemical, MI) was determined using ε_{252nm} = 8400 M^{-1} cm^{-1}.[7]

Measuring reaction rates between Cu(II)-Nmar1307 and NO at pH8.0

Cu(II)-Nmar1307 solution in 50 mM Tris at pH8.0 was degassed and equilibrated with argon on a Schlenk line and was then transferred into an anaerobic chamber (Coy Laboratories). To 1.0 ml of 58 μM Cu(II)-Nmar1307 solution, 6.5, 10.0, 13 or 16 molar equivalents of Proli NONOate solution (Cayman Chemicals, from 60 mM Proli stock solution in 10 mM NaOH) was added at room temperature under stirring. The UV-vis spectra were monitored on an Agilent 8453 photodiode array spectrometer located inside the anaerobic chamber. The extinction coefficient at 558 nm (ε_{558nm} = 2290 M^{-1} cm^{-1}) was used to calculate the concentration of Cu(II)-Nmar1307 solution. The concentration of Proli NONOate (Cayman Chemical, MI) was determined using ε_{252nm} = 8400 M^{-1} cm^{-1}.[7] The concentration of NO was calculated to be twice of the concentration of Proli NONOate (each Proli NONOate dissociates to form one proline and two NO molecules). The absorption decreasing at 558 nm was fitted as pseudo-first-order kinetics. The second-order reaction rates between Cu(II)-Nmar1307 and NO was calculated upon linear fitting of pseudo-first-order reaction rates at different concentrations of NO.

Oxidation of Cu(I)-Nmar1307 to Cu(II)-Nmar1307 with ferricyanide

Cu(II)-Nmar1307 solution in 50 mM Tris at pH8.0 was degassed and equilibrated with Ar on a Schlenk line and was then transferred into an anaerobic chamber (Coy Laboratories). To 1.0 ml of 116 μM Cu(II)-Nmar1307 solution, 6.5 molar equivalents of Proli NONOate solution (Cayman Chemicals, from 60 mM Proli stock solution in 10 mM NaOH) was added at room temperature under stirring. After the color of the solution changed from purple to colorless, 130 molar equivalents of potassium ferricyanide (from 1 M

stock of potassium ferricyanide in water) was added under stirring. The UV-vis spectra were monitored on an Agilent 8453 photodiode array spectrometer located inside the anaerobic chamber. The extinction coefficient at 558 nm (ε_{558nm} = 2290 M^{-1} cm^{-1}) was used to calculate the concentration of Cu(II)-Nmar1307 solution. The concentration of Proli NONOate (Cayman Chemical, MI) was determined using ε_{252nm} = 8400 M^{-1} cm^{-1}.[7]

Determination of the product of Cu(II)-Nmar1307 reaction with NO by Griess assay

The Griess reagent kit for nitrite quantitation containing *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilic acid and nitrite standard solution was purchased from Life technologies and stored refrigerated at 4 °C, protected from light. Sodium nitrite standard solutions with concentrations between 1-100 µM were prepared by diluting the nitrite stock solution with Millipore water. Equal volumes of *N*-(1-naphthyl)ethlyenediamine and sulfanilic acid were mixed to form the Griess reagent. A mixture of 10 µL of the Griess reagent, 30 µL of the nitrite-containing sample and 260 µL of Millipore water were incubated for 30 minutes at room temperature. To obtain the standard curve, concentration of nitrite was plotted against the absorbance of the mixture at 548 nm, measured relative to the reference sample containing 10 µL Griess reagent and 290 µL Millipore water.

To investigate whether the protein produces nitrite, Cu(II)-Nmar1307 was mixed with 6.5 molar equivalents Proli NONOate (Cayman Chemicals, MI) solution in 100 mM Tris buffer pH 8.0 according to the procedure described above. After the solution turned colorless, 30 µL of the solution was mixed with 10 µL of the Griess reagent and 260 µL of Millipore water. In the meantime, a solution of only Proli NONOate in 100 mM Tris buffer pH 8.0 was used as a control, also incubated with Griess reagent at room temperature for 30 min. The absorbance at 548 nm was then measured and nitrite concentrations were determined by comparing the sample absorbance with those from the standard plot.

Figures:

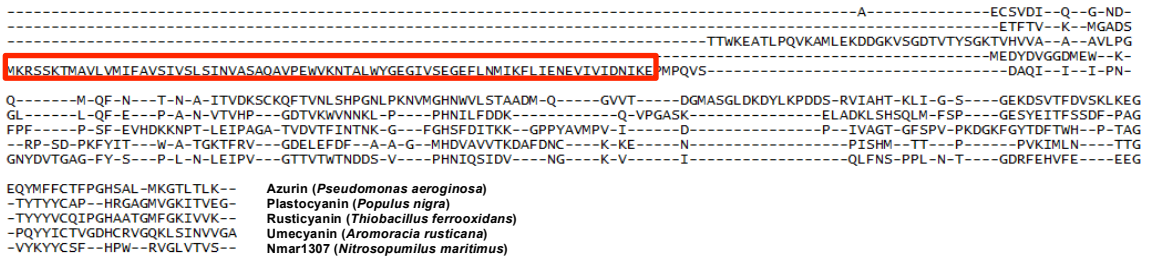

Figure S1. Structure-based sequence alignment of Nmar1307 and a number of other cupredoxins. The initial sequence shown in a red box is the membrane helix that was excluded from the sequence to express the protein in a soluble form.

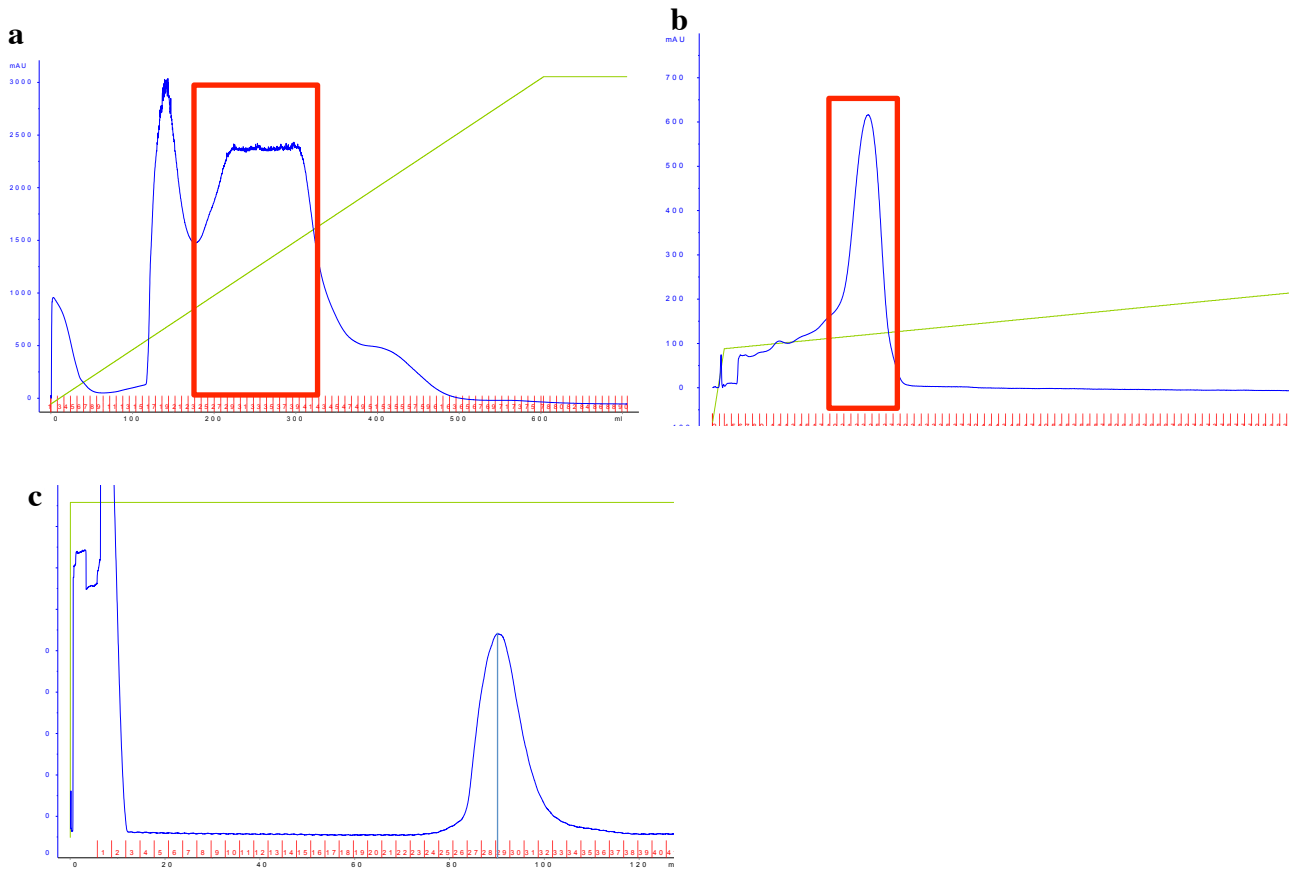

Figure S2. (a) FPLC trace of metal free N. mar-1307 on a Q-column. After metal reconstitution and EDTA treatment, the holo-protein is loaded into a second Q FF column. The trace is shown in (b). (c) shows the SEC trace of the final protein. In all figures, the blue line indicates absorption at 280 nm and the green line represents the concentration of buffer B. Collected fractions are shown in red box.

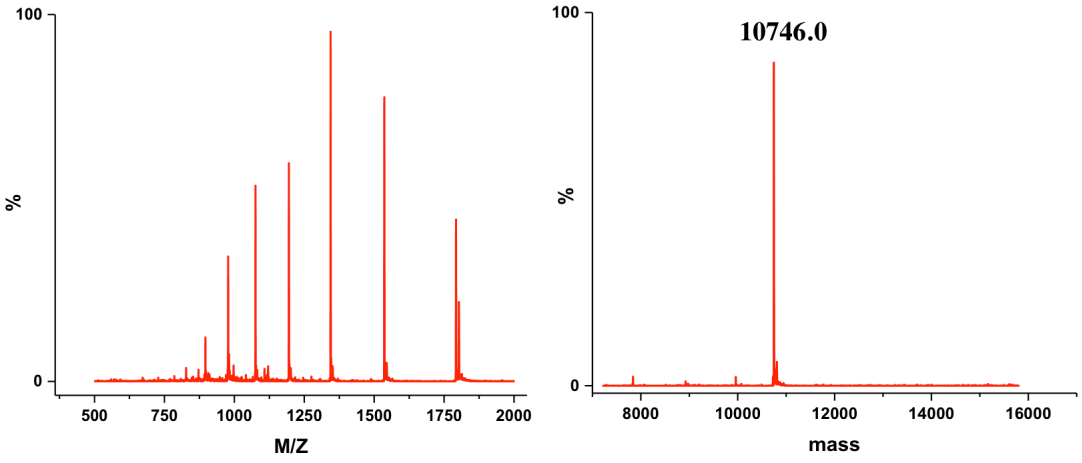

Figure S3. Representative ESI-MS of Nmar1307. The observed mass of apo-N.mar_1307 is 10,746 Da and the calculated mass is 10,747 Da.

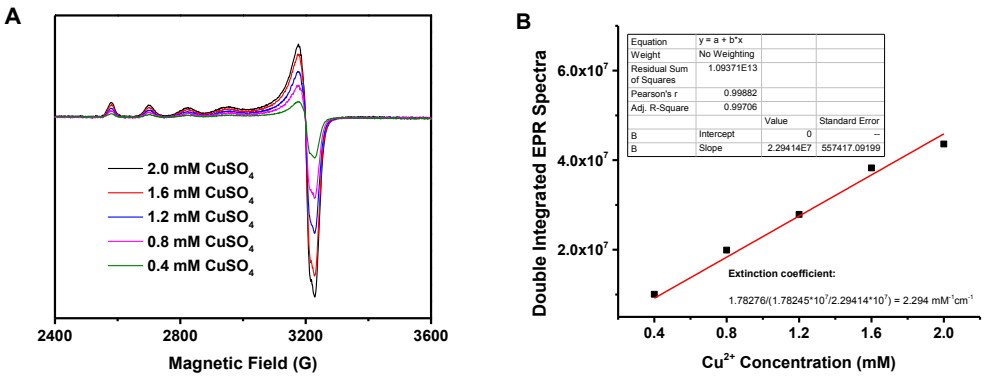

Figure S4. Cu(II)-Nmar1307 extinction coefficient determination by spin-counting EPR. (A) The EPR spectra of standard $CuSO_4$ solution with known concentrations collected at 77 K. (B) The total spins in samples were quantified by double integration of the spectrum. A standard curve was obtained by linear fitting of double integrated EPR spectra versus the $CuSO_4$ concentrations. The concentration of Cu(II)-Nmar1307 was calculated by dividing the double integrated EPR spectrum of Cu(II)-Nmar1307 by the slope of the linear fitting. UV-vis spectrum of Cu(II)-Nmar1307 was taken and its extinction coefficient (2294 $M^{-1}cm^{-1}$ at 558 nm) was calculated based on the absorption intensity and concentration of Cu(II)-Nmar1307.

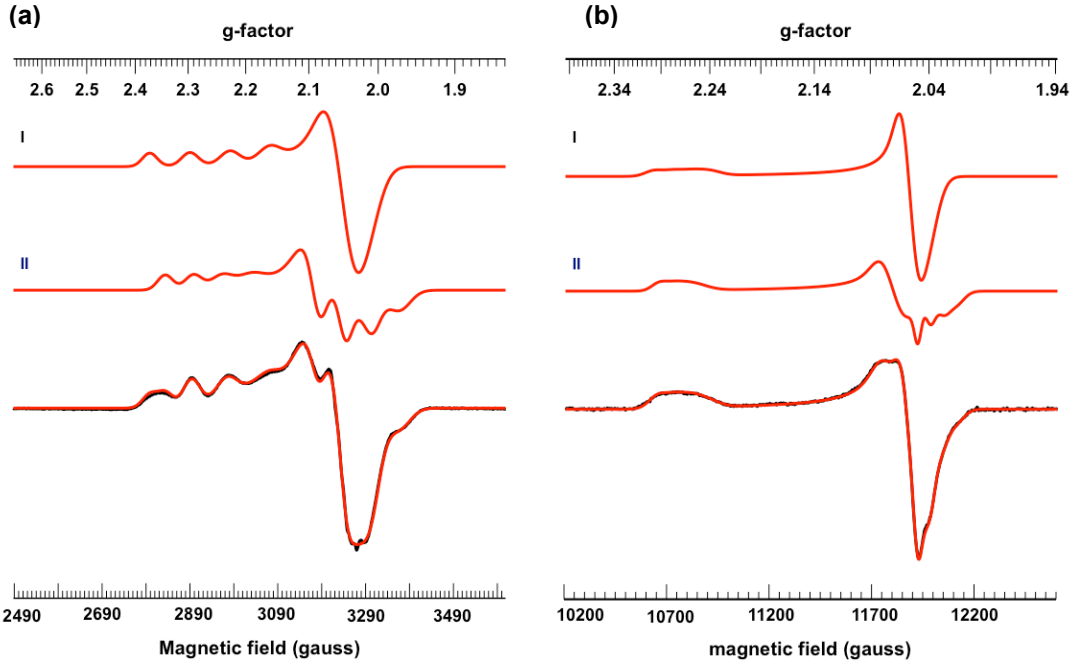

Figure S5. (a) EPR simulation of species I and II observed in X-band EPR of Nmar1307. The overall simulation and the experimental data are overlaid at the bottom of the figure. X-band EPR was collected at 77 K, power of 1.0 mW and modulation of 4 gauss. (b) EPR simulation of species I and II observed in Q-band EPR of Nmar1307. The overall simulation and the experimental data are overlaid at the bottom of the figure. Q-band EPR was collected at 77 K, power of 0.046 mW and modulation of 8 gauss.

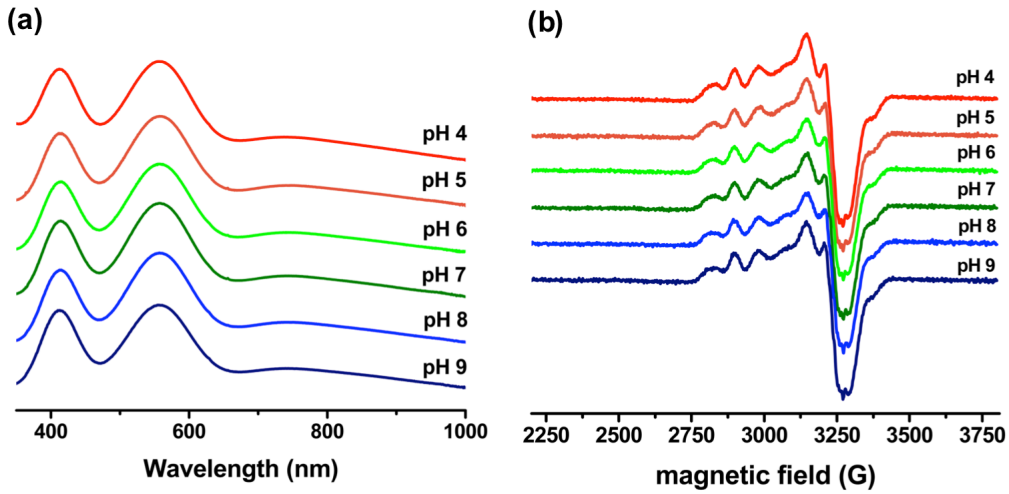

Figure S6. (a) UV-vis absorption spectra of Cu(II)-Nmar1307 at different pH values. Cu(II)-Nmar1307 was exchanged into universal buffer (UB buffer, containing 50 mM NH_4OAc, 40 mM MOPS, 40 mM MES and 40 mM Tris) which spans a wide range of pH with the same composition of buffer ions. (b) X-band EPR spectrum of Cu(II)-Nmar1307 at different pH values. 0.77 mM Cu(II)-Nmar1307 at different pHs with 20% glycerol was quickly frozen in liquid nitrogen. Frequency: 9.29 GHz. Power: 1.0 mW. Field modulation: 4.0 G. Temperature: 77 K.

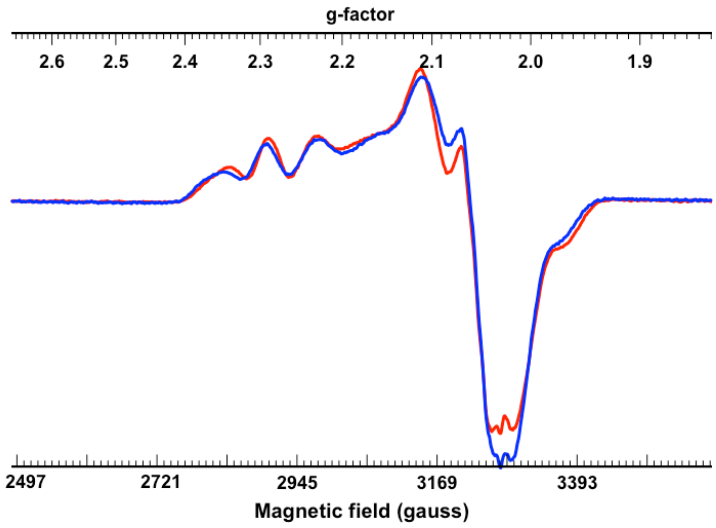

Figure S7. EPR of Cu(II)-Nmar1307 at tris HCl pH 8 with 0% (blue) and 60% (red) glycerol. The data was collected at 77 K, power of 1.0 mW and modulation of 4 gauss. The spectra are scaled to have the same double integrals.

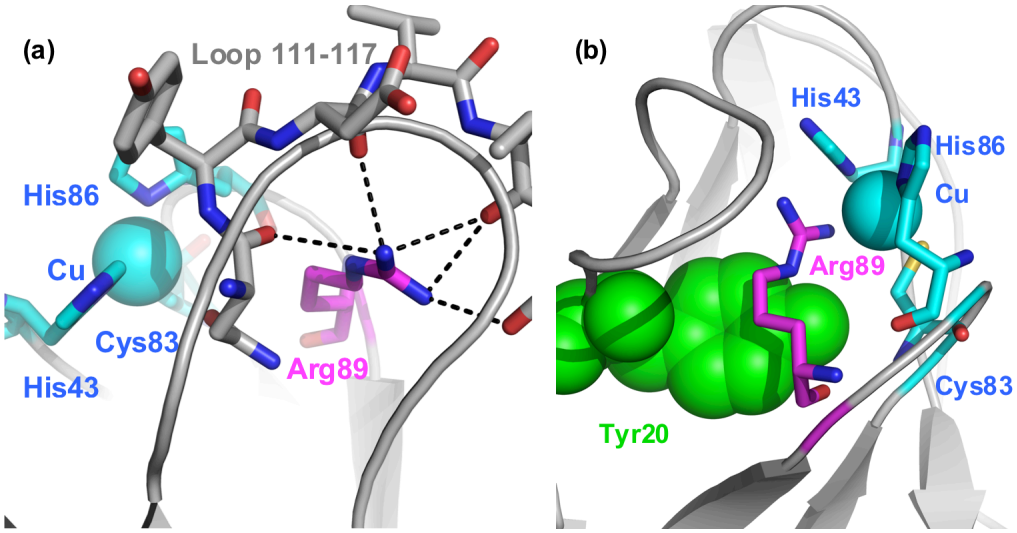

Figure S8. (a) The axial Arg (shown in magenta) is locked in a position away from the Cu binding site through multiple electrostatic interactions (shown as dashed lines) with residues in an adjacent loop (shown as gray sticks). (b) Tyr20 (green spheres) sterically blocks the axial Arg. The copper ion is shown as a cyan sphere and its ligands as cyan sticks.

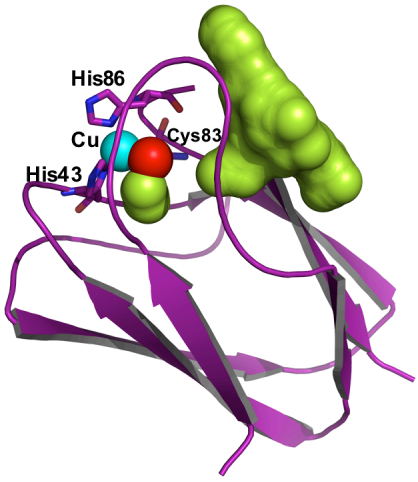

Figure S9. Possible entrance of water into the Cu binding site. The Cu ion is shown as a cyan sphere and the primary ligands are shown as magenta sticks. The red sphere is the axial water ligand. The green spheres show a "hole" in the protein that can fit an average atom in them. The collection of these empty holes demonstrates a possible "tunnel" for entrance of the axial water (red sphere) into the Cu binding site.

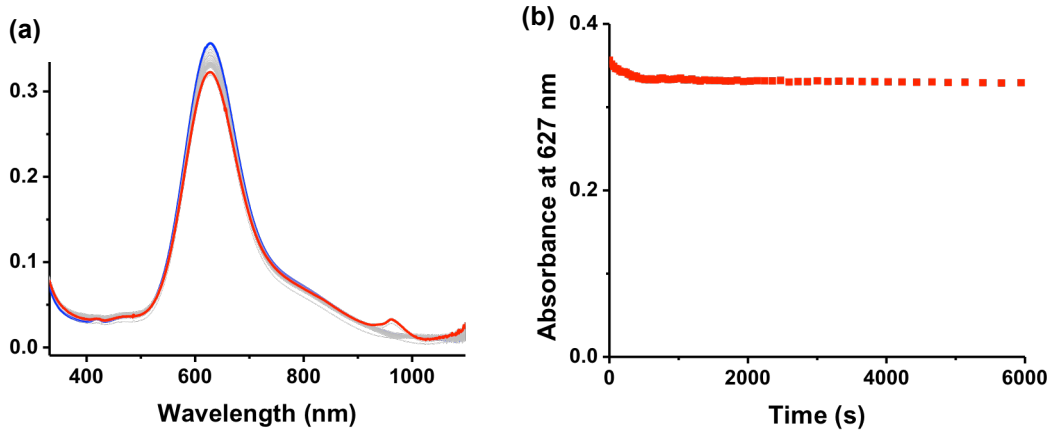

Figure S10. UV-vis spectroscopic study of Cu(II)-WTAz reaction with NO. (a) UV-vis spectra of 70 μM Cu(II)-WTAz reacting with 5 eq. NO in 50 mM BisTris pH7.0 buffer. (b) Time trace of the reaction based on monitoring the absorbance at 625 nm.

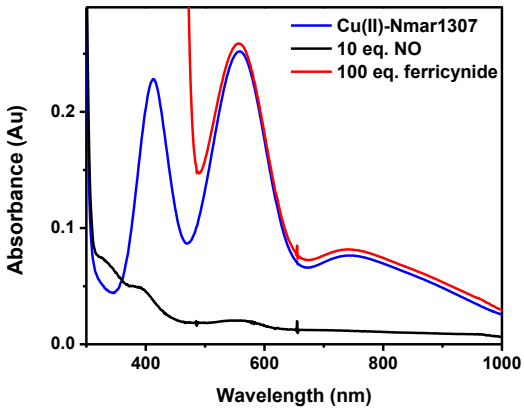

Figure S11. Reaction of Cu(I)-Nmar1307, generated from reaction with NO, with ferricyanide. An almost quantitative recovery of Cu(II) absorbance suggests that the site is intact. To 1.0 ml of 116 μM Cu(II)-Nmar1307 solution (shown in blue), 6.5 molar equivalents of Proli NONOate solution (from 60 mM Proli stock solution in 10 mM NaOH) was added at room temperature under stirring. After the color of the solution changed from purple to colorless (shown in black), 130 molar equivalents of potassium ferricyanide (from 1 M stock of potassium ferricyanide in water) was added under stirring (shown in red).

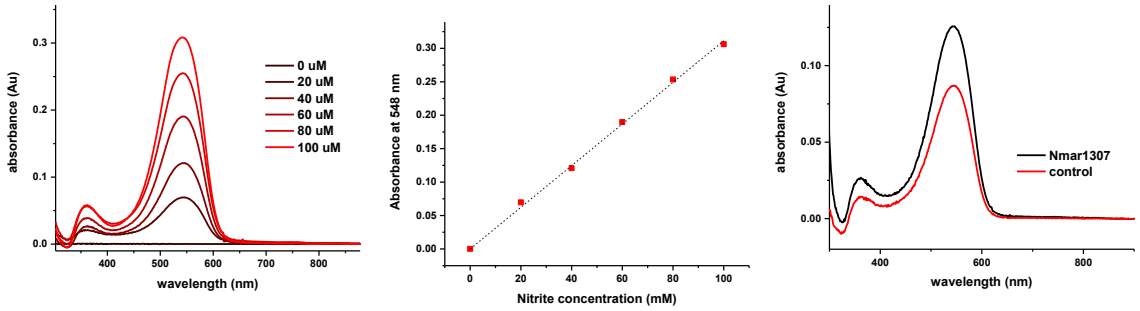

Figure S12. (a) , (b) show standard curve of Griese reagent for NO_2^- production. (c) Production of NO_2^- in buffer control (27.9 µM) compared with Nmar1307 (40.2 µM) after 30 min time with starting 200 µM concentration of NO. The background NO_2^- production in buffer is due to side reaction of NONOate.

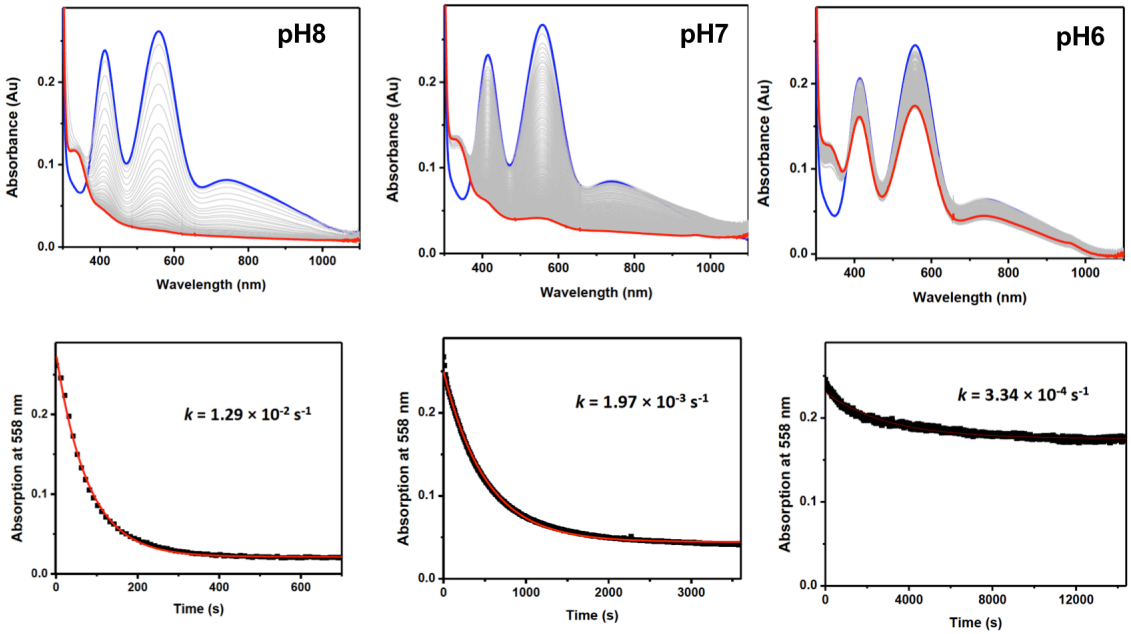

Figure S13. PH-dependent activity assay of Nmar1307. All the reactions are performed at saturated NO concentrations (2 mM) with 150 uM protein. To 1.0 ml of 116 µM Cu(II)-Nmar1307 solution in 50 mM MES at pH6.0, 50 mM BisTris at pH7.0 or 50 mM Tris at pH8.0, 13 molar equivalents of Proli NONOate solution (from 60 mM Proli NONOate stock solution in 10 mM NaOH) was added at room temperature under stirring. The absorption decreasing at 558 nm was fitted in pseudo-first-order kinetics.

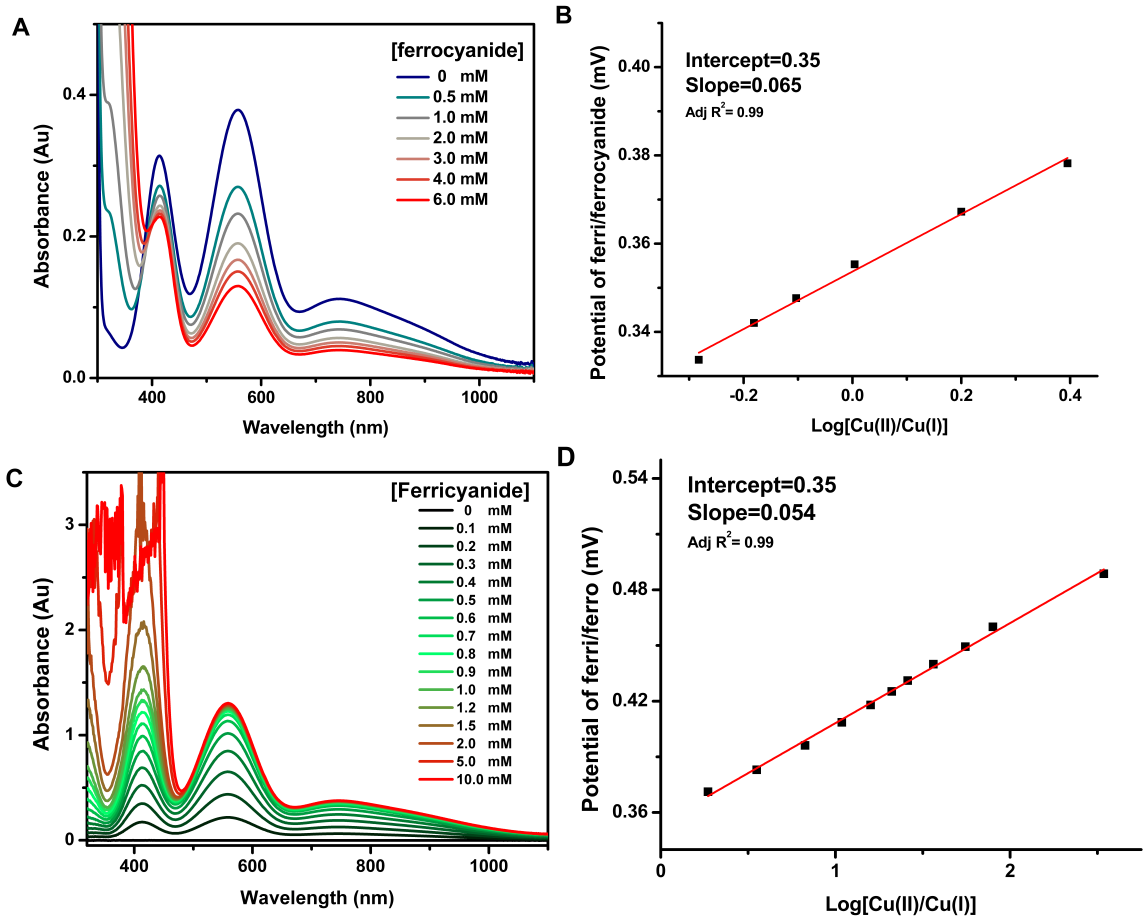

Figure S14. (A) Redox titration of Cu(II)-Nmar1307 with different concentrations of ferrocyanide monitored by decrease in the absorption at 558 nm due to reduction of Cu(II)-Nmar1307 by ferrocyanide at room temperature. (B) The reduction potential of Cu(II)-Nmar_1307 was calculated based on Nernst equation by linear plotting the potentials of ferri/ferrocyanide redox pair versus log([Cu(II)/Cu(I)]). The intercept (354 mV) represents the reduction potential of Cu(II)-Nmar1307 at pH 8.0. The slope of the fitting was 65 mV, close to the theoretic value 59 mV. (C) Redox titration of Cu(I)-Nmar1307 with different concentrations of ferricyanide monitored by increase in the absorption at 558 nm due to oxidation of Cu(I)-Nmar1307 by ferricyanide at room temperature. (D) The reduction potential of Cu(II)-Nmar1307 was calculated based on Nernst equation by linear plotting the potentials of ferri/ferrocyanide redox pair versus log([Cu(II)/Cu(I)]). The intercept (354 mV) represents the reduction potential of Cu(II)-Nmar1307 at pH 8.0. The slope of the fitting was 65 mV, close to the theoretic value 59 mV.

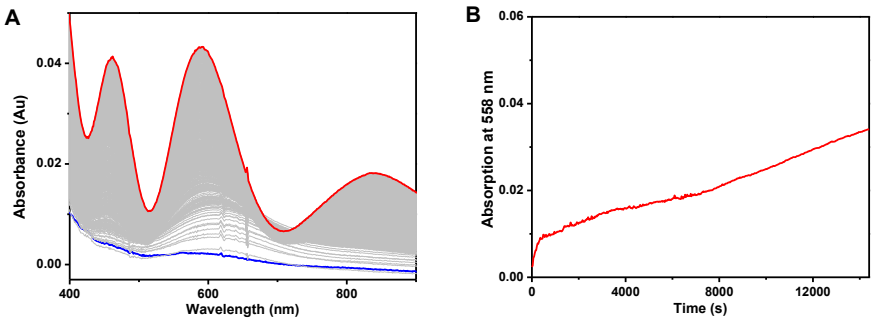

Figure S15. (A) Kinetic UV-vis of Cu(I)-Nmar1307 upon reaction with 13 molar equivalents of NO_2^- at pH 6. The blue trace is the initial spectrum and the red trace is the final spectrum after 4 hours. (B) Monitoring the increase in LMCT bands at 558 nm.

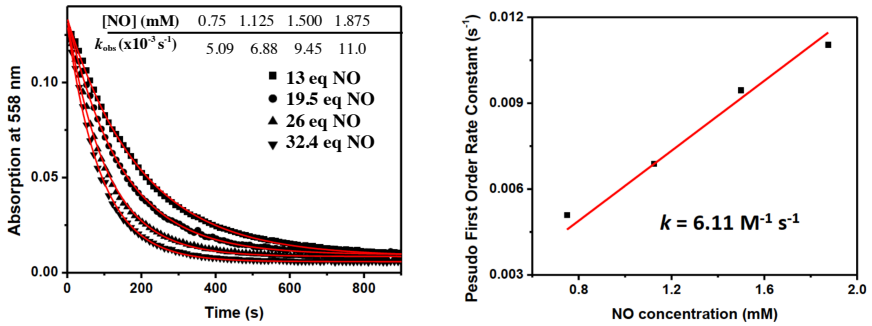

Figure S16. Reaction of Nmar1307 with different concentrations of NO under single turnover conditions and the observed k_{obs} for each concentration. Plotting the k_{obs} values vs concentration gives a second order rate constant of 6.11 $M^{-1}s^{-1}$ for the reaction. To 1.0 ml of 58 μM Cu(II)-Nmar1307 solution, 6.5, 9.7, 13 or 16.2 molar equivalents of Proli NONOate solution (from 60 mM Proli stock solution in 10 mM NaOH) was added at room temperature under stirring. The concentration of NO was calculated to be twice of the concentration of Proli NONOate (each Proli NONOate dissociates to form one proline and two NO molecules). The absorption decreasing at 558 nm was fitted in pseudo-first-order kinetics. The second-order reaction rates between Cu(II)-Nmar1307 and NO was calculated upon linear fitting pseudo-first-order reaction rates under varies concentrations of NO.

Tables:

Table S1. Data collection and refinement statistics of Nmar1307 crystal.

Resolution range (Å)	45.87 - 1.6 (1.657 - 1.6)
Space group	P 4_1 2_1 2
Unit cell	68.887 68.887 184.45/ 90 90 90
Unique reflections	59603 (5817)

Completeness (%)	99.97 (99.74)
Mean I/sigma(I)	17.74 (5.31)
Wilson B-factor	18.70
R-work	0.1703 (0.2032)
R-free	0.1918 (0.2411)
Number of non-hydrogen atoms	3609
Macromolecules	3012
Ligands	6
Water	591
Protein residues	384
RMS(bonds)	0.006
RMS(angles)	1.07
Ramachandran favored (%)	97
Ramachandran outliers (%)	0
Clashscore	2.57
Average B-factor	22.90
Macromolecules	20.80
Ligands	24.10
Solvent	33.40

Statistics for the highest-resolution shell are shown in parentheses.

Table S2. Relevant bond length near the Cu binding site of Nmar1307

Atoms	Chain A (Å)	Chain B (Å)	Chain C (Å)	Chain D (Å)	Average distance (Å)
S^{γ}_{Cys83}-Cu	2.23	2.26	2.26	2.24	2.25

N^{δ}_{His43}-Cu	1.97	2.03	2.00	2.04	2.01
N^{δ}_{His86}-Cu	2.05	2.05	2.07	2.09	2.07
O_{Pro42}-Cu	4.24	4.29	4.21	4.28	4.25
O^{δ}_{Asn11}-Cu	3.95	3.90	3.95	3.88	3.92
O^{δ}_{Asn11}-O_{water}	2.56	2.54	2.51	2.56	2.54
O^{η}_{Tyr20}-Cu	4.92	4.77	4.85	4.84	4.85
O^{η}_{Tyr20}-O_{water}	3.05	2.96	2.95	2.92	2.97
O_{water}-Cu	2.24	2.21	2.26	2.27	2.25

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