Electronic Supplementary Material (ESI) for Inorganic Chemistry Frontiers

Supporting Information for:

Modulating the Copper-Sulfur Interaction in Type 1 Blue Copper Azurin by Replacing Cys112 with Nonproteinogenic Homocysteine

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Experimental Procedure

Materials. All amino acids and resins were purchased from Chem-Impex Int. Co. Other chemicals were obtained from Acros or Sigma-Aldrich.

Computational Modeling of C112Hcy mutation. Molecular dynamics simulations were performed using NAMD 2.6 using the CHARMM force field under periodic boundary conditions. A time step of 2 fs was used and 1 ns simulation were performed with triplicate. Distances were sampled every 2 ps during equilibration to obtain an average and standard deviation.

Expressed Protein Ligation. The gene encoding azurin lacking its C-terminal 17 amino acids but with an intein and chitin binding domain at its C-terminus ("Δ17-Azurin-intein-CBD") was constructed in the pTXB1 plasmid as previously described.[1] *E. coli* BL21 (DE3) cells were transformed with the plasmid and the cells were grown with shaking at 210 rpm in LB at 37 °C for 16 h in the presence of 100 mg/L of ampicillin. The cells were harvested by centrifugation at 9000 × g and stored at -20 °C.

For EPL, the cells were thawed and lysed by sonication in lysis buffer (25 mM HEPES, 250 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X100, 1 M urea) for a total work time of 6 min. The final lysate was centrifuged at 13, 500 × g for 30 min at 4 °C. The resulting supernatant was mixed with chitin beads (bed volume 40 mL, from New England Biolabs) pre-equilibrated with ligation buffer (25 mM HEPES, 250 mM NaCl, 1 mM EDTA), and stirred at 4 °C for 2 h. The chitin resin was then poured into a column and the column headspace was purged with Ar. The column was then washed with 5 column volumes of ligation buffer by cannulation under Ar pressure.

Ligations were initiated by the addition of the 17-mer peptide (1.02 mM, 60 mg) and 2 equiv tris-(2-carboxyethyl)phosphine (TCEP) (3 mM, 30 mg) in 40 mL of degassed buffer 1 (35 mL) containing ~50 mM mercaptophenyl acetic acid (MPAA) (50 mM, 350 mg) to the column under Ar pressure via cannulation. The chitin resin was re-suspended in the column and the entire column was agitated gently at 4 ºC for 64 h.

After the above ligation procedure, the protein was eluted with 3 column volumes of the ligation buffer, concentrated, and refolded by rapidly diluting the protein in guanidine solution into buffer without guanidine.[2] Then the buffer was exchanged to 50 mM NH_4OAc pH 5.1 using a Millipore Centricon Unit with 10,000 MWCO for further study.

The protein was titrated with slow addition of sub-equivalent amounts of Cu^{2+} until the LMCT band at 618 nm stopped increasing, as monitored by UV-vis spectroscopy. The resulting protein was purified by anion exchange chromatography using a protocol reported previously.[2] The final purified holo protein was exchanged into 50 mM NH_4OAc buffer for further study.

UV-Vis and EPR characterization. UV-vis spectra were obtained on either an HP diode array spectrometer or a Cary 5000 spectrometer. X-band EPR spectra of samples containing 20% glycerol were collected on a Varian E-122 spectrometer at the Illinois EPR Research Center (IERC) at ~30 K using liquid He and an Air Products Helitran cryostat. Magnetic fields were calibrated with a Varian NMR gaussmeter, and the frequencies were measured with an EIP frequency counter. The extinction coefficients of absorption bands were calculated based on EPR spin counting, using $CuSO_4$ solutions with known concentrations as calibration. The total spins in samples were quantified by double integration of the spectrum and the concentration of Cu(II) in the protein was estimated based on a standard curve. UV-vis spectra of the proteins were taken

and their extinction coefficients were calculated based on the absorption intensity and concentration of Cu(II).

Electrochemical study. The reduction potential of each mutant was determined by cyclic voltammetry after verifying the WT azurin reduction potential using a CH Instruments 617A potentiostat equipped with a picoamp booster and a Faraday cage. A pyrolytic graphite edge (PGE) electrode was polished, and 2-3 μL of protein solution was applied directly to the electrode following previously described methods.[3] After a short incubation time, the electrode was immersed in either 50 mM NaOAc, pH 4.0 with 100 mM NaCl, or 50 mM NH_4OAc, pH 7.0 with 100 mM NaCl before data collection. The reduction potentials were measured against Ag/AgCl and converted to values against NHE.

Reference

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2. K. M. Clark, Y. Yu, N. M. Marshall, N. A. Sieracki, M. J. Nilges, N. J. Blackburn, W. A. van der Donk and Y. Lu, *J. Am. Chem. Soc.*, 2010, **132**, 10093-10101.
3. J. Hirst and F. A. Armstrong, *Anal. Chem.*, 1998, **70**, 5062-5071.