Supporting information

Probing the Role of the Backbone Carbonyl Interaction with the Cu_A Center in Azurin by Replacing the Peptide Bond with an Ester Linkage

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

SPPS of 19-mer peptide by Boc chemistry. Synthesis of the 19-mer peptide using Boc chemistry was completed on H-Lys(2-Cl-Z)-PAM resin using the Boc amino acid derivatives listed in the experimental section. Trial cleavages of a shorter peptide (H_2N-ALMKGTLTLK-OH) using TMSOTf or TFMSA were completed before the full length (FL) peptide was synthesized to verify that cleavage procedures were sufficient to fully cleave the peptide.

The ester was installed using DIC/DMAP chemistry identically to the methods used in Fmoc-based SPPS and the FL peptide was cleaved from the resin in a 10% TFMSA cocktail in TFA for 2-4 h. Precipitation of the FL peptide was difficult as extraneous organics in the cleavage cocktail prevented peptide trituration, instead yielding a golden oil. Therefore, the cleavage cocktail was taken up in THF/TFA and extracted with dH_2O. The aqueous layer was then extracted with Et_2O, resulting in peptide yields after lyophilization of the aqueous layer that corresponded to the expected yield of crude peptide (150-250 mg crude material for a 0.1 mmol synthesis). MALDI-MS characterization of the peptide synthesized *via* Boc chemistry showed less than 5% hydrolyzed product and the correct m/z of the 19-mer peptide.

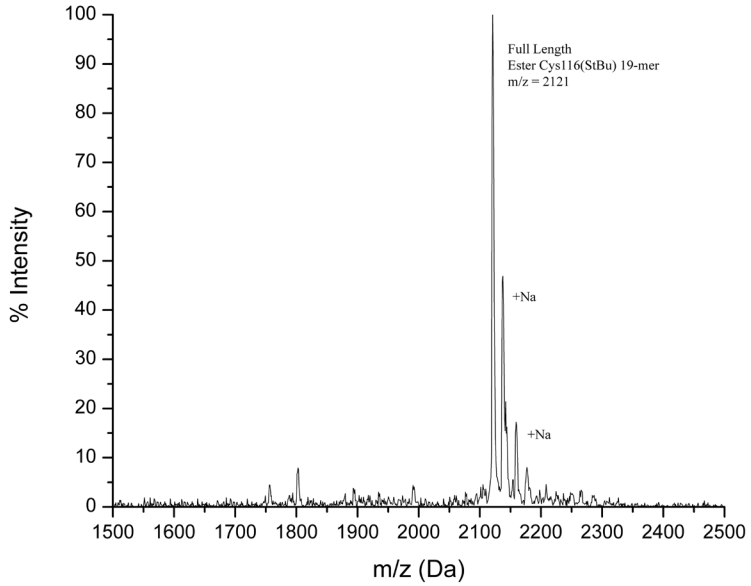

Figure S1. MALDI-MS analysis of the 19-mer peptide synthesized via Boc-SPPS.

Semi-synthesis of ester-Cu_A Az by EPL. The 19-mer peptide was purified by reversed phase HPLC and used in EPL reactions following the same methodology described for WT Cu_A Az reactions.[1] Noteworthy however is the sensitivity of the ester linkage (both the peptide and the subsequent FL protein) in buffers at pH > 7.5. No ligated products were observed using buffers at pH > 7.5 during the course of the EPL reaction. MALDI-TOF MS analysis of EPL elutions in buffer at pH > 7.5 showed peptide that was fully hydrolyzed between Glu114 and Leu115. To increase the rates of transthioesterification and reduce hydrolysis, buffers at pH 7.2 were used for all ester Cu_A EPL reactions. Full length apo protein from EPL reactions was refolded immediately following ligation and titrated with 1 equiv. $CuSO_4$ when the protein solution turned purple in color.

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 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.

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.[2] 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.

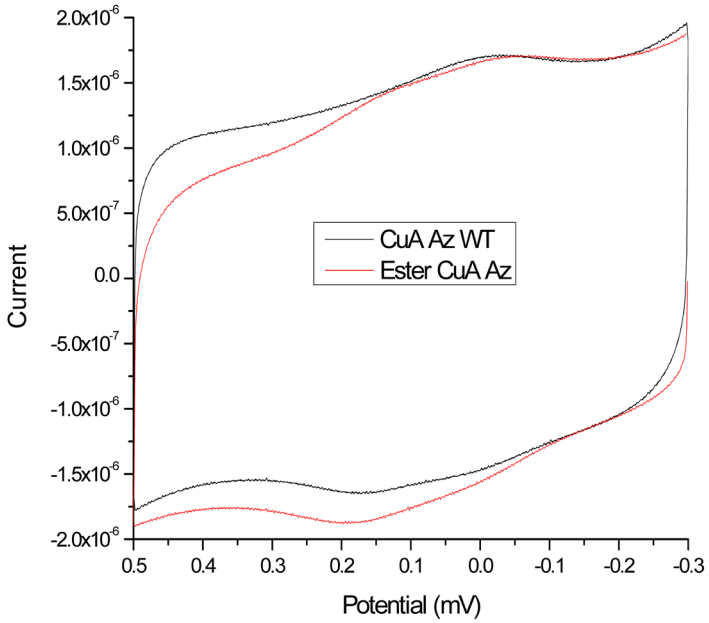

Figure S2. CV of Ester Cu_A azurin overlaid with WT Cu_A azurin. The additional peak in the ester spectrum at ~174 mV is attributed to background noise from the electrode and can also be seen in the WT spectrum.

Table S1. Simulated EPR parameters of WT Cu_A and ester Cu_A azurin.

Species	g values	Hyperfine coupling constants ($\times 10^{-4}$ cm^{-1})
WT Cu_A azurin	2.02, 2.01, 2.17	Cu_{A1}: 34, 16, 59 Cu_{A2}: 18, 9, 53
Ester Cu_A azurin	2.02, 2.01, 2.17 (40 %)	Cu_{A1}: 27, 14, 62 Cu_{A2}: 21, 8, 51
	2.04, 2.04, 2.29 (60 %)	21, 9, 157

References:

1. K. M. Clark, PhD. Thesis "Probing the Roles of Metal Binding Ligands in Cupredoxins: Incorporating Nonproteinogenic Amino Acids into Azurin and Cu_A Azurin" University of Illinois at Urbana, Champaign, 2010.
2. H. J. Hwang, S. M. Berry, M. J. Nilges and Y. Lu, *J. Am. Chem. Soc*., 2005, **127**, 7274-7275