Electronic Supplementary Material (ESI) for Metallomics.

The Functional Roles of The Three Copper Sites Associated with The Methionine-Rich Insert in the Multicopper Oxidase CueO from *E. coli*

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Electronic Supplementary Data

Table S1. Primer sequences for variant forms of CueO [a]

Variant	Site variation	Primer sequence (5'- 3')	
Cu5-m1	D360M	F	CAACTCTCTATGGACCCGATGCTC**ATG**ATG ATGGGGATGCAGATGCTAATG
		R	CAT**CAT**GAGCATCGGGTCCATAGAGAGTTG
Cu5-m2	M355L,D360N	F	GTACGCAAGCTGCAACTCTCT**CTG**GACCCG ATGCTC**AAT**ATGATGGGGATGCAGATGCTA ATG
		R	GAGCATCGGGTC**CAG**AGAGAGTTGCAGCT TGCGTAC
Cu6-m	M358,362S	F	CAACTCTCTATGGACCCG**AGT**CTCGATATG **AGT**GGGATGCAGATGCTAATGGAGAAA
		R	ATCGAG**ACT**CGGGTCCATAGAGAGTTG
Cu7-m	M364,368S	F	CAACTCTCTATGGACCCGATGCTCGATATG ATGGGG**AGT**CAGATGCTA**AGT**GAGAAATA TGGCGATCAGGCGATG
		R	CCC CAT CAT ATC GAG CAT CGG GTC
Cu6,7-m	M358,362, 364,368S	F	CAACTCTCTATGGACCCG**AGT**CTCGATATG **AGT**GGG**TCG**CAGATGCTA**TCG**GAGAAATA TGGCGATCAGGCGATG
		R	ATCGAG**ACT**CGGGTCCATAGAGAGTTG

[a] Forward and reverse primers are indicated by the letters F and R, respectively. Sequences corresponding to site-specific mutations are in bold-face and underlined.

Table S2. Mass Spectrometry Data for isolated *apo*-CueO and its variants

Protein	Site Variations	Molar Mass (Da)	
		Calced	Found
wt-CueO	–	53420.40	53418.27
Cu5-m1	D360M	53436.50	53436.48
Cu5-m2	M355L,D360N	53401.39	53401.99
Cu6-m	M358,362S	53332.18	53331.57
Cu7-m	M364,368S	53332.18	53332.78
Cu6,7-m	M355,362,364,368S	53243.96	53243.78

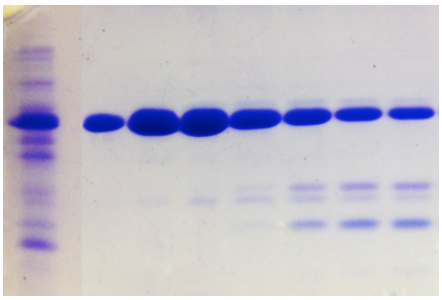

Fig. S1 Representative SDS-PAGE gel of NaCl-gradient elution fractions from an anion-exchange DE-52 column in Tris-HCl buffer (20 mM, pH 8.5). wt-CueO protein and its variants were expressed in *E. coli* BL21(DE3) Codon-Plus(+) cells (see experimental section).

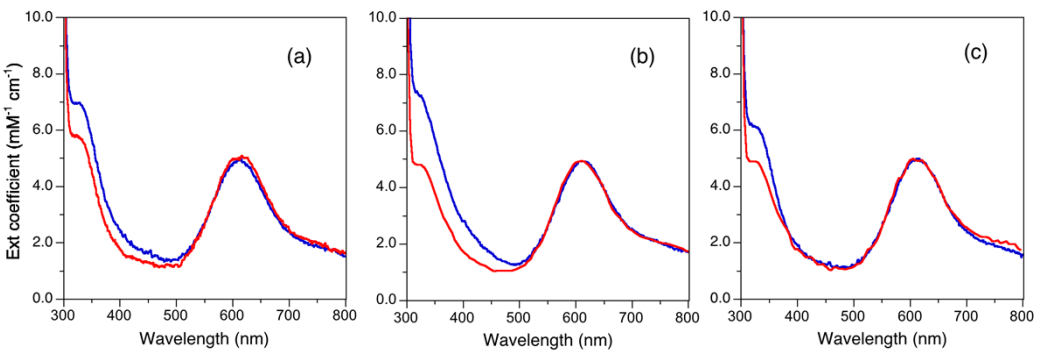

Fig. S2 Representative solution spectra of Cu-activated CueO samples isolated in Mops buffer (20 mM, pH 7.0; shown in blue) and then after buffer-change to BisTris buffer (20 mM, pH 7.0; shown in red): (a) wt-CueO; (b) Cu5-m2; (c) Cu6,7-m.

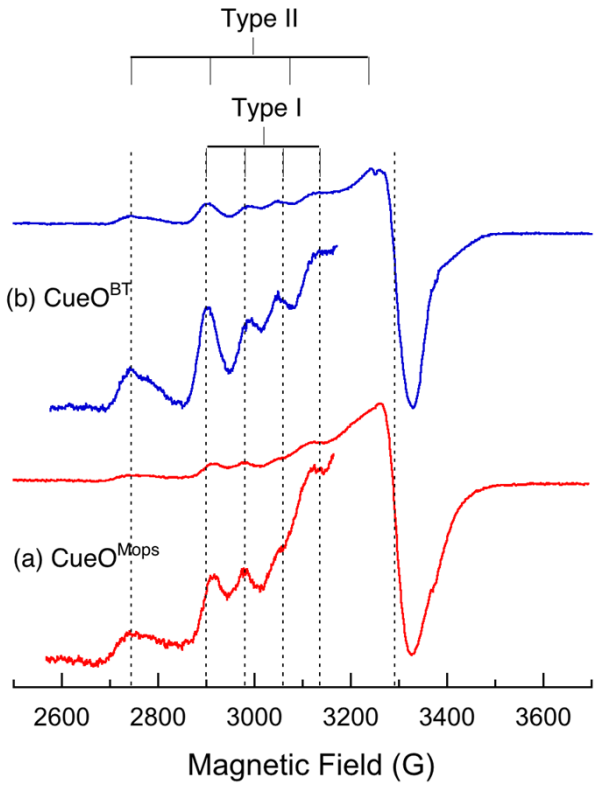

Fig. S3 EPR spectra of Cu-activated wt-CueO enzyme (~100 μM): (a) as isolated form in Mops buffer (50 mM, pH 7.0) designated as $CueO^{Mops}$; (b) after buffer-change to BisTris (50 mM, pH 7.0) designated as $CueO^{BT}$. ~10% glycerol was included in the buffer. EPR recording conditions: microwave frequency 9.461 GHz, microwave power 0.63 mW, modulation frequency 100 kHz; modulation amplitude 4 G, sweep time 50 s, time constant 20 ms, average number of scan 10.

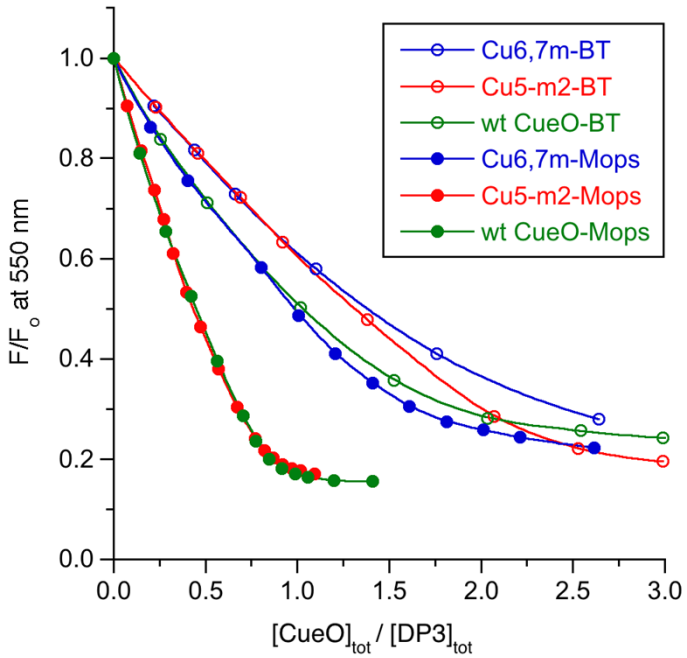

Fig. S4 Change in fluorescence intensity (expressed as F/F_o at 550 nm) of Cu(II) probe DP3 (2.0 μM) in Mops buffer (50 mM, pH 7.4) upon titration with variant forms of Cu-activated CueO proteins (expressed as $[CueO]_{tot}/[DP3]_{tot}$). see inset for each titration curve label

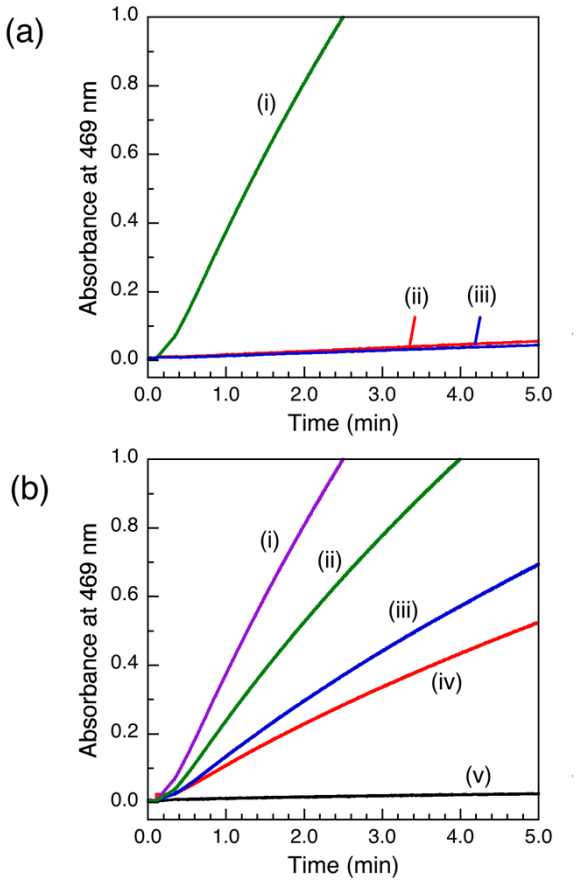

Fig. S5 Phenol oxidase activity of wt-CueO on model substrate DMP (5.0 mM) in air-saturated buffers (50 mM, pH 7.0):

(a) Buffer effect demonstrating a requirement for loosely bound copper for DMP oxidation: (i) reaction with wt-CueO (0.05 μM) in MOPS buffer; (ii) same reaction as (i) but with addition of 5.0 mM BisTris buffer (pH 7.0) into the MOPS buffer; (iii) same reaction as (i) but with the wt-CueO pre-treated with 20 mM BisTris buffer (pH 7.0) for 10 min and then buffer-changed back to MOPS only, by a desalting column, for the reaction.

(b) Reaction in MOPS buffer with different concentrations of wt-CueO at 0.05 μM (i), 0.04 μM (ii), 0.025 μM (iii), 0.02 μM (iv) and control without the enzyme (v). A plot of the reaction rates versus CueO concentrations is shown in Figure 2b(i).