Electronic Supplementary Material (ESI) for Metallomics.

pH-Regulated Metal-Ligand Switching in the HM Loop of ATP7A: A New Paradigm for Metal Transfer Chemistry

Chelsey D. Kline†, Benjamin F. Gambill†, Mary Mayfield†, Svetlana Lutsenko§, Ninian J. Blackburn†*

†Institute of Environmental Health, Oregon Health and Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97239, United States

§ Department of Physiology, The Johns Hopkins University, Baltimore, Maryland 21205, USA

Supporting Information

Materials and Methods

General
All buffers used were reagent grade, purchased from Sigma-Aldrich at a minimum purity of 99%. Distilled deionized water used throughout the experiments was purified to a resistivity of 17−18 MΩ with a Barnstead Nanopure II system.

HM Loop Constructs
The HM Loop was constructed as a chimeric protein by replacing the copper-binding CXXXC motif of BSco with lumenal loop Histidine- Methionine (HM) rich peptide (^{674}MDHHFATLHHNQNMSKEEMINLHSSM^{699}) from Menke's protein (ATP7A), as previously reported [1,2]. The HM loop was further constructed by polymerase chain reaction (PCR) amplification to include His_6 and a Tev cleavage site at the N-terminus (for ease of purification with the nickel-NTA affinity column) in the petDuet vector using SacI/KpnI restriction enzymes. The construct was checked by DNA sequencing and termed WT HM Loop. Plasmids were transformed into *Escherichia coli* strain BL21 DE3.

Three of the four methionine residues were mutated to isoleucine (M234I, M134I, M124I, and M123I termed M1, M2 M3, and M4 respectively) in the HM rich sequence peptide in order to identify the sulfur (Met) residue that binds copper. Triple Met mutants were individually constructed in the petDuet vector using Splicing by Overlap Extension (SOEing). Sense and antisense oligonucleotide primers encoding about 15 bases downstream and upstream of the mutations were used for site-directed mutagenesis and paired with primers upstream and downstream of two restriction enzyme sites, SacI & KpnI. Plasmid mutants were transformed into BL21 DE3, while simultaneously transforming M1 into B834 DE3 for expression as a methionine auxotroph referred to as SeM1. The series of six double Met to Ile mutants were constructed using similar protocols.

Expression His_6 WT HM Loop and mutants
Expression was carried out using protocols described previously [1-3]. Ten mL of culture medium (LB and 50 mg/mL ampicillin) was inoculated early morning from a freshly streaked plate of BL21 DE3 cells and used to inoculate a 1 L flask of culture medium mid afternoon. The culture was induced by addition of isopropyl-ß-D- thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM and incubated overnight at 17 °C once the culture reached an OD_{600} of 0.6-0.9 with shaking. Cells were harvested by pelleting via centrifugation in a Sorvall GS-3 rotor at 5000g for 30 min and frozen at −80 °C (as needed).

Expression of His_6 Selenomethionine (SeM) labeled HM Loop
Expression was carried out using protocols described previously [3]. Ten mL of culture medium (LB and 50 mg/mL ampicillin) was inoculated from a freshly streaked plate of B834 DE3 cells and incubated at 37 °C for a few hours before inoculating 100 µL of this culture into a 10 mL flask of minimal culture medium which included L-methionine for overnight incubation at 37 °C with shaking. A 1 L flask of minimal medium that contained SeM in place of methionine was inoculated with the 10 mL minimal culture until an OD_{600} of 0.6-0.9 was reached. The 1 L culture was then induced by addition of IPTG and incubated overnight at 17 °C. Cells were harvested as stated above.

Protein Purification
Purification was carried out using protocols described previously [1-3]. Briefly, apoproteins were purified from the soluble lysate by re-suspending the pelleted cells in 50 mM Tris buffer, 400 mM NaCl at pH 8.0 containing EDTA-free protease inhibitor (Roche). Cells were lysed in a French Press at 1000 psi and centrifuged at 8500g for 30 min. The supernatant was passed

through a 0.45 μm syringe filter and purified by chromatography on a nickel-NTA affinity column. The His_6 tag was cleaved by overnight incubation with recombinant tobacco etch virus (r-TEV) protease followed by dialysis against imidazole-free buffer, and a second metal affinity chromatography step removed the cleaved His_6 tag. Eluted protein fractions were assayed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) analysis on an Amersham Biosciences PHAST system using gradient 8−25% (GE Healthcare) and stained with Coomassie brilliant blue R-250 which showed a single band ~23 kDa. Following purification, the protein was dialyzed in 20 mM phosphate buffer, pH 8.0 and stored at −80 °C. Protein concentrations were determined by the bicinchoninic acid (BCA) assay, and a Perkin-Elmer Optima 2000 DV inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to accurately determine selenium content in the SeM labeled HM Loop constructs.

Copper Reconstitution

Purified protein was reconstituted with cupric sulfate by slow addition of ~1 mol equiv Cu(II) per protein followed by exhaustive dialysis to remove unbound cupric ions. Protein concentrations were determined using the BCA assay and copper concentrations were determined using ICP-OES [2,3]. Cu(I) containing sample preparation is described in the next section.

Sample Preparation for Cu (I) XAS

Cu(I) containing samples were prepared under anaerobic conditions by 5-fold dilution with pH-adjusted anaerobic buffers (buffers contained equal volumes of 50 mM MES, HEPES, CHES, and formic acid adjusted to pH pH 8.0 and pH 3.5 with sodium hydroxide) to which 5 mM ascorbate had been added along with ~20% ethylene glycol [4]. Samples were transferred to XAS cuvettes via a syringe and flash-frozen in liquid nitrogen. Final concentrations of copper varied from 300 to 1200 μM, being careful not to exceed 3mM in selenium concentrations for the SeM labeled HM Loop samples.

Collection and Analysis of X-ray Absorption Data (XAS) Data

X-ray absorption data were collected at the Stanford Synchrotron Radiation Lightsource as previously reported [3,4]. The extended X-ray absorption fine structure (EXAFS) and X-ray absorption near-edge structure (XANES) of Cu (8.9 keV) and Se (12.6 keV) were measured on beamline 9-3 and 7-3 using a Si 220 monochromator with crystal orientation φ = 90° and a Rh-coated mirror located upstream of the monochromator set to 13 keV (Cu) or 15 keV (Se) cutoff to reject harmonics. Samples were measured as frozen aqueous glasses in ~20% ethylene glycol at temperatures between 7 and 15 K, and the XAS was detected as Kα fluorescence using either a 100 element (beamline 9-3) or 30 element (beamline 7-3) Canberra Ge array detector. A Z-1 metal oxide filter (Ni, As) and Soller slit assembly were placed in front of the detector to attenuate the elastic scatter peak. Four to six scans of a buffer blank were measured at each absorption edge and subtracted from the raw data to produce a flat pre-edge and eliminate residual Ni/ As Kβ fluorescence of the metal oxide filter. Energy calibration was achieved by placing a Cu or Se metal foil between the second and third ionization chamber. Data reduction and background subtractions were performed using the program modules of EXAFSPAK.31. Data from each detector channel were inspected for dropouts and glitches before being included into the final average. Spectral simulation was carried out using the program EXCURVE 9.2 as previously described [5-10]. Refinement of structural parameters included distances (R), coordination numbers (N), Debye−Waller factors (2σ2) and included simulations using a mixed-shell model consisting of imidazole from histidine residues and sulfur/selenium for methionine coordination.

Figure S1. XAS data comparing the Fourier transforms of ascorbate-reduced WT HM Loop and triple met variants at the Cu K-edge. Samples contained Cu(I) at 1:1 metal to protein ratio. Top panel compares WT HM Loop at pH 8.0 (black) and pH 3.5 (red). The middle panel compares SeM-substituted M1 FT data at pH 8.0 (black) and pH 3.5 (red). The bottom figure compares SeM-substituted M1, M2, M3, and M4 data at pH 3.5 representing the loss of Cu-Met coordination for all four triple mutants (green, purple, orange, and blue traces correspond to the triple met mutants M1 to M4 respectively).

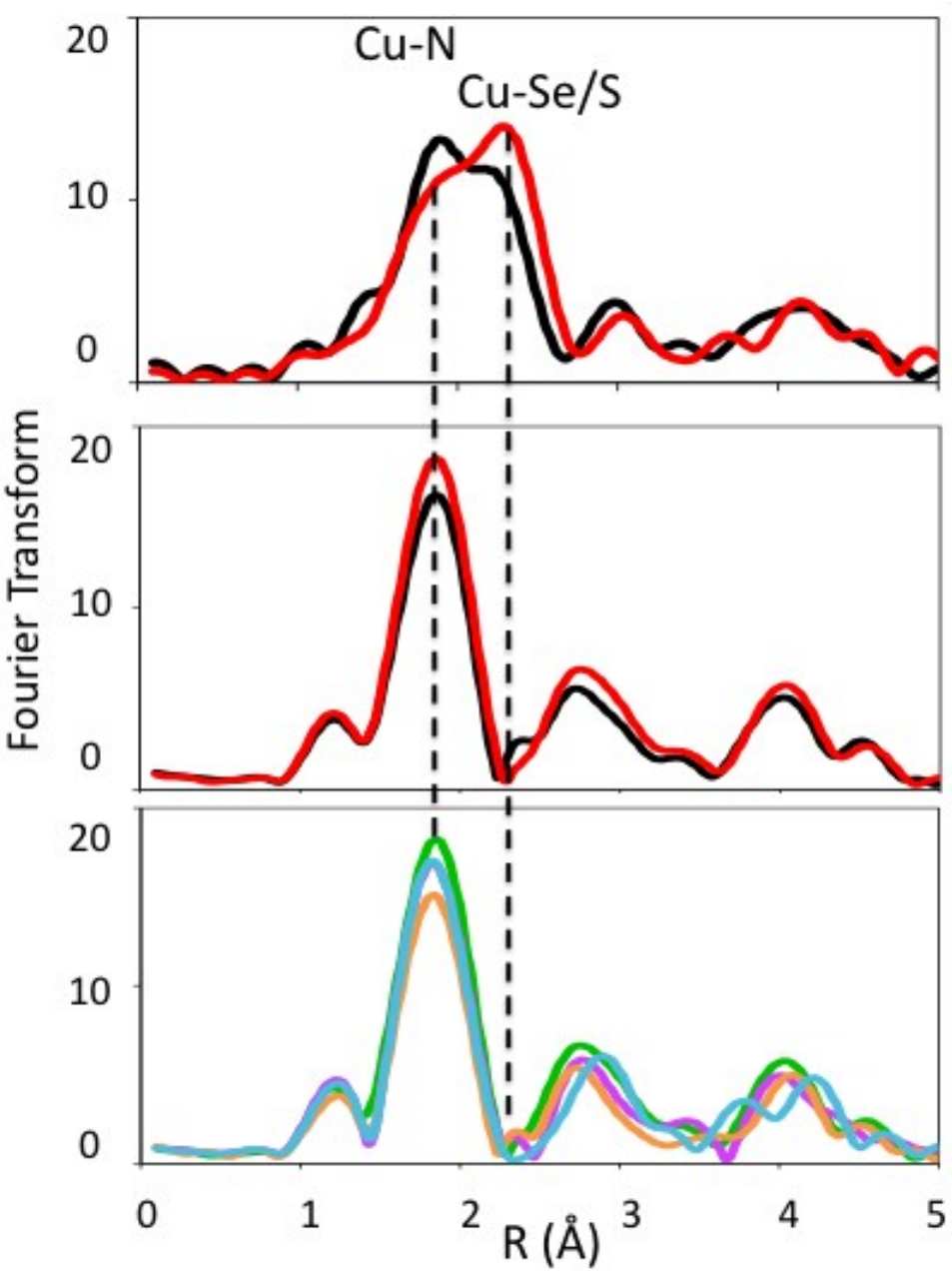

Figure S2. pH dependence of the Fourier transforms and EXAFS (insets) for the series of double Met to Ile variants of the HM loop. Samples contained Cu(I) at 1:1 metal to protein ratio. Top panels pH 8, bottom panels pH 3.5. Left panel is M34 (M3&4 retained) and right panel is M24 (M2&4 retained). Parameters used in the fits are given in Table S3.

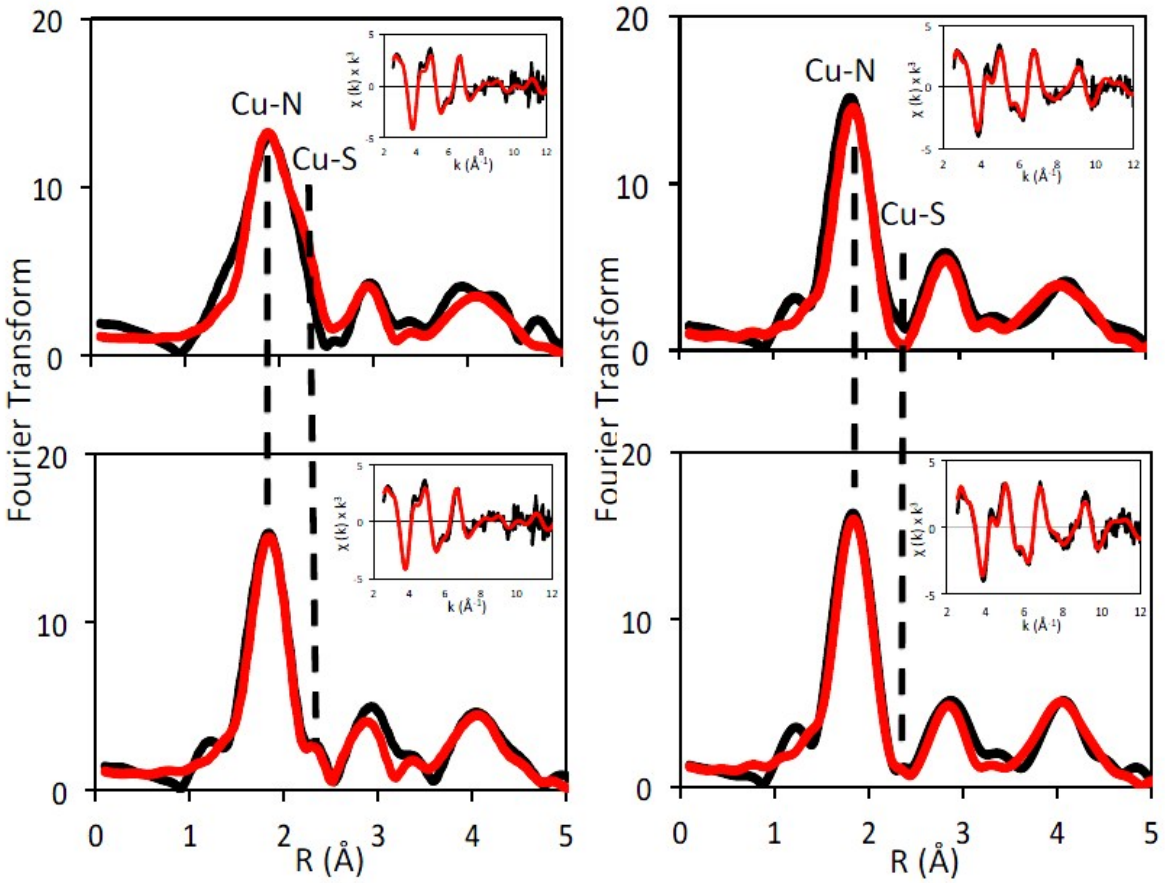

Figure S3. pH dependence of the Fourier transforms and EXAFS (insets) for the series of double Met to Ile variants of the HM loop. Top panels pH 8, bottom panels pH 3.5. Left panel is M23 (M2&3 retained) and right panel is M14 (M1&4 retained). Parameters used in the fits are given in Table S3.

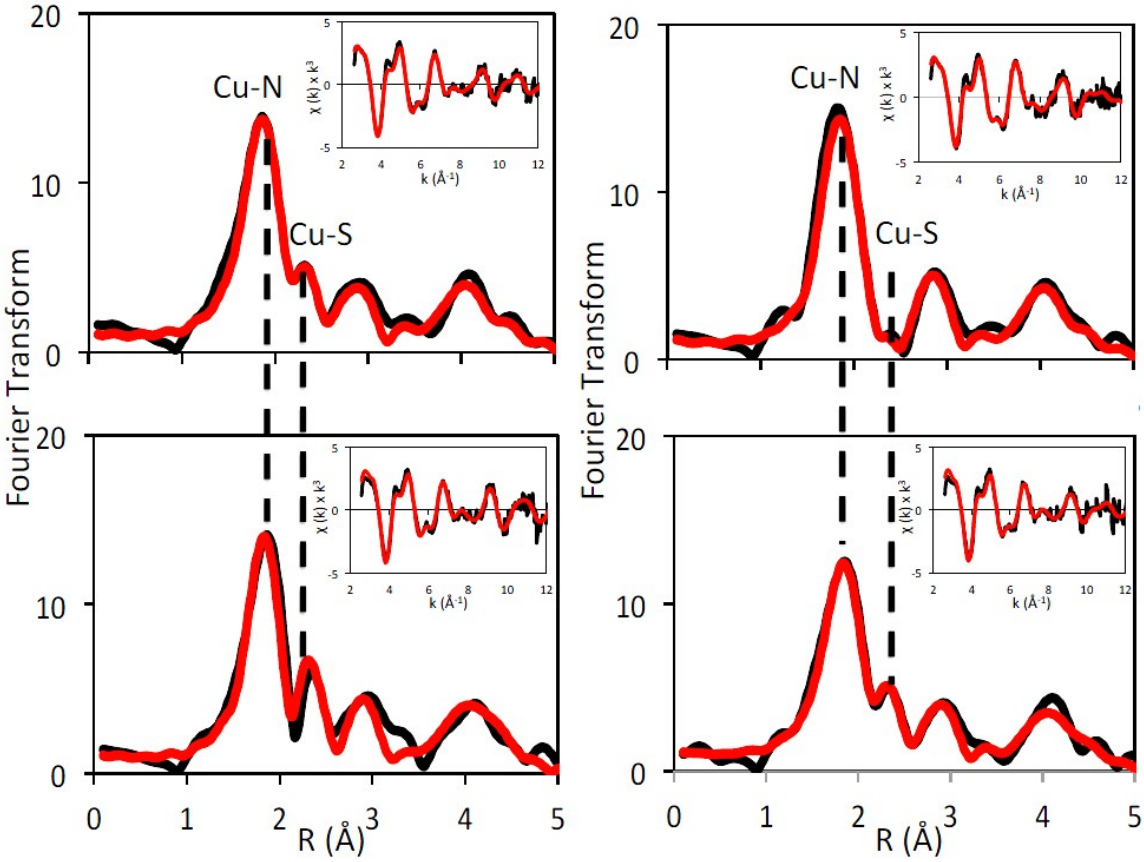

Figure S4. pH dependence of the Fourier transforms and EXAFS (insets) for the series of double Met to Ile variants of the HM loop. Samples contained Cu(I) at 1:1 metal to protein ratio. Top panels pH 8, bottom panels pH 3.5. Left panel is M13 (M1&3 retained) and right panel is M12 (M1&2 retained). Parameters used in the fits are given in Table S3.

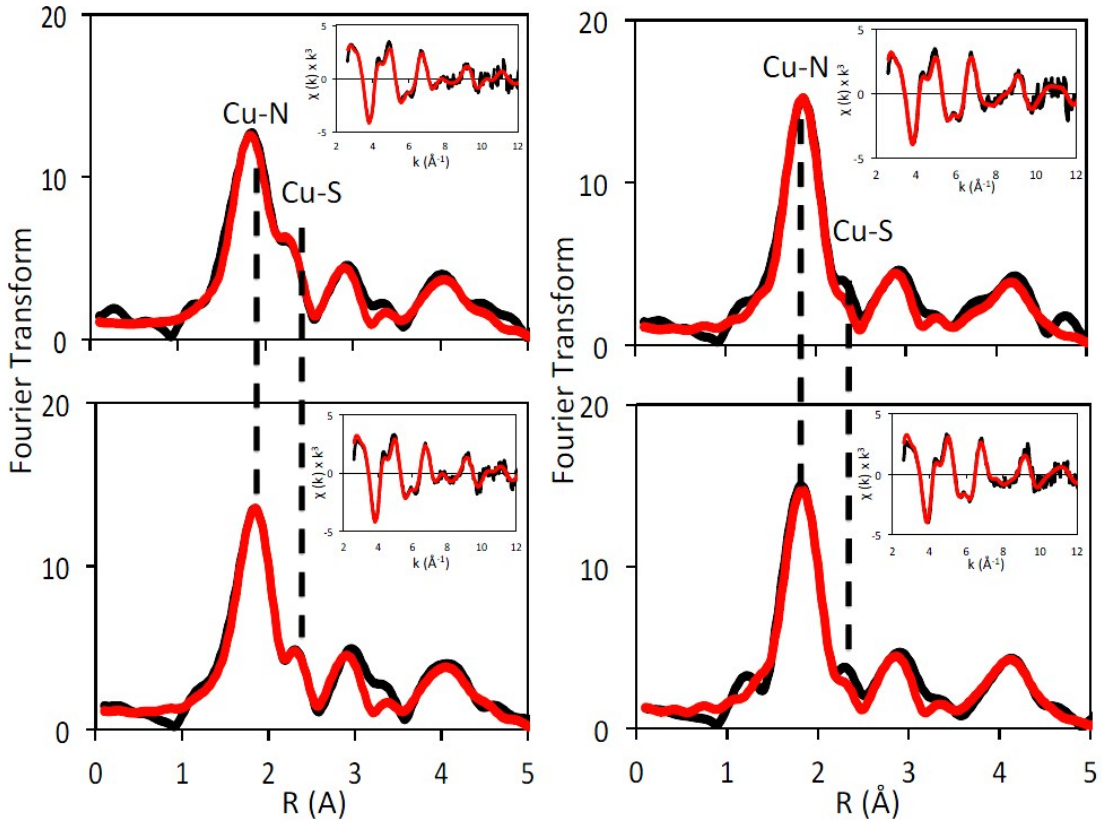

Fig. S5. Comparison of the absorption edges (XANES) of WT HM loop at pH 8 and pH 3.5. Samples contained Cu(I) at 1:1 metal to protein ratio.

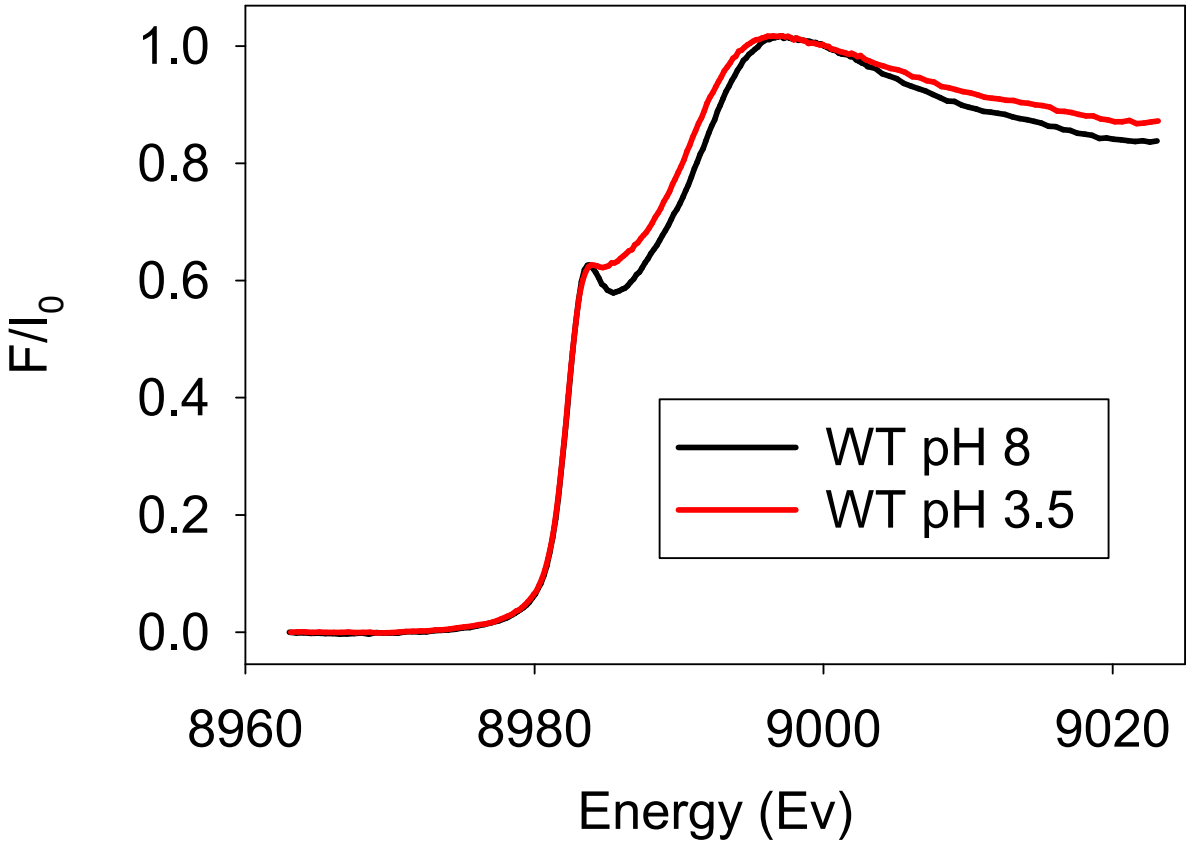

Fig. S6. Absorption edges (pH 3.5) of triple mutants (M1 – M4) and double mutants, Mxy, where x and y represent the Met residues (Table S1) that are retained. All samples contained Cu(I) at 1:1 metal to protein ratio.

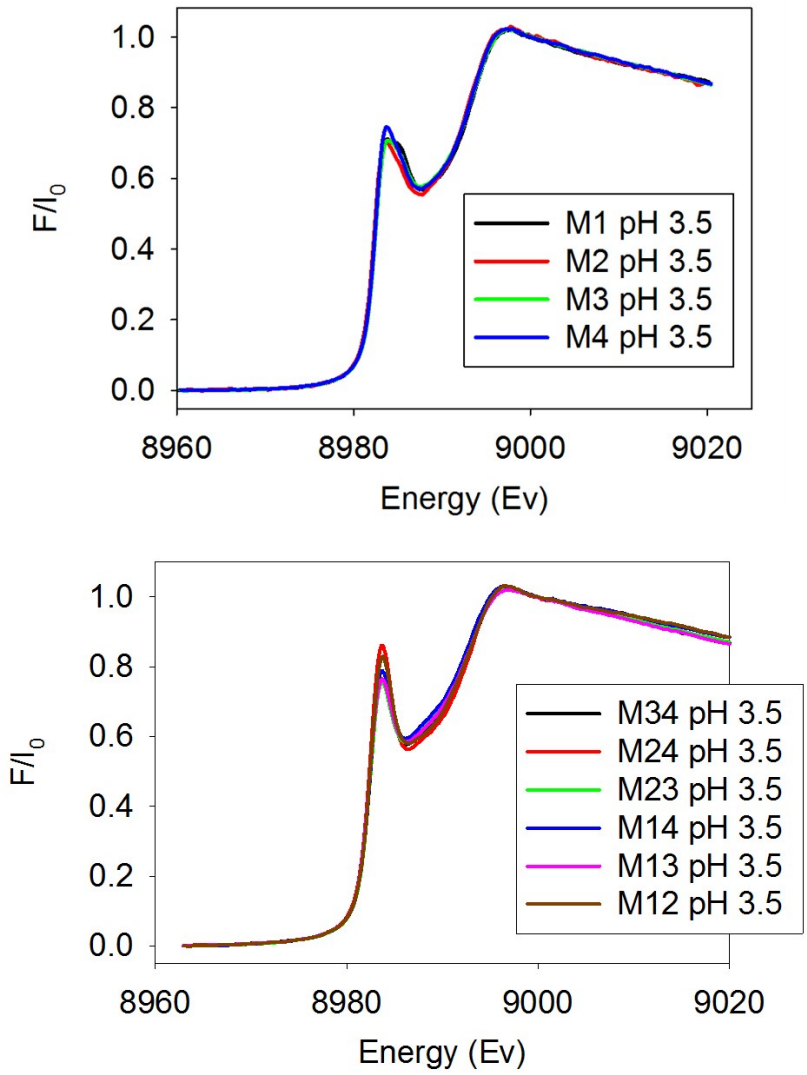

Table S1. HM Loop triple met mutant nomenclature. M1, M2, M3 and M4 denotes the methionine that is retained in the HM Loop sequence (highlighted in red), while the other three methionine residues are mutated to isoleucine (bold).

HM Loop Triple Met Mutant Nomenclature	
	Sequence
M1	**M**DHHFATLHHNQNISKEEIINLHSSI
M2	IDHHFATLHHNQN**M**SKEEIINLHSSI
M3	IDHHFATLHHNQNISKEE**M**INLHSSI
M4	IDHHFATLHHNQNISKEEIINLHSS**M**

	F[a]	No[b]	R(Å)[c]	DW(Å^2)	No[b]	R(Å)[c]	DW(Å^2)	No[b]	R(Å)[c]	DW(Å^2)	-E_0
		Cu-N(His)[d]			Cu-S						
HM Loop WT Cu Edge (S-Met)											
Reduced pH 8.0	0.245	2.0	1.942	0.012	1.0	2.268	0.012				-1.7
Reduced pH 3.5	0.288	1.0	1.912	0.008	2.0	2.251	0.021				0.06
HM Loop WT Cu Edge (Se-Met)											
								Cu-Se			
Reduced pH 8.0	0.298	2.0	1.932	0.016				1.0	2.433	0.018	-1.8
Reduced pH 3.5	.300	1.0	1.944	0.003				2.0	2.414	0.017	-4.1
HM Loop WT Se Edge (Se-Met)											
		Se-C						Se-Cu[e]			
Reduced pH 8.0	0.374	2.0	1.962	0.005				0.8	2.415	0.017	-5.8
Reduced pH 3.5	0.341	2.0	1.960	0.004				2.0	2.414	0.017	-5.6

Table S2. Fits obtained for Cu(I) HM Loop (1:1) with and without SeM labeling at pH 8.0 and pH 3.5 for both the Cu and Se K-edges. [a]F is a least-squares fitting parameter defined as $F^2 = (1/N)\Sigma^N_{i=1}\, k^6(\text{data} - \text{model})^2$. [b]Coordination numbers are generally considered accurate to ±25%. [c]In any one fit, the statistical error in bond lengths is ± 0.005 Å. However, when errors due to imperfect background subtraction, phase shift calculations, and noise in the data are compounded, the actual error is closer to ±0.02 Å. [d]Fits modeled histidine coordination by an imidazole ring, which included single and multiple scattering contributions from the second-shell (C2/C5) and third-shell (C3/N4) atoms, respectively. The Cu– N–Cx angles were as follows: Cu–N–C2, 126°; Cu–N–C3, –126°; Cu–N–N4, 163°; Cu–N–C5, –163°. [e]Samples were prepared at Cu to protein ratios of 1:1. Since there are 4 Met residues in the HM Loop sequence, such samples will have Se:Cu ratios of 4:1, with some of the Se-containing sites in the apo form. The values of the Se–Cu coordination numbers determined from EXAFS simulation at the Se edge have therefore been renormalized by multiplying the calculated Se:Cu shell occupancy by 4. This process renders the Cu–Se and Se–Cu coordination numbers determined from the Cu and the Se edges comparable.

	F[a]	No[b]	R(Å)[c]	DW(Å^2)	No[b]	R(Å)[c]	DW(Å^2)	No[b]	R(Å)[c]	DW(Å^2)	-E_0
		Cu-N(His)[d]			Cu-S			Cu-Se[e]			
Se-M1 HM Loop at pH 3.5											
Reduced pH 3.5	0.322	2.0	1.877	0.007				0.0			-2.0
M2 HM Loop at pH 3.5											
Reduced pH 3.5	0.354	2.0	1.872	0.008	0.1	2.372	0.009				-1.4
M3 HM Loop at pH 3.5											
Reduced pH 3.5	0.324	2.0	1.881	0.011	0.1	2.300	0.008				-1.5
M4 HM Loop at pH 3.5											
Reduced pH 3.5	0.320	2.0	1.869	0.009	0.0						-1.3

Table S3. Fits obtained for Cu(I)HM loop (1:1) triple Met mutants at pH 3.5. [a]F is a least-squares fitting parameter defined as $F^2 = (1/N)\Sigma^N_{i=1}\ k^6(\text{data} - \text{model})^2$. [b]Coordination numbers are generally considered accurate to ±25%. [c]In any one fit, the statistical error in bond lengths is ± 0.005 Å. However, when errors due to imperfect background subtraction, phase shift calculations, and noise in the data are compounded, the actual error is closer to ±0.02 Å. [d]Fits modeled histidine coordination by an imidazole ring, which included single and multiple scattering contributions from the second-shell (C2/C5) and third-shell (C3/N4) atoms, respectively. The Cu–N–Cx angles were as follows: Cu–N–C2, 126°; Cu–N–C3, −126°; Cu–N–N4, 163°; Cu–N–C5, −163°. [e]Samples were prepared at Cu to protein ratios of 1:1.

	F[a]	No[b]	R(Å)[c]	DW(Å²)	No[b]	R(Å)[c]	DW(Å²)	-E_0
		Cu-N(His)[d]			Cu-S			
M34 HM Loop (Met3,4 retained)								
Reduced pH 8.0	0.331	2.0	1.927	0.013	0.5	2.234	0.010	-1.4
Reduced pH 3.5	0.270	2.0	1.898	0.011	0.2	2.244	0.008	-1.6
M24 HM Loop (Met2,4 retained)								
Reduced pH 8.0	0.266	2.0	1.899	0.011	0.0			-2.1
Reduced pH 3.5	0.273	2.0	1.883	0.010	0.0			-2.5
M23 HM Loop (Met2,3 retained)								
Reduced pH 8.0	0.238	2.0	1.911	0.012	0.5	2.279	0.014	-2.0
Reduced pH 3.5	0.244	2.0	1.908	0.011	0.5	2.299	0.008	-2.5
M14 HM Loop (Met1,4 retained)								
Reduced pH 8.0	0.276	2.0	1.896	0.012	0.1	2.221	0.008	-1.7
Reduced pH 3.5	0.299	2.0	1.900	0.015	0.5	2.279	0.014	-1.6
M13 HM Loop (Met1,3 retained)								
Reduced pH 8.0	0.269	2.0	1.911	0.014	0.5	2.264	0.012	-1.8
Reduced pH 3.5	0.239	2.0	1.902	0.013	0.5	2.275	0.015	-1.9
M12 HM Loop (Met1,2 retained)								
Reduced pH 8.0	0.234	2.0	1.906	0.010	0.2	2.249	0.008	-1.7
Reduced pH 3.5	0.239	2.0	1.881	0.011	0.2	2.245	0.008	-2.1
			Cu-N(His)[d]		Cu-S			
Se-M13 HM Loop Cu Edge (Se-Met 1,3 retained)								
Reduced pH 8.0	0.273	2.0	1.899	0.013	0.5	2.406	0.016	-3.3
Reduced pH 3.5	0.258	2.0	1.892	0.012	0.5	2.400	0.016	-1.7
			Se-C		Se-Cu[e]			
Se-M13 HM Loop Se Edge (Se-Met 1,3 retained)								
Reduced pH 8.0	0.327	2.0	1.959	0.005	0.6	2.422	0.016	-5.5
Reduced pH 3.5	0.310	2.0	1.959	0.005	0.6	2.400	0.016	-5.4

Table S4. Fits obtained for Cu(I) HM Loop (1:1) double mutants with and without SeM labeling at pH 8.0 and pH 3.5 for both the Cu and Se K-edges. Nomenclature is the same as that used for the triple mutants where Mxy refers to the Met residue retained in the sequence. [a]F is a least-squares fitting parameter defined as $F^2 = (1/N)\sum^{N}_{i=1} k^6(\text{data} - \text{model})^2$. [b]Coordination numbers are generally considered accurate to ±25%. [c]In any one fit, the statistical error in bond lengths is ± 0.005 Å. However, when errors due to imperfect background subtraction, phase shift calculations, and noise in the data are compounded, the actual error is closer to ±0.02 Å. [d]Fits modeled histidine coordination by an imidazole ring, which included single and multiple scattering contributions from the second-shell (C2/C5) and third-shell (C3/N4) atoms, respectively. The Cu– N–Cx angles were as follows: Cu–N–C2, 126°; Cu–N–C3, −126°; Cu–N–N4, 163°; Cu–N–C5, −163°. [e]Samples were prepared at Cu to protein ratios of 1:1. Since there are 2 Met residues in the HM Loop sequence, such samples will have Se:Cu ratios of 2:1, with some of the Se-containing sites in the apo form. The values of the Se–Cu coordination numbers determined from EXAFS simulation at the Se edge have therefore been renormalized by multiplying the calculated Se:Cu shell occupancy by 2. This process renders the Cu–Se and Se–Cu coordination numbers determined from the Cu and the Se edges comparable.

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