

Supplementary Information for

An Atypical Linear Cu(I)-S₂ center Constitutes the High-affinity Metal-Sensing Site in the CueR Metalloregulatory Protein, Kui Chen, Saodat Yuldasheva, James E. Penner-Hahn, Thomas V. O'Halloran

Mutagenesis, Expression, and Protein Purification. The wild-type CueR protein was prepared as previously described.¹ The CueR mutant genes were prepared by site-directed mutagenesis (QuikChange[®] Site-Directed Mutagenesis Kit, Stratagene) of the over-expression vector pET24a inserted with the wild-type *cueR* gene. The sequences of the oligonucleotides used are shown below, with the modified base shown in lower case: C112S, 5'-CACTGGCGAATGCCaGCCCTGGCGATGACAGC-3'; C120S, 5'-GCGATGACAGCGCCGACaGCCCCGATTATCGAAAATCTC-3'; C129S/C130S, 5'-CGAAAATCTCTCCGGCaGCaGTCATCATCGGGCAGGGTG-3'. The sequence of each mutated gene was confirmed by DNA sequencing analysis of both strands of the mutant gene. Similar over-expression and purification procedures as those described for the wild-type CueR¹ yielded the mutant proteins with > 90% purity. The molecular mass of each of the CueR mutant proteins was determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI, PerSpective Biosystems Voyager-DE), by amino acid hydrolysis (AAA, Protein Chemistry Laboratory, Texas A&M University), and by calculation based on the sequence (with N-terminal Met) as follows: CueRC112S, 15218 Da (MALDI), 15219 (AAA), 15219 (calc); CueRC120S, 15216 Da (MALDI), 15218 (AAA), 15219 (calc); CueRC129S/C130S, 15202 Da (MALDI), 15203 (AAA), 15203 (calc).

Protein-Metal Ratio Determination. The protein concentration was determined initially by quantitative amino acid hydrolysis (Protein Chemistry Laboratory, Texas

A&M University) and later by the Bradford assay (Bio-Rad, with IgG as the calibration standard) for the same sample. Comparison of protein concentrations revealed that the Bradford assay overestimated the concentration of the wild-type CueR by 3.05 fold, CueRC112S by 2.68 fold, CueRC120S by 2.70 fold, and CueRC129S/C130S by 2.27 fold. These correction factors were then used routinely for the analysis of protein concentrations by the Bradford assay. Metal concentrations were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Thermo Jarrell Ash AtomScan 25 ICP-AE spectrometer.

Cu(I) Binding Assays. Cu(I) binding assays were performed under an N₂ atmosphere in a glove box in an Amicon ultrafiltration cell with a YM-10 membrane. 1~5 equivalents (as indicated) of [Cu(I)(CH₃CN)₄]PF₄ (CH₃CN stock) was added to 2~10 μM CueR or its mutant in 10 mL of 50 mM Tris/Cl, pH 8.0, 5.0 mM DTT (as indicated). The solution was stirred at 20⁰ C for 30 min and then washed four times with buffer to remove excess metal.

Preparation of EXAFS Samples. All EXAFS samples were prepared as duplicates, transferred to an EXAFS cuvette (Lucite body, Kapton windows), and frozen at -20⁰ C under N₂ atmosphere in a glove box. The samples were then kept in liquid N₂ until analysis. The samples were prepared by addition of 0.5~1.0 equivalent of [Cu(I)(CH₃CN)₄]PF₄ (CH₃CN stock) to 10~15 μM WT-CueR or CueRC129S/C130S in 10 mL 50 mM Tris/Mes, pH 8.0, 5.0 mM DTT. The solution was stirred at 20⁰ C for 30 min and then washed four times with 10 mL 50 mM Tris/Mes, pH 8.0 to remove excess metal and DTT. Glycerol was added to a final concentration of 30%. The protein concentration and the Cu(I)/protein ratio for the duplicates were following: 1Cu(I)-WT-

CueR, 0.91 mM protein with 0.90 Cu per monomer, or 0.68 mM protein with 0.95 Cu per monomer; : 0.5Cu(I)-WT-CueR, 1.51 mM protein with 0.54 Cu per monomer, or 1.19 mM protein with 0.60 Cu per monomer; 1Cu(I)-CueRC129S/C130S, 0.53 mM protein with 0.91 Cu per monomer, or 1.1 mM protein with 0.92 Cu per monomer; 0.5Cu(I)-CueRC129/C130S, 1.2 mM protein with 0.62 Cu per monomer, or 1.7 mM protein with 0.49 Cu per monomer.

Thiol Modification. Thiol modification was performed under an N₂ atmosphere in a glove box with the cysteine-specific reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS, Molecular Probes). The mass of an AMS-labeled Cys residue increases by 535 Da upon covalent bond formation. For each sample, 2.5 mg/mL CueR protein (no DTT) was incubated with 8 equivalents of AMS (66 μM) and 400 equivalents of bathocuproine sulfonate (BCS, 3.3 mM) as indicated in 20 μL 50 mM Tris/Cl, pH 8.0, 0.25% SDS at 37⁰C for 2 hr. The sample was then removed from N₂ atmosphere and run on a 15% SDS-PAGE under non-reducing conditions, with protein bands detected by Coomassie staining.

X-Ray Absorption data collection. X-ray absorption data were measured at SSRL beamline 9-3. (3.0 GeV, 55-100 mA) using a Si(220) double crystal monochromator. The monochromator was run fully tuned and a Rh-coated Pt mirror was used for harmonic rejection. Samples were held at 10K using an Oxford Instruments helium cryostat. Spectra were measured using 10 eV steps in the pre-edge, 0.25 eV steps in the edge (9639-9709 eV), and 0.05 Å⁻¹ increments in the extended x-ray absorption fine structure (EXAFS) region, integrating for 1s, 1s, and k^3 weighted 1-25s, respectively, for a scan length of ca. 35 minutes. X-ray energies were calibrated by simultaneous

measurement of a Cu foil absorption spectrum, assigning the first inflection point as 8980.3 eV. Fluorescence excitation spectra were recorded using a 30-element Ge solid-state detector array (incident count rate < 110 kHz; windowed Cu K α count rate 6-19 kHz). Each channel of each scan was examined for glitches prior to averaging.

XAS data analysis. XANES data were normalized by fitting the data below and above the edge to the McMaster X-ray absorption cross-sections. EXAFS data reduction used a first-order polynomial in the pre-edge and a two-region cubic spline through the EXAFS. Data were converted to k -space using $E_0=9000$ eV. Fits to both Fourier filtered ($R=0.9-3.0$ Å) and unfiltered data gave equivalent structural parameters. EXAFS data were fit using amplitude and phase functions calculated with FEFF v8.10² for Cu-S at 2.15 Å. A scale factor of 0.89 and ΔE_0 of 13.0 were calibrated by fitting EXAFS data for crystallographically characterized complexes. Coordination numbers were fixed at integer values, and R and σ^2 were freely variable parameters.

In vitro Run-Off Transcription. Run-off transcription reactions were performed as previously described¹ except the incubation buffer (pH = 8.0) was treated with Chelex to remove exogenous metals. 1.0 mM NaCN (ACS reagent) was present to sequester residual copper in the incubation buffer which could contribute to the background transcription level (see Text reference 5 for details). The [Cu(I)(CH₃CN)₄]PF₄ (ACS reagent) stock solution in CH₃CN was kept under N₂ atmosphere just before the reaction and the reduced metal state was maintained by 1.0 mM dithiothreitol (DTT) in the reaction solution.

- (1) Outten, F. W.; Outten, C. E.; Hale, J. A.; O'Halloran, T. V. *J. Biol. Chem.* **2000**, *275*, 31024-31029.
- (2) Zabinsky, S. I.; Rehr, J. J.; Ankudinov, A.; Albers, R. C.; Eller, M. J. *Phys. Rev. B* **1995**, *52*, 2995-3009.

Table S1. Cu(I) incorporation into CueR and its mutants in the presence (+) or absence (-) of a Cu(I)-competitor, DTT.^a

DTT	WT		C129S/C130S		C112S		C120S	
	+	-	+	-	+	-	+	-
5 equiv Cu(I)	1.0	3.0	0.9	1.0	< 0.1	1.7	< 0.1	1.9
1 equiv Cu(I)	1.0	0.9	1.0	1.0	< 0.1	ppt ^b	< 0.1	ppt ^b
as-purified	< 0.1		< 0.1		< 0.1		< 0.1	

^aAll dialyses were performed under N₂. ^bProtein precipitated during the dialysis.

Table S2. EXAFS fitting results^a

	Cu-S first shell		MS ^b	Cu-C second shell			χ^2_{red} ^d
	R(Å)	$\sigma^2 \times 10^3$ (Å ²)	$\sigma^2 \times 10^3$ (Å ²)	R(Å)	$\sigma^2 \times 10^3$ (Å ²)	$\theta_{\text{Cu-S-C}}$ (°) ^c	
CueR	2.141	2.1					8.6
0.54:1	2.141	2.1	1.9				4.1
	2.141	2.1	2.0	3.179	10.5	106.5	3.7
CueR	2.141	1.6					9.1
0.60:1	2.141	1.6	4.2				7.2
	2.141	1.6	4.8	3.233	0.7	109.1	6.0
CueR	2.143	1.6					10.0
0.90:1	2.143	1.6	1.1				7.6
	2.143	1.6	1.3	3.191	8.0	107.0	7.4
CueR	2.143	2.1					11.4
0.95:1	2.144	2.1	2.2				7.9
	2.144	2.1	2.5	3.202	5.0	107.5	7.4
CueR-CCSS	2.138	1.4					9.7
0.62:1	2.138	1.4	4.8				7.8
	2.138	1.4	5.2	3.220	5.8	108.6	7.5
CueR-CCSS	2.142	2.1					9.6
0.49:1	2.142	2.1	2.5				6.2
	2.142	2.1	2.8	3.202	4.0	107.6	5.6
CueR-CCSS	2.141	2.3					5.7
0.91:1	2.141	2.3	2.7				3.8
	2.141	2.3	3.3	3.221	2.1	108.5	3.2
CueR-CCSS	2.140	1.9					17.0
0.92:1	2.140	1.9	3.2				12.1
	2.140	1.9	3.5	3.245	2.8	109.8	11.4

^a Fitting results for Cu-S coordination numbers constrained to 2. Fits with unconstrained coordination number and Debye-Waller factor give apparent Cu-S coordination numbers ranging from 1.72 to 2.04, with corresponding Debye-Waller factors from 0.8 to 2.2, respectively.

^b Cu-S first-shell multiple scattering shell. Coordination number and distance were constrained by first-shell Cu-S fit and only Debye-Waller factor was varied.

^c Calculated Cu-S-C angle assuming a S-C distance of 1.82 Å.

^d Mean-square deviation between data and fit, normalized to the number of free parameters.