Supporting Information for:

A Highly Sensitive and Selective Gold(I)

Recognition by a Metalloregulator in

Ralstonia metallidurans

Xing Jian[*], Erik C. Wasinger[†], Jenny V. Lockard[†], Lin X. Chen[†‡], and Chuan He[§]*

[*]Department of Chemistry, 5735 South Ellis Avenue, The University of Chicago, Chicago, Illinois 60637, [*]Chemistry Division, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439, [‡] Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

Expression and purification of recombinant CupR protein from *E. coli*

The expression plasmid of *Ralstonia metallidurans* CH34 *cupR* gene is a generous gift from Dr. Daniel van der Lelie at Brookhaven National Laboratory. The gene was cloned into pET30a vector (Novagen) between *Nde*I and *BamH*I sites. Then the plasmid was transformed into *E. coli* BL21(DE3)plysS cells. A single colony of transformed *E. coli* cells was cultured in 10 mL LB media at 37 ºC with kanamycin (30 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}). On the second day the culture was transferred to 1 L LB media with kanamycin and chloramphenicol and shaken vigorously at 37 ºC. Cells were induced with 1 mM isopropyl-beta-D-thiogalactopyranoside at 30 ºC when OD$_{600}$ reached 0.6. After shaking for another 4 hr the bacteria were harvested, frozen by liquid nitrogen and stored at -80 ºC until use. Cell pellet was re-suspended into lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM β-ME (beta-mercaptoethanol) and 5% glycerol. After sonication, the lysate was centrifuged at 12, 000 rpm for 20 min. The supernatant was loaded on to Q Sepharose column pre-equilibrated with buffer A (10 mM Tris-HCl, 10 mM β-ME, pH 7.4), after which unbound proteins were washed with buffer A. The protein was eluted with a 10-30% gradient of buffer B (1 M NaCl, 10 mM β-ME, 10 mM Tris-HCl, pH 7.4). The fractions containing CupR were concentrated by Amicon Ultra -15 centrifugal filter devices to 5 mL and diluted with 1 volume of 1.5 M (NH$_4$)$_2$SO$_4$, before loading onto Phenyl HP column. The column was washed with 0.75 M (NH$_4$)$_2$SO$_4$, and the bound protein was eluted with a 5-100% gradient of 50 mM sodium phosphate buffer (pH 7.4). The fractions containing CupR were concentrated to 2 mL and loaded onto Superdex-200 column pre-equilibrated with degassed buffer GF [100 mM NaCl, 10 mM Tris-HCl, 0.5 mM DTT (dithiothreitol), pH 7.4]. After this purification step the protein was over 95% pure (Figure S1). The

fractions were concentrated to 1 mL before adding 300 μL of glycerol. The protein can be stored at -80 ºC for up to one month.

Northern hybridization

Ralstonia metallidurans CH34 was grown in medium 869 at 30 ºC until OD_{600} reached 1.0.[1] Metal salt was added into 1 mL culture, and the bacteria were harvested after 30 min. Total RNA was isolated by RNeasy Mini kit (Qiagen), and transferred to nitrocellulose membrane (GE Healthcare) after denaturing agarose gel electrophoresis. A specific radioactive probe for the interested gene was generated by DECAprime II kit (Ambion), and used to hybridize with the membrane at 42 ºC overnight. After washing away excessive amounts of the probe, the membrane was exposed to Fuji film for autoradiography. The signal was detected by Bio-Rad FX Pro Plus imager. Primer3 was used to design the primers to generate the probes.[2]

Gel mobility shift assay

A 72-mer dsDNA containing the *cupA* promoter was labeled with P^{32} by T4 polynucleotide kinase (New England Biolabs) and γ-P^{32}-ATP (Perkin Elmer). In a solution containing 40 mM KCl, 1 mM DTT, 10 mM Tris-HCl (pH 7.4), 1 nM labeled DNA was mixed with 0, 6.25, 12.5, 25, 37.5, 50, 62.5 and 75 nM CupR at 4 ºC. The use of too much protein would lead to aggregation. Either 10 μg/mL calf thymus DNA (Invitrogen) or 1 mM $K[Au(CN)_2]$ was added to the solution containing 1 nM DNA and 50 nM protein in separate experiments. The formation of the protein-DNA complex was resolved on a 6% native poly-acrylamide gel. The radio-isotope signal

was detected by a Fuji phosphor imager plate and visualized by a Bio-Rad FX Pro Plus imager.

Mutation

All mutants were constructed by QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The mutation primers were designed according to the manual of the kit. The mutant proteins were purified by the same procedure as the wild-type protein.

UV-visible spectroscopy

The UV-visible spectra of protein samples were measured by Agilent 8453 spectrometer. To make gold(I)-or copper(I)-loaded protein, an excess amount of K[Au(CN)$_2$] or [Cu(CH$_3$CN)$_4$]PF$_6$ was added to the degassed protein solution and incubated at 4 ºC for 30 min. The holo-protein was then purified by Superdex-200 column. To estimate the dissociation constant of the copper(I)-CupR complex, 100 μM of copper(I)-loaded protein was titrated with bcs. Copper(I) and bcs form a stable 1:2 complex with an association constant of logβ_2 = 19.8.[3] The absorption of the complex at 483 nm (ε = 13 300 M^{-1} cm^{-1}) was monitored and used to calculate the dissociation constant for copper(I). To estimate the dissociation constant of the gold(I)-CupR complex, a solution containing 100 μM freshly purified protein in 200 mM Tris-HCl (pH 7.7) was mixed with a buffered gold(I) solution containing KCN and K[Au(CN)$_2$]. The free gold(I) concentration was calculated based on the proton dissociation constant of CN$^-$ (log$K$ = 9.04, 25 ºC, 0.1 M ionic strength),[4] and the association constant of [Au(CN)$_2$]$^-$ (logβ_2 = 36.6).[5] The concentration of KCN and K[Au(CN)$_2$] were kept at least 10 times higher than the concentration of the protein. The mixed solution was equilibrated at 20 ºC for 30 min under N$_2$, and loaded onto a

Micro Bio-Spin 6 column (Bio-Rad), and spun down immediately. The increase of A_{255}/A_{283} ratio was used to calculate the occupancy of gold(I) in the protein. The dissociation constant was obtained by fitting data to the Hill equation. To confirm the results, the gold(I) content of each sample was further confirmed by ICP-MS.

ICP-MS

ICP-MS analysis was conducted by the ICP-MS analytic research laboratory at the University of Notre Dame.

Metal exchange experiment

E. coli CueR was expressed and purified as described in ref 4. The *cupR* gene was cloned between *Nde*I and *Hind*III sites into pET28a vector (Novagen) to generate CupR with an N-terminal his$_6$-tag. The wild-type protein was expressed and purified with a HisTrap column (GE Healthcare). In one reaction copper(I)-CupR (his$_6$-tagged) was mixed with apo-CueR, while for the other, apo-CupR (his$_6$-tagged) was mixed with copper(I)-CueR. The exchange reaction was performed at 4 ºC overnight, and the two proteins were separated by Ni-NTA resin (Qiagen) before UV-visible spectroscopic analysis.

X-ray absorption data collection and EXAFS curve fitting

Protein (1 mM) loaded with copper(I) was used for the study. CupR XAS spectra were collected at copper K-edge on the bending magnet beam line 12BM at the Advanced Photon Source, Argonne National Laboratory, and internally calibrated with an inline copper foil. The details of the beamline parameters have been

described in a previous publication.[6] A sample temperature of 15 K was maintained with a Janis displex cryostat (CCS-100) throughout the experiment. Thirty scans were accumulated with a Canberra 13-element solid-state germanium detector array and averaged. A pre-edge Gaussian function modeling elastically scattered beam was removed from the data below 8947 eV before the data were normalized to an edge jump of one at 9857 eV. EXAFS data were extracted by fitting a three-segment spline to the normalized data between 9000 and 9857 eV. Although CupR data were collected to k=15 Å^{-1}, EXAFS data were fit over the range k=1-11Å^{-1} due to Zn K-edge contamination. EXAFS data were fit by the EXAFSPAK program[7] using phases and amplitudes computed from the FeFF7.0 program.[8] Several different input models were used with different coordination numbers of S/N/O and Cu-S distances ranging from 2.10-2.40 Å and Cu-O/N distances ranging from 1.95-2.45 Å in 0.10 Å increments as input parameters for the FeFF calculations.

All fits were restricted to a coordination number of three in accordance with the results from XAS edge fitting. Fits consisting of a single shell of either three nitrogen- or oxygen-based ligands or, alternatively, of three sulfur-based ligands could not adequately fit the data (Table S4, Fits 1 and 2, respectively) resulting in large residual errors and an unreasonable ΔE_0. Fits consisting of two sulfur ligands at 2.12 Å and either a nitrogen- or oxygen-based ligand at 2.50 Å (Table S4, Fit 3) or a sulfur ligand (Table S4, Fit 4) both successfully fit the data. Attempts to fit the data modeling a histidine at the active site would imply significant multiple scattering which is largely absent in the FT of the data. Thus, histidine ligation is not likely supported by the EXAFS fit. A fit consisting of a single shorter nitrogen- or oxygen-based ligand, a single sulfur, and a single longer nitrogen- or oxygen-based ligand was also unsuccessful resulting in physically unreasonable negative Debye-Waller

factors (Table S4, Fit 5). A fit of two shorter nitrogen- or oxygen-based ligands and a longer sulfur ligand resulted in a reasonable fit, but with a significantly higher residual error (Table S4, Fit 6). When the copper(I)-CupR137del mutant was used, a different edge spectrum was obtained, and the EXAFS data are best fit with 2 sulfur based ligands at 2.13Å (Figures 5D and S8). Adding extra sulfur ligands or nitrogen ligands either reduces the quality of the fit (Table S5, Fit 2), results in physically unreasonable parameters (Table S5, Fit 3: ΔE_0, Fits 5 and 6: σ^2), or simply does not improve the fit; thereby it does not justify the additional parameters (Table S5, Fit 4). Thus, the EXAFS data are consistent with a three-coordinate site, including two sulfur-based ligands at 2.12Å and a third, longer interaction, quite possibly a third sulfur ligand at 2.26Å (Figure 3C and 3D).

Reaction of apo- and holo-protein with fluoresin-5-maleimide

50 μL of 125 μM apo-, copper(I) or gold(I)-CupR were mixed with 4 fold excess of 1 mM fluoresin-5-maleimide (Pierce) in thiol free buffer on ice for 1 h. The free dye and the labeled protein were separated by a Micro Bio-Spin 6 column (Bio-Rad). The concentration of protein and fluoresin were calculated from UV-vis spectrum according to manufacturer's instruction.

Supplementary Tables

Table S1. Titration of Copper(I)-CupR Complex with bcs.

protein	CupR					CupR137del			
[copper(I)-P]$_t$, μM	48.9	48.2	47.8	35.0	34.0	52.6	51.5	50.4	48.8
[bcs]$_t$, mM	0.06	0.08	0.1	1.0	1.5	0.1	0.14	0.18	0.25
[Cu(bcs)$_2$$^{3-}$], μM	11.4	11.6	12.7	26.6	30.0	7.7	9.3	11.0	12.1
K_d, × 10^{-19} M	1250	590	470	5.1	7.4	33	22	18	10

Table S2. ICP-MS Analysis of Gold Concentration in Samples Used to Determine the K_d of the Gold(I)-CupR Complex.

sample #	1	2	3	4	5
mearsured by UV-Vis (μM)	48	50	34	7.5	15
measured by ICP-MS (μM)	54.4	45.8	30.6	12.3	21.6

Table S3. Quantification of the Surface Exposed Free Thiols by Fluoresin-maleimide.

sample	protein (μM)	fluoresin-maleimide (μM)	exposed –SH per protein
apo-CupR	44	42	0.96
copper(I)-CupR	45	25	0.55
gold(I)-CupR	45	30	0.67

Table S4. EXAFS Fits of Copper(I)-CupR. (^{a} The error is given by $\Sigma[(\chi_{obsd}-\chi_{calc})^{2}k^{6}]/[\ \Sigma[(\chi_{obsd})^{2}k^{6}])$

Fit	Ligands	R (Å)	σ^{2} (Å^{2})	ΔE_0	F^{a}
1	3 N/O	2.18	0.00069	-61.9	0.78
2	3 S	2.28	0.00459	-79.0	0.47
3	2 S	2.12	0.00538		
	1 N/O	2.50	0.00372	-19.3	0.29
4	2 S	2.12	0.00480		
	1 S	2.26	0.00936	-16.9	0.31
5	1 N/O	1.93	-0.00060		
	1 S	2.17	0.00115		
	1 N/O	2.53	-0.00000	-12.1	0.28
6	2 N/O	1.98	0.00434		
	1 S	2.16	0.00540	-9.0	0.37

Table S5. EXAFS Fits of Copper(I)-CupR137del.

Fit	Ligand @ R (Å)	σ^2 (Å^2)	ΔE_0 (eV)	F
1	2S @ 2.13	0.00358	-20.0	0.48
2	3S @ 2.13	0.00654	-18.8	0.80
3	3N @ 2.04	0.00095	0.19	0.49
4	2S @ 2.13	0.00300	-15.1	0.48
	2N @ 2.11	0.00595		
5	1S @ 2.13	0.00320	-9.3	0.44
	1N @ 2.11	-0.00180		
6	2S @ 2.15	0.00232	-17.8	0.45
	1N @ 2.21	-0.00100		
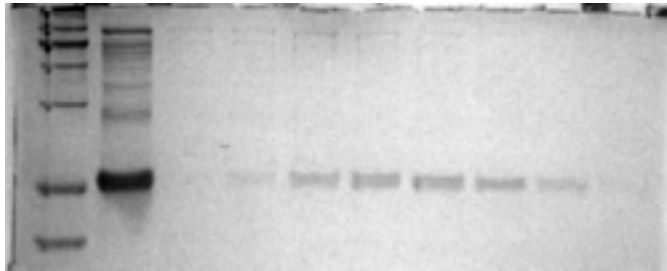
Figure S1. Purified CupR protein. Lane 1: 10-225kD protein marker (Promega). Lane 2: sample before the gel-filtration column. Lane 3-10: fractions 10-17 from gel-filtration.
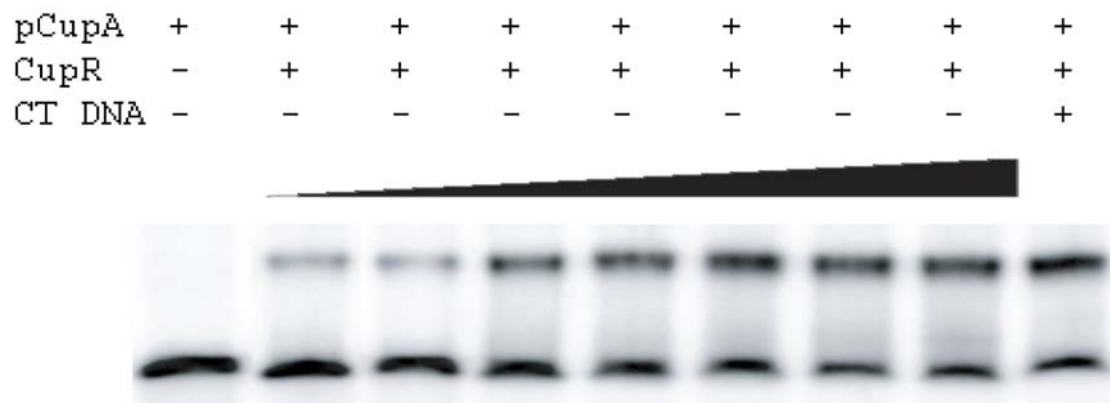
Figure S2. Gel mobility shift assay showing complex formation between the *cupA* promoter DNA and the CupR protein. A 72-mer dsDNA (1 nM) containing *cupA* promoter sequence and CupR (6.25 nM to 75 nM in first 8 lanes) were used in the assay. The addition of 10 μg/mL calf thymus DNA (CT DNA) had little effect on the complex formation (last lane).
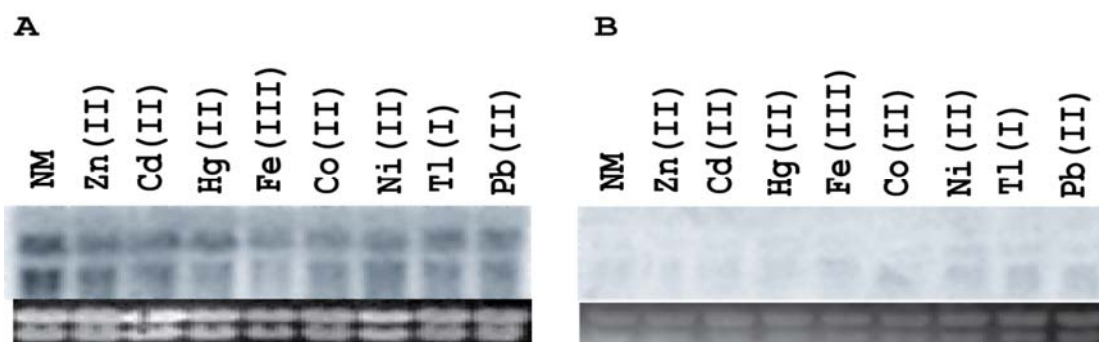
Figure S3. No induction of *cupC* (A) or *cupA* (B) gene by Zn(II), Cd(II), Hg(II), Fe(III), Co(II), Ni(II), Tl(I) or Pb(II) in *R. metallidurans*. Concentrations used were the same as Figure 2A. NM: no metal. Loading controls were shown at the bottom.
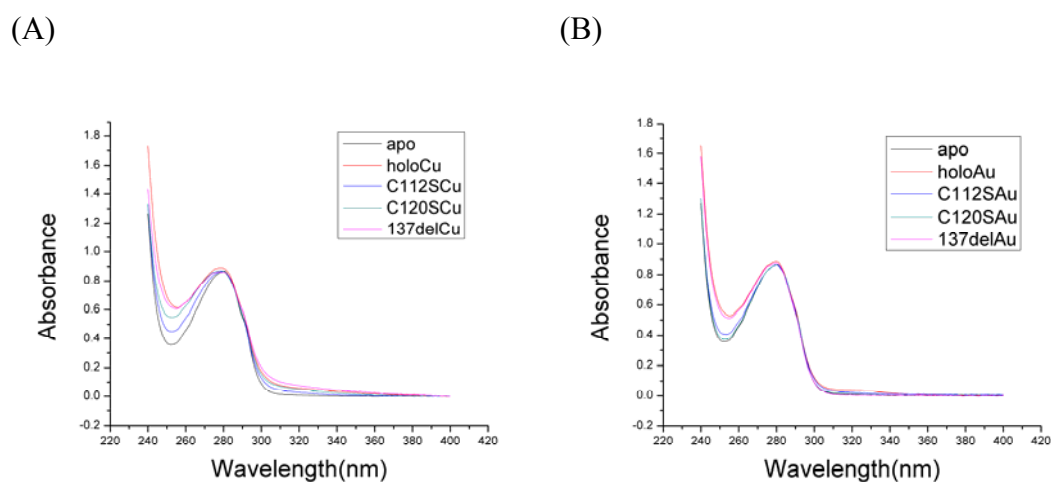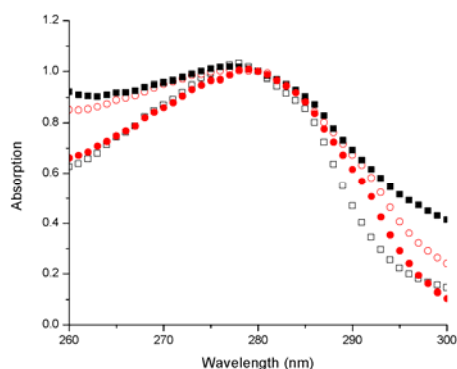
(A)   (B)

Figure S4. (A) UV-visible spectra of apo-CupR, copper(I)-CupR and copper(I)-bound forms of three mutants C112S, C120S and CupR137del. (B) UV-visible spectra of apo-CupR, gold(I)-CupR and gold(I)-bound forms of three mutants C112S, C120S and CupR137del.
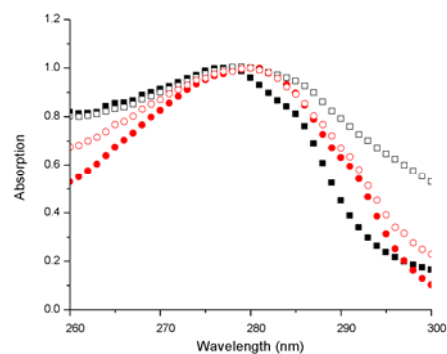
(A)　　　　　　　　　　　　　　　　　　　　(B)

Figure S5. (A) Metal exchange from copper(I)-CupR (black square) to CueR (red dot). (filled dots: before the exchange reaction; empty dots: after the reaction). A transfer of copper(I) from copper(I)-CupR to CueR was observed. (B) The same experiment between copper(I)-CueR (black square) and apo-CupR (red dots) (filled dots: before reaction, empty dots: after reaction). CupR could not compete for copper(I) from copper(I)-CueR.
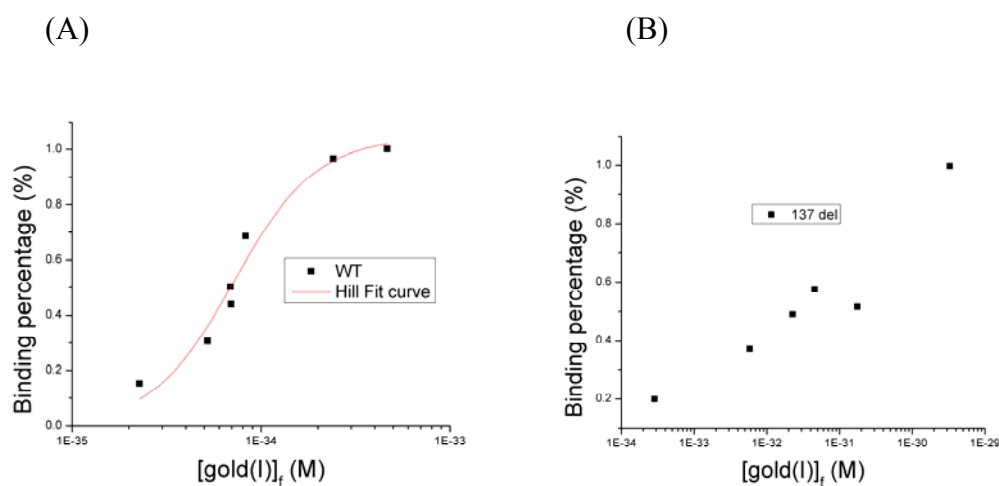
(A)　　　　　　　　　　　　　　　　　　　　(B)

Figure S6. (A) Binding of gold(I) to CupR in a cyanide-buffered gold(I) solution. Red curve shows that the binding can be fit into a sigmoidal function. (B) Estimation of the K_d for gold(I)-CupR137del. The free gold(I) concentration is at the upper limit of the gold(I)-CN⁻ buffered system, which gives errors that are larger than the errors that the experiments with the wild-type protein produced.
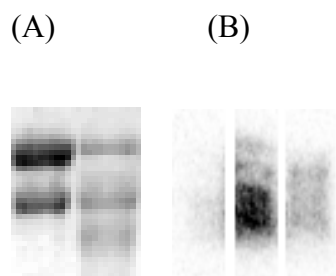
(A)   (B)

Figure S7. (A) Electrophoresis of RNA sample after adding 50 μM HAuCl₃ (left lane) or 50 μM AgNO₃ (right lane) into *R. metallidurans* culture. The degradation of rRNA (internal standard) is evident in the second lane. (B) Detecting induction level of *cupR* gene with no additional metal (lane 1), 50 μM HAuCl₃ (lane 2) or 50 μM AgNO₃ (lane 3) by northern hybridization. As silver(I) is very toxic, the death of bacteria and the massive degradation of bacterial RNA upon silver(I) treatment prevented data quantification.
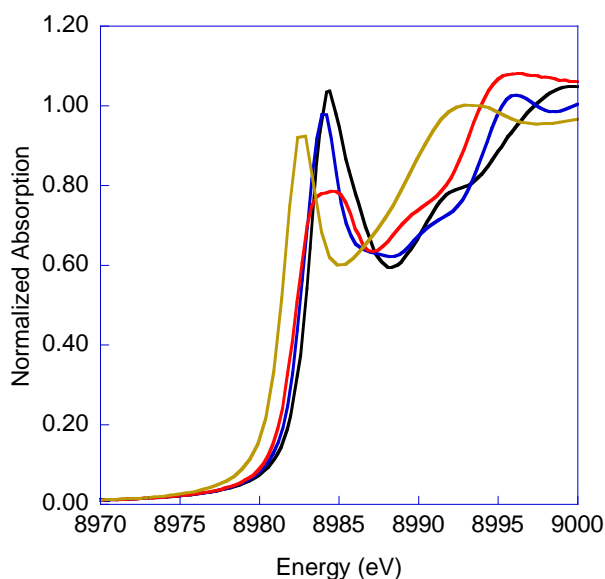

Figure S8. XANES spectra of $[Cu(xypz)]_2(BF_4)_2$ (black), $[Cu_2(EDTB)](ClO_4)_2$ (blue), $[Cu(BBDHp)](BF_4)_{0.34}(PF_6)_{0.66}$ (red), and copper(I)-CupR137del (yellow). Copper(I) in CupR137del is in a 2-cooridnate linear geometry.

Reference

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