Redox Tuning of Two Biological Copper Centers through Non-covalent Interactions: Same Trend but Different Magnitude†

Siu Yee New,[†a] Nicholas M. Marshall,[†b] T. S. Andy Hor,[ac] Feng Xue[a] and Yi Lu*[b]

[a] *Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543.*
[b] *Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 (USA).Email: yi-lu@illinois.edu*
[c] *Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR), 3 Research Link, Singapore 117602.*
†These authors contributed equally.

This supplementary information is an extension of the communication with the same title, containing further discussion on the experimental section, tabulated spectroscopic data (Table S1), UV-vis spectra (Figure S1), EPR spectra (Figure S2), ESI-MS spectra (Figure S3), cyclic voltammograms (Figure S4) and plots of redox titration (Figure S5-6).

Methods

Protein Expression. The Asn47Ser and Glu114Pro mutants were constructed, expressed and purified following the reported procedure.[1-3]

Electrochemistry. Cyclic voltammetry was performed on CH Instrument model 620A Electrochemical Analyzer (Austin, TX), following the reported procedure for Cu_A Az.[4] Pyrolytic graphite edge (PGE) electrodes assembled following a previously published procedure[5] were polished with alumina (0.3 and 0.05 μm), rinsed and coated using DDAB in chloroform (10 μL, 10 mM) as co-adsorbent. The DDAB-film PGE electrode was kept in a closed tube for slow drying overnight. A platinum wire was used as auxiliary electrode and a Ag/AgCl electrode (Bioanalytical Systems, West Lafayette, 1 N, 3 M NaCl) was used as a reference. The electrodes were immersed in protein samples, 3 mL of 0.1 mM protein that were kept on ice and bubbled with nitrogen prior to measurement to remove exogenous O_2. All reported readings were converted to NHE potentials by adding 210 mV to the value obtained *vs.* the Ag/AgCl reference.

Redox titration. 0.1-0.2 mM of protein samples in degassed 50 mM NH_4OAc buffer (pH 5.1) were titrated with appropriate aliquots of 2-6 mM $[Ru(NH_3)_5Py](ClO_4)_2$ under a flow of argon.[4] The absorption change of A_{max} at ψ→ψ* transition (~780 nm) region is monitored. The reduction potential of the protein can be calculated using equation (1):[6]

$$E = E_{Ru} + \frac{RT}{nF} \ln K \qquad (1)$$

E_{Ru} = reduction potential of the ruthenium complex (E_m = 284 mV),
R = Avogadro's constant
T = temperature
n = the number of electrons
F = Faraday constant
K = equilibrium constant
where K can be obtained from

$$K = \left[\frac{(A^{\circ}_{max} - A_{max})}{A_{max}}\right]\left[\frac{\varepsilon_{max}[Ru]_t}{(A^{\circ}_{max} - A_{max})} - 1\right]^{-1} \qquad (2)$$

A_{max} = maximum absorbance of Cu_A azurin at $\psi \rightarrow \psi^*$ transition region
A°_{max} = maximum absorbance of fully oxidized Cu_A azurin at $\psi \rightarrow \psi^*$ transition region
ε_{max} = extinction coefficient of A_{max}
$[Ru]_t$ = total concentration of ruthenium ions

By extrapolating to zero concentration of $[Ru(NH_3)_5Py](ClO_4)_2$, the y-intercept was used to calculate E_m. Reduced variants were then re-oxidized by titrating with $[(NH_4)_2Ce(NO_3)_6]$ ($E_{mCe4+/3+}$ = 1.72 V), indicating that the observed redox event was reversible. In the case of both variants, the level of re-oxidation was observed to be less than the originally oxidized protein, which was also seen in previous reports of the unmodified protein using these same redox titrants.[4] The lower level of re-oxidation is due to the use of $[(NH_4)_2Ce(NO_3)_6]$ as the oxidant, which is known to be a strong denaturant for many proteins.

Electronic absorption and EPR spectroscopy. UV-visible absorption spectra were recorded in buffer containing 50 mM NH_4OAc (pH5.1) on Cary 5000 UV-Vis-NIR spectrophotometer at ambient temperature. Mass Spectra were measured on Macromass Quattro II Mass Spectrometer and processed by Waters MassLynx data system. EPR spectra were recorded using a Varian E-line 12" Century Series X-Band CW spectrometer at 30 K. The protein samples were prepared in buffers mixed with 50% glycerol. Simulation of EPR spectra was performed using the SIMPOW software package to determine g and $A_{\parallel}$ values.[7]

Spectroscopic Data

Table S1 Tabulated spectroscopic data for Cu_A Az variants.

Variants	λ_{max} (nm)	Calculated Mass (Da)	Experimental Mass (Da)	EPR g (A) in TIP (pH 7)
Cu_A Az	483, 530, 760	14173.0	14176.0	g_1 = 2.021 (28.0 G) g_2 = 2.027 (12.1 G) g_3 = 2.173 (56.2 G)
Asn47Ser	483, 530, 753	14140.0	14144.0	g_1 = 2.027 (24.3 G) g_2 = 2.027 (14.2 G) g_3 = 2.176 (55.8 G)
Glu114Pro	473, 535, 784	14141.0	14140.0	g_1 = 2.017 (29.5 G) g_2 = 2.021 (10.9 G) g_3 = 2.169 (56.6 G)

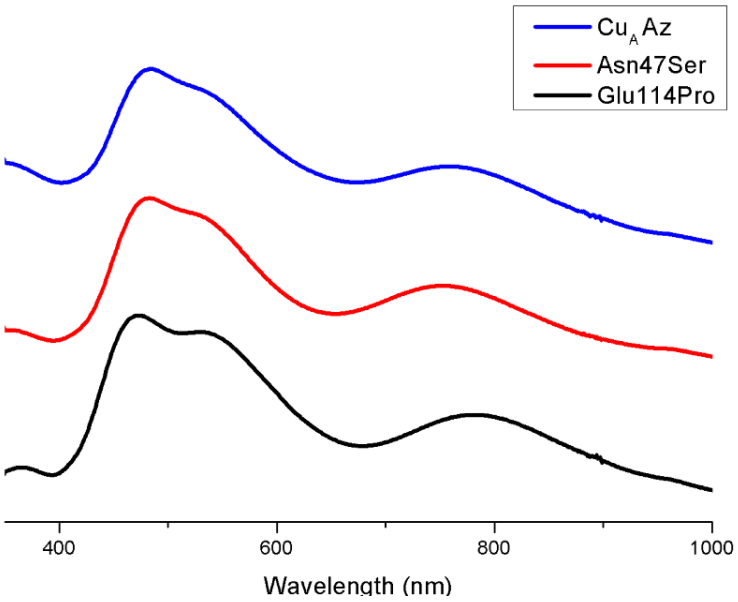


Figure S1 UV-vis spectra of Cu_A Az, Asn47Ser and Glu114Pro mutants at room temperature in 50 mM NH4OAc (pH5.1) buffer.

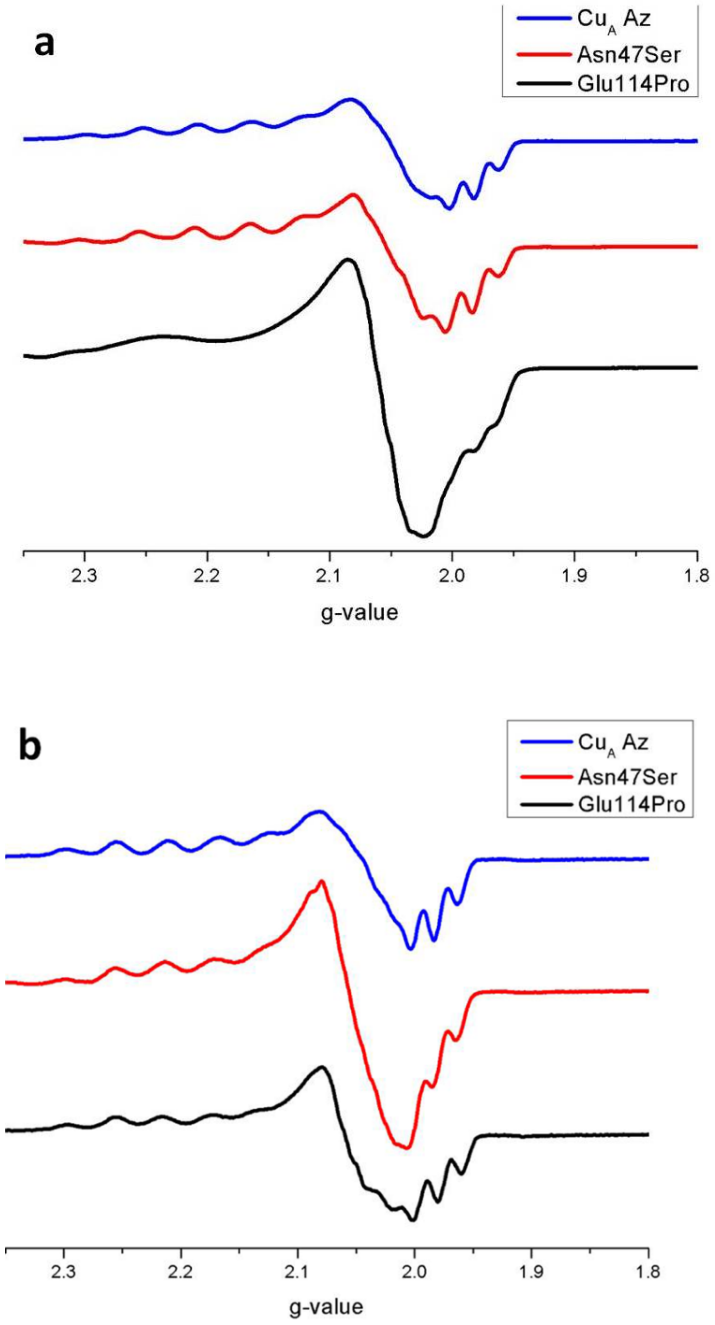
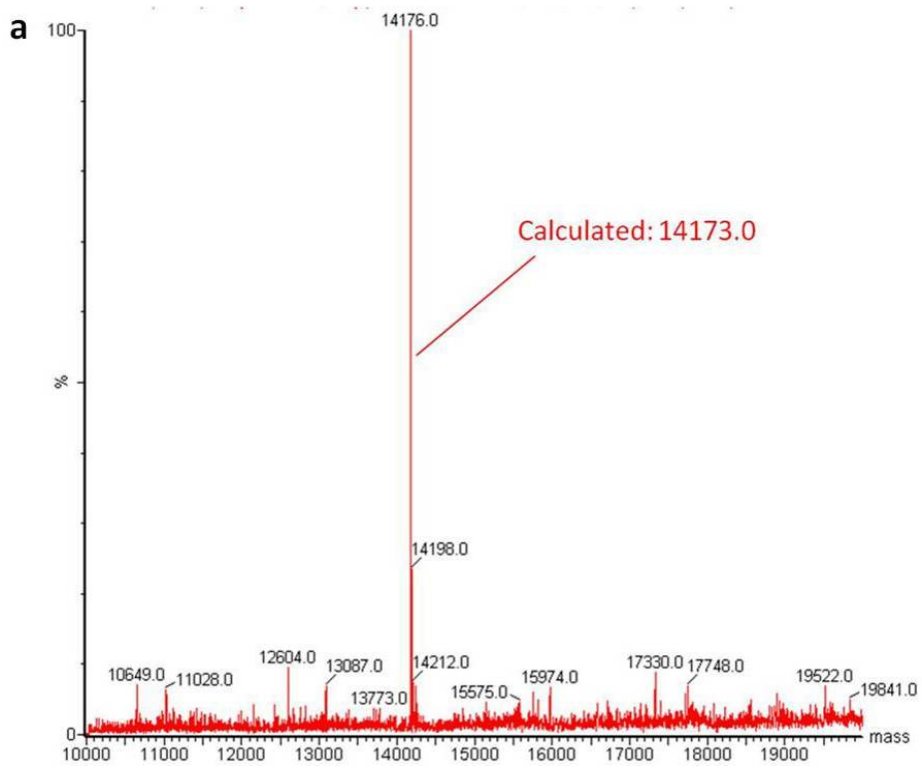
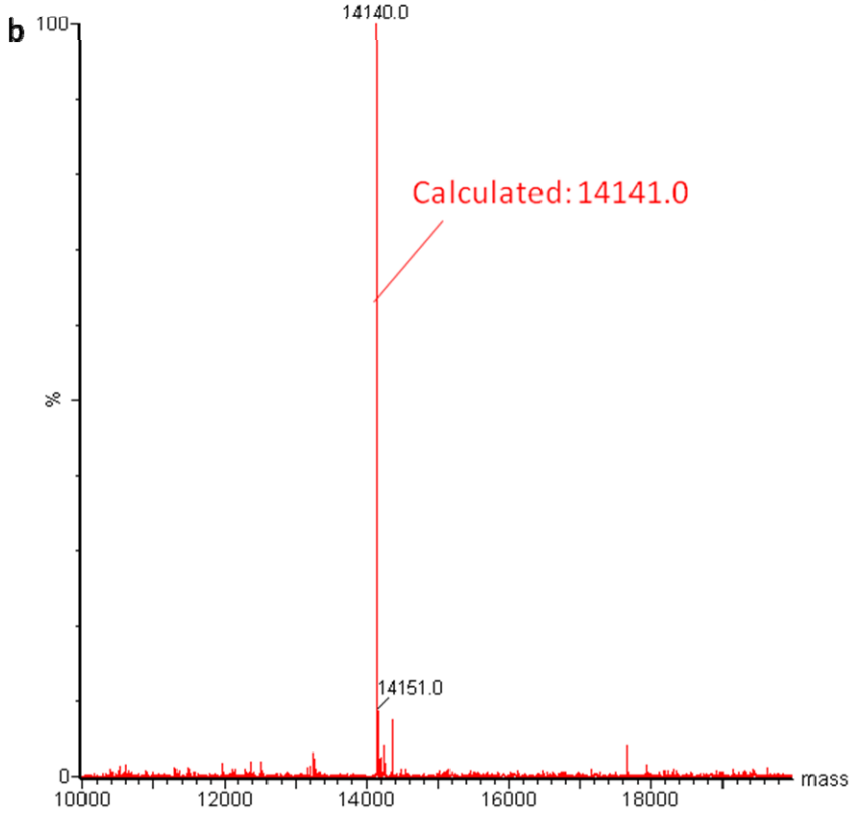


Figure S2 Low temperature (at 30 K) X-band EPR spectra of Cu_A Az, Asn47Ser and Glu114Pro mutants in (a) 50 mM NH_4OAc (pH5.1) buffer and (b) temperature independent pH buffer at pH 7 (TIP7) buffer (pH7.0). At pH 5.1, while the UV-vis spectrum of the Glu114Pro mutant at room temperature is very similar to that of unmodified Cu_A Az under the same condition (see Figure S1), the sample showed obvious color change upon frozen in EPR sample preparation, which is likely due to changes in the effective pH when cooled to cryogenic temperatures. To eliminate this pH effect due to cryogenic temperatures, all EPR samples were re-prepared in aTIP7 buffer for comparison, and the main conclusion of the paper is based on the data collected at pH 7.0.


a
100
%
0
14176.0
Calculated: 14173.0
14198.0
10649.0
11028.0
12604.0
13087.0
13773.0
14212.0
15575.0
15974.0
17330.0
17748.0
19522.0
19841.0
10000
11000
12000
13000
14000
15000
16000
17000
18000
19000
mass


b
100
%
0
14140.0
Calculated: 14141.0
14151.0
10000
12000
14000
16000
18000
mass

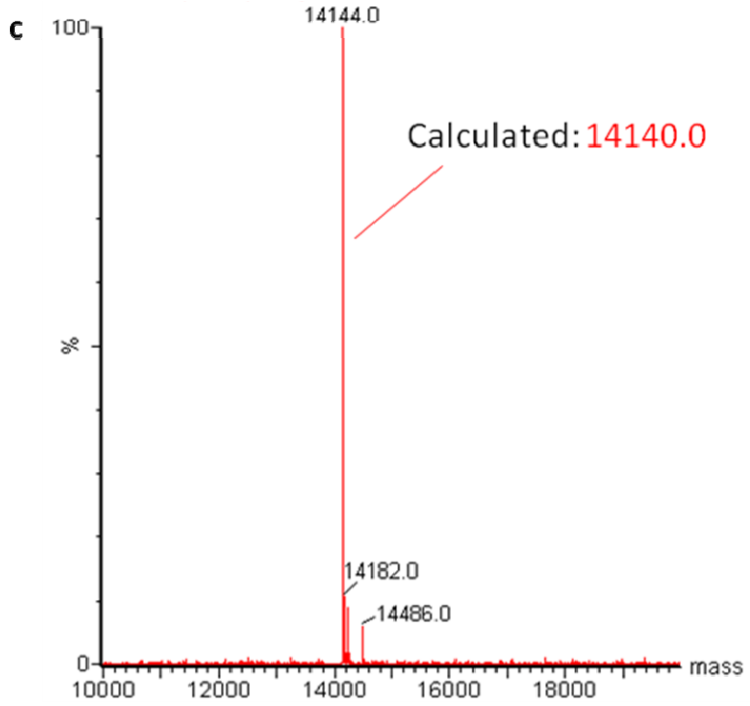


Figure S3 ESI-MS spectra of (a) Cu_A Az, (b) Glu114Pro and (c) Asn47Ser mutants.

Cyclic Voltammograms

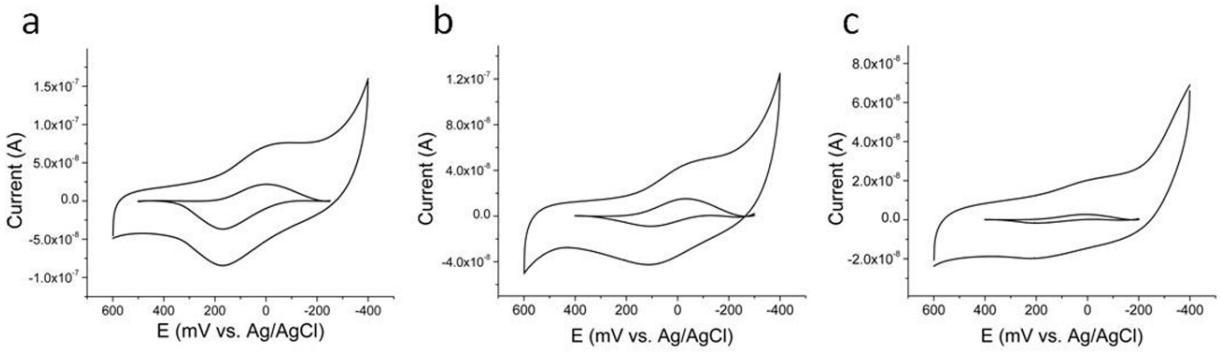


Figure S4 Representative cyclic voltammograms of (a) Cu_A Az, (b) Glu114Pro and (c) Asn47Ser in 100 mM KPi buffer (pH 7.0). The inset corresponds to the background subtracted voltammogram.

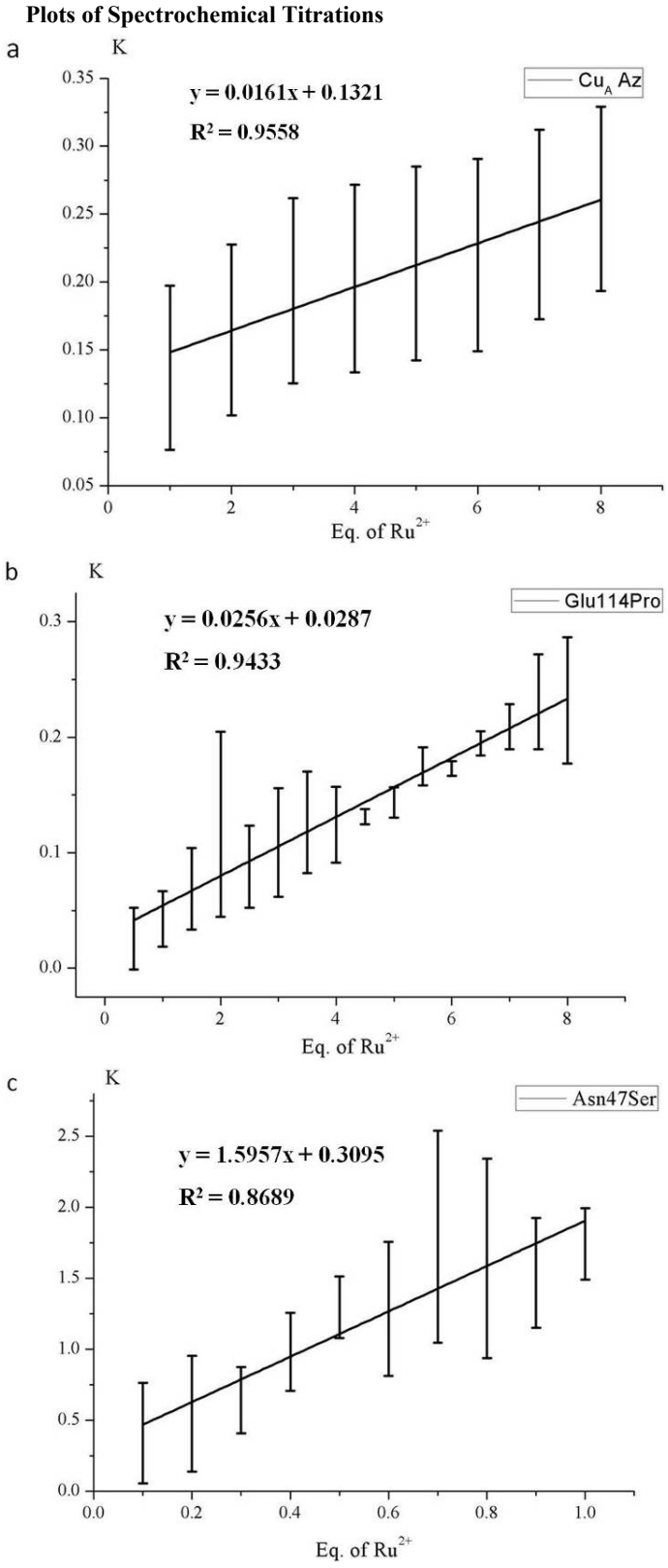
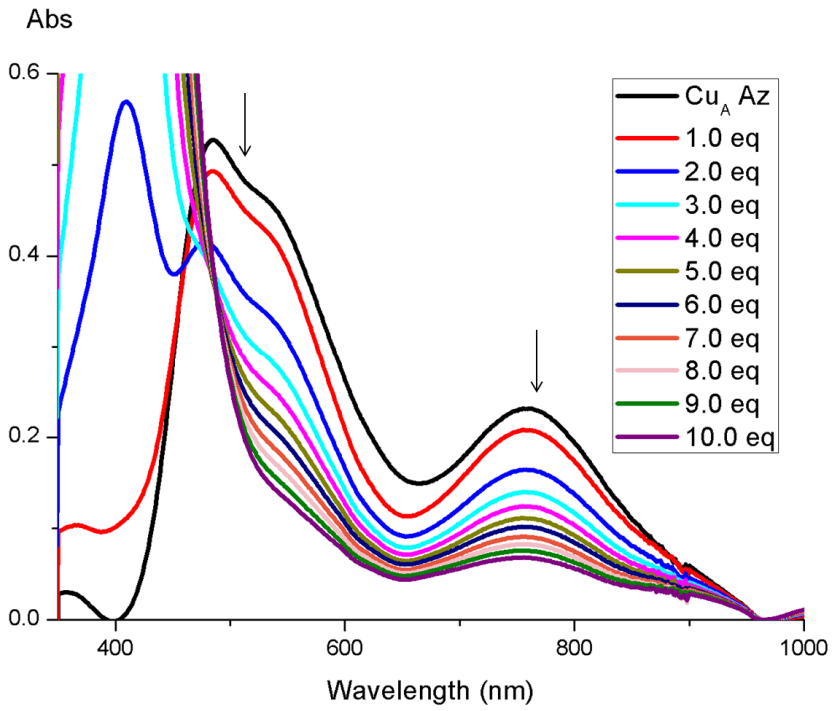


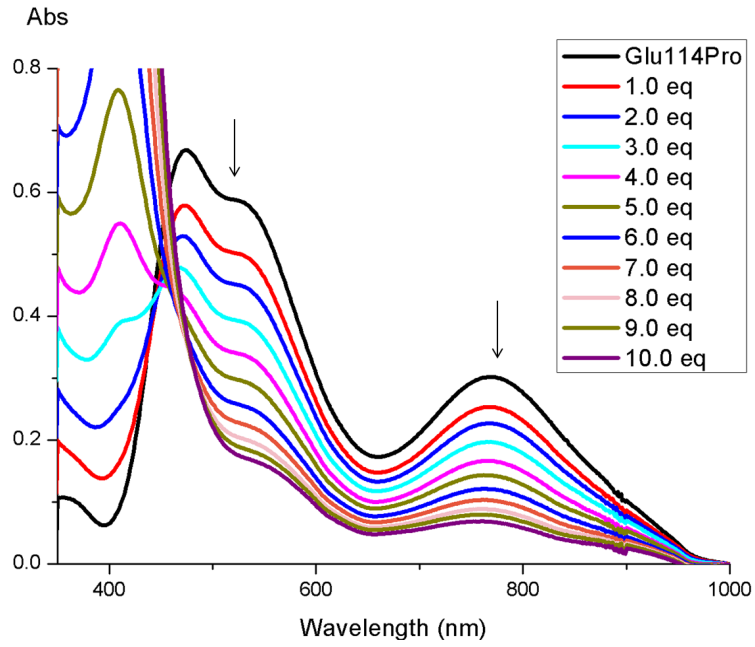
Figure S5. Plots of redox titration for (a) Cu_A Az, (b) Glu114Pro and (c) Asn47Ser.

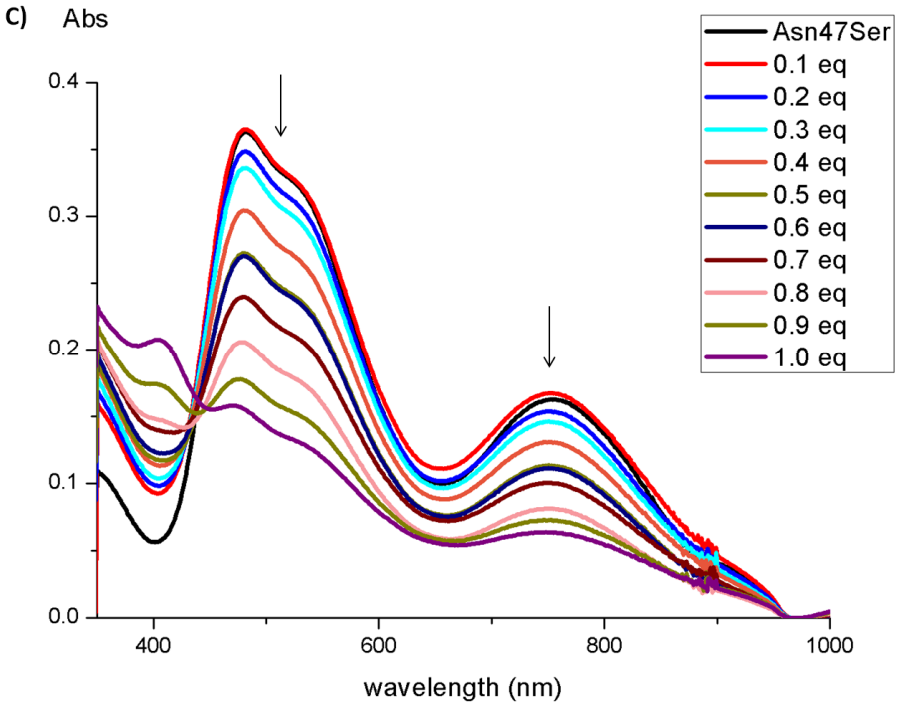
Spectrochemical Titration

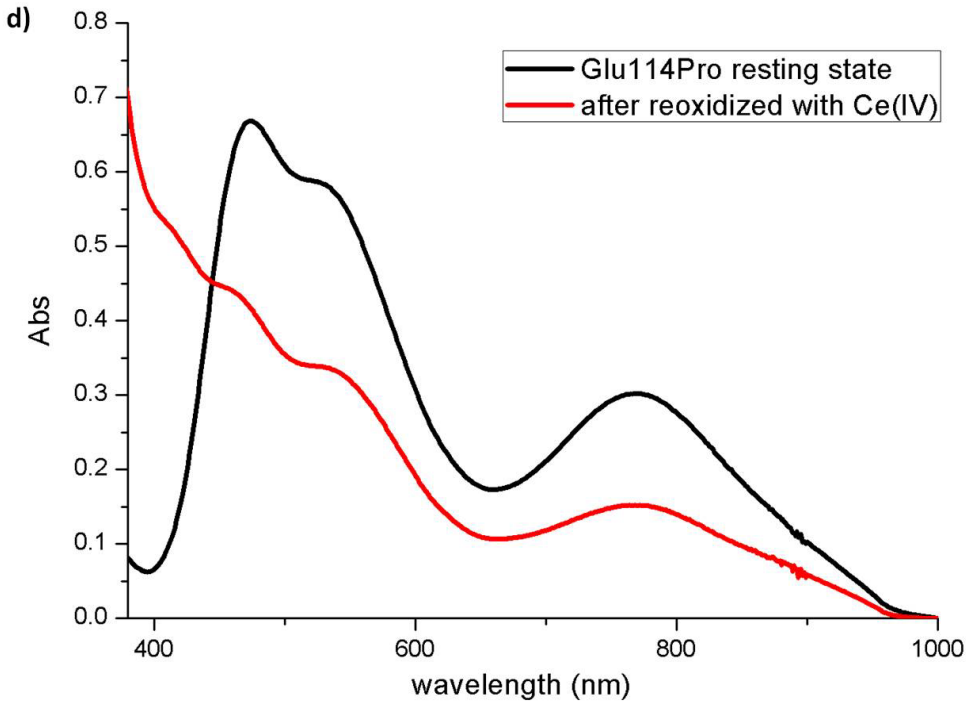
a)



b)







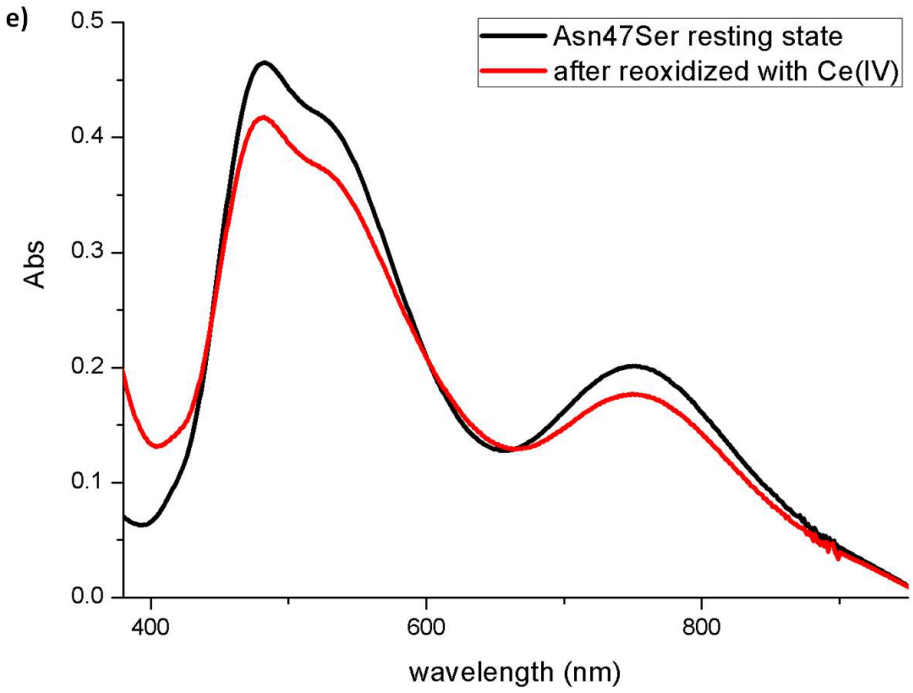


Figure S6 UV-vis spectra of redox titration for (a) Cu_A Az, (b) Glu114Pro and (c) Asn47Ser using $[Ru(NH_3)_5Py](ClO_4)_2$. Reduced (d) Glu114Pro and (e) Asn47Ser were re-oxidized back by titrating with $[(NH_4)_2Ce(NO_3)_6]$.

References

1 M. T. Hay, J. H. Richards and Y. Lu, Proc. Natl. Acad. Sci. USA, 1996, 93, 461-464.
2 M. T. Hay, M. C. Ang, D. R. Gamelin, E. I. Solomon, W. E. Antholine, M. Ralle, N. J. Blackburn, P. D. Massey, X. Wang, A. H. Kwon and Y. Lu, Inorg. Chem., 1998, 37, 191-198.
3 H. J. Hwang and Y. Lu, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 12842-12847.
4 H. J. Hwang, M. C. Ang and Y. Lu, *J. Biol. Inorg. Chem.*, 2004, **9**, 489-494.
5 L. J. C. Jeuken and F. A. Armstrong, *J. Phys. Chem. B*, 2001, **105**, 5271-5282.
6 O. Farver, Y. Lu, M. C. Ang and I. Pecht, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 899-902.
7 M. J. Nilges, K. Matteson and R. L. Belford, in *ESR Spectroscopy in Membrane Biophysics*, eds. M. Hemminga and L. Berliner, Springer, New York, 2007, vol. 27, pp. Appendix 2.