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Altered coordination in a blue copper protein upon association with redox partner revealed by carbon-deuterium vibrational probes

Claire C. Mammoser, Ryan E. Agh, Nicholas M. Garcia, Yiqi Wang, and Megan C. Thielges*

Department of Chemistry, Indiana University Bloomington, Bloomington, IN, 47405, United States

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Protein Expression and Purification

Plasmids pEAP and pEAF, constructed by Miguel A. de la Rosa (University of Seville) for the expression of *Nostoc* Pc (excluding cellular transit sequence) and cyt *f* (soluble domain, residues 1-254), respectively, were provided by Marcellus Ubbink (Leiden University).[1–3] The Pc gene was modified to add the non-native precursor sequence MAA- which has been excluded from numbering for clarity of comparison to literature.[4]

Unlabeled Pc was expressed in BL21(DE3) in lysogeny broth supplemented with 200 μM $CuSO_4$ and 50 μg/mL kanamycin. d_2Cys-labeled Pc was expressed in JM15(DE3), a cysteine auxotrophic strain, in defined media, by the same procedures. Defined media consisted of M9 salts (3 g/L KH_2PO_4, 6 g/L K_2HPO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 3 mg/L $CaCl_2$), 400 mg/L each amino acid excluding cysteine, 2 g/L glycerol, 200 μM $CuSO_4$, 50 μg/mL kanamycin, 1 mM $MgSO_4$, 1.5 μM thiamine, and 140 mg/L d_4-DL-cystine (Cambridge Isotopes, Tewskbury, MA). Expression was carried out at 1 L culture volume in 4 L baffled flasks. Cells were grown to OD_{600} = 0.6 at 37° C, 250 rpm, and expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were allowed to express under the same conditions for 4-5 hours, at which point they were harvested by centrifugation for 25 minutes at 7,550*g*.

Cells were frozen and resuspended in 2 mM potassium phosphate buffer, pH 7, then supplemented with 200 μM $CuSO_4$, 1 mM phenylmethylsulfonylfluoride (PMSF), 20 mM NaCl, 2 mM $MgCl_2$, and 2 U/mL benzonase. Cells were lysed by sonication (Q500, QSonica, Newtown, CT) using eight 30-second pulses at 60% amplitude with 2 minute rest periods between each pulse. Following sonication, cells were rocked gently on ice for 20 min, then centrifuged for 25 minutes at 31,000*g.* The supernatant was dialyzed into 2 mM potassium phosphate buffer, pH 7, with 200 μM $CuSO_4$ overnight, exchanging the buffer at least once. Over this period, the lysate turns from

colorless or pale yellow to dark blue. Another exchange is performed into the same buffer without $CuSO_4$ prior to purification. Dialyzed lysate was loaded to a 25 mL S-sepharose column (BioRad, Hercules, CA) equilibrated in the dialysis buffer. Protein was eluted using a gradient of 2-200 mM potassium phosphate, and blue fractions were checked for purity. Protein was pooled, concentrated using centrifugal filters, and loaded to a S100HR sephacryl column (GE Life Sciences, Niskayuna, NY). Fractions with A_{280}/A_{600} less than 1.5 were brought to 25% w/v glycerol, and stored at -80° C. Zn(II)-substituted Pc was generated as described previously.[4]

Cyt *f* was coexpressed with pEC86, containing a c type cytochrome maturation cassette, in DH5α. Cultures were 1.7 L of lysogeny broth with 100 µg/mL ampicillin, 20 µg/mL chloramphenicol, 30 µL/L Antifoam SE-15 (Sigma, St. Louis, MO), in 2 L unbaffled flasks. Cells were grown to OD_{600} = 0.6 at 30° C, 200 rpm, and expression was induced with 1 mM IPTG and 0.5 mM δ-aminolevulinic acid (GoldBio, St. Louis, MO). Expression proceeded at 28-30° C, 150 rpm for 40 hours, after which cells were harvested by centrifugation for 20 minutes at 7,550*g*.

Cell pellets were frozen and resuspended in 4x (w/v) 10 mM Tris-Cl, pH 8.2, with 1 mM PMSF. Cells were lysed by freezing in liquid nitrogen and thawing in room temperature water three times. Lysate was clarified by centrifugation at 31,000*g* for 30 min, brought to 20 mM NaCl and 2 mM $MgCl_2$, and treated with 2 U/mL benzonase for 10 min. Pellets were resuspended in 10 mM Tris-Cl, pH 8.2 and the freeze-thaw procedure and centrifugation were repeated. The resulting lysate was similarly treated with benzonase and lysate from both rounds was pooled. Protein was precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation and recovered by centrifugation at 31,000*g*, after which the pellets were redissolved in half of the original volume of 10 mM Tris-Cl, pH 8.2. Lysate was then dialyzed into the same buffer overnight, with a minimum of two buffer exchanges. Dialyzed lysate was applied to a 30 mL DEAE-cellulose column (Sigma, St. Louis, MO) and eluted under a gradient of 0-2 M NaCl. Fractions containing cyt *f* were pooled and dialyzed into 10 mM 2-ethanesulfonic acid (MES) buffer, pH 6. Sample was then applied to a 20 mL Q-sepharose column (Bio-Rad, Hercules, CA) and eluted with a gradient of 0-2 M NaCl. Fractions with A_{280}/A_{556} less than 15 were pooled and applied to a S100HR sephacryl column (GE Life Sciences, Niskayuna, NY). Following gel filtration, fractions with A_{280}/A_{556} less than 5 were pooled and stored at -80° C.

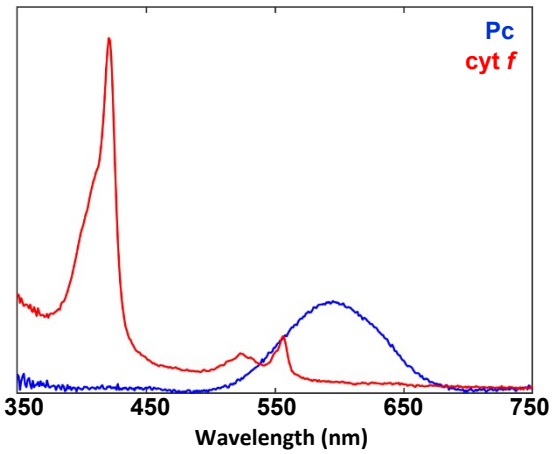

Fig. S1. Visible spectra of purified Cu(II) Pc and Fe(II) cyt *f*.

FTIR Spectroscopy

Pc was exchanged into 2 mM potassium phosphate buffer, pH 7. FTIR spectra were taken at concentrations of 3 mM for free Pc or 1.5 mM Pc with greater than 2 mM cyt *f* exchanged into the same buffer for the complex in order to ensure greater than 90% Pc bound to cyt *f* based on a K_D of 72.9 μM.[5] The redox states were prepared by addition of two equivalents of cobalt phenanthroline for the oxidized proteins or four equivalents of sodium ascorbate for the reduced proteins. Spectra of Cu(II) Pc were collected without inclusion of a chemical oxidant. Redox state of all species was verified by UV-Visible spectroscopy (Fig. S1).[1,3,6]

Samples were loaded between calcium fluoride windows separated by a 38.1 μm Teflon spacer. Spectra were collected at room temperature on an Agilent Cary 670 FTIR spectrometer with a liquid nitrogen-cooled MCT detector. Background and sample transmission spectra were averages of 8000 single-sided scans taken after a 25-minute purge of the sample chamber with dry nitrogen gas. Light incident on the detector was limited through bandpass filtering (BBP-4000-5000 nm, Spectrogon, Mountain Lakes, NJ, or FB 4500-500, Thorlabs, Newton, NJ). Collected interferograms were processed using a 4-term Blackman-Harris apodization function with a zero-filling factor of 8.

Absorption spectra were generated from the ratio of the sample transmission spectra to reference transmission spectra of unlabeled Pc at the same concentration. Myoglobin (equine skeletal muscle, Sigma, Burlington, MA) was added to the unlabeled Pc in place of cyt *f* to obtain reference spectra for the complex. Slow variation in the baseline was removed by fitting and subtracting a polynomial function, and resulting spectra were modeled by fitting a Gaussian band to each absorption. Spectra and Gaussian fits represent an average of three replicate samples.

Spectra of d_2Cys89 Zn Pc were collected alone, in complex with Fe(III) cyt *f*, and in complex with Fe(II) cyt *f*. Each set of spectra yielded absorbances at roughly the same frequencies (Fig. S2).

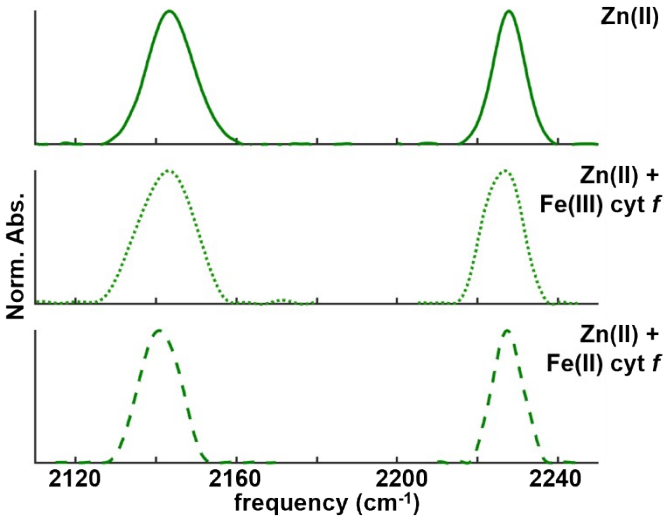

Fig. S2. Spectra of CD bonds of d_2Cys89 Zn Pc alone and in complex with oxidized and reduced cyt *f*

Redox titrations

Midpoint potentials for Pc and Pc-cyt *f* complex were determined by chemical redox titration. For Pc or cyt *f* alone, a solution of 20 mM HEPES, 50 mM sodium chloride, 5 μM Pc or cyt *f*, 1.5 mM potassium ferricyanide, 10 μM diphenylamine sulfonic acid, and 2.5 μM ruthenium hexamine, pH 6.8, was titrated with 50 mM sodium ascorbate. Each point was obtained by visible difference spectroscopy of the solution against a background of the initial (fully oxidized) solution, using the absorbance at 600 nm (oxidized Pc) to calculate the ratio of reduced and oxidized Pc. The potential of the solution was simultaneously measured using an Ag/AgCl electrode (MTC301, Hach, Loveland, CO). A linear fit to these data points was obtained through the Nernst equation, giving a midpoint potential and number of electrons transferred (Fig. S3).

Redox titration of the complex proceeded by the same general method, but due to the high concentrations of both proteins needed to form the complex, several modifications were necessary. Pc and cyt *f* were both concentrated in 20 mM HEPES, 50 mM sodium chloride, 30 mM potassium ferricyanide, 333 μM diphenylamine sulfonic acid, and 83 μM ruthenium hexamine, pH 6.8. Samples of the complex were prepared by mixing concentrated proteins so that the final concentrations were 950 μM Pc and 1.4 mM cyt *f* in 10 μL solution (estimated to give at least 88% of Pc in complex with cyt *f*) with varying concentrations of sodium ascorbate (Fig. S4).[5] Visible spectra were taken with a path length of 50 μm. Spectra were obtained of oxidized Pc, oxidized cyt *f*, and reduced cyt *f* individually. The spectra acquired during the titration were fit to a sum of these three component spectra. Potential of the cell at each point was determined by calculating the ratio of reduced to oxidized ferricyanide based on the added reductant since low solution volume prohibited use of an electrode.

Midpoint potentials determined by this method for each protein were 360 ± 3 mV (Pc), 330 ± 2 mV (cyt *f*), and 356 mV (Pc in complex) vs. NHE.

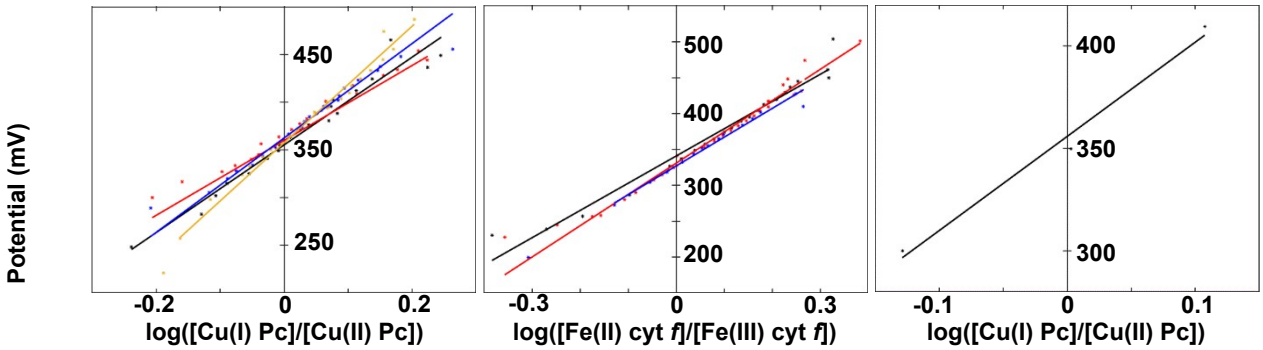

Fig. S3. Fits to redox titration data for Pc (left), cyt *f* (center) and Pc in complex with cyt *f* (right). Individual points are computed from spectra at each potential, solid lines of the same color are linear fits to the corresponding data points. Midpoint potentials are the y-intercept of these fits and reported values are an average of all replicates shown.

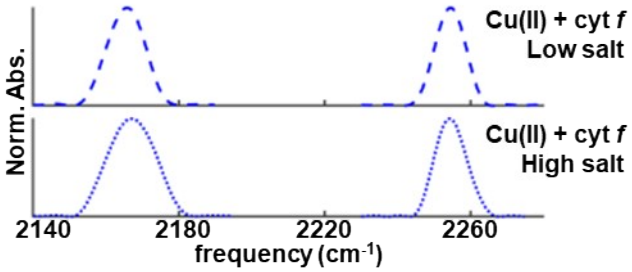

Fig. S4. Spectra of CD bonds of d_2Cys89 Pc/cyt *f* complex in low (2 mM potassium phosphate) and high (50 mM potassium phosphate, 100 mM potassium chloride) salt buffers. The similarity of these spectra indicates that under the higher salt conditions used in finding the midpoint potential of the complex Pc is still at least 88% bound to cyt *f*.

Spectral interpretation

Comparison of the C-D vibrations of d_2Cys89 for Cu(I) and Zn(II) Pc provides empirical support that stronger interaction of the Cys89 ligand with the positively charged metal ion results in higher frequencies. Application of MO theory provides an explanation for this effect. If two orbitals have compatible symmetries then the strength of their interaction will depend on overlap and energy difference. In this case the S p orbitals have the correct symmetry to interact with both the C-H/D σ and σ* orbitals, with the result being three orbitals with varying amounts of S p, C-H/D σ and C-H/D σ* character. If we assume that the C-H/D σ and σ* orbitals have similar spatial overlap with the S π space, then the main factor determining the extent to which these orbitals interact is the energy differences between them. Interaction with the point charge lowers the energy of the S p orbital, allowing for increased interaction with the C-H/D σ orbitals, and reducing the C-H/D σ* character (Figure S5).[4] Supporting this model, calculations of methyl ethyl sulfide interacting with a point charge at the B3LYP cc-pVDZ level of theory indicate that the HOMO of sulfide is almost entirely S p in character but takes on increasing C-H/D σ character as the magnitude of the point charge is increased (Figure S6).[4] Thus, the experimentally observed shift in C-D stretching frequency is well described as an effect stemming from perturbation of the S-Cu interaction.

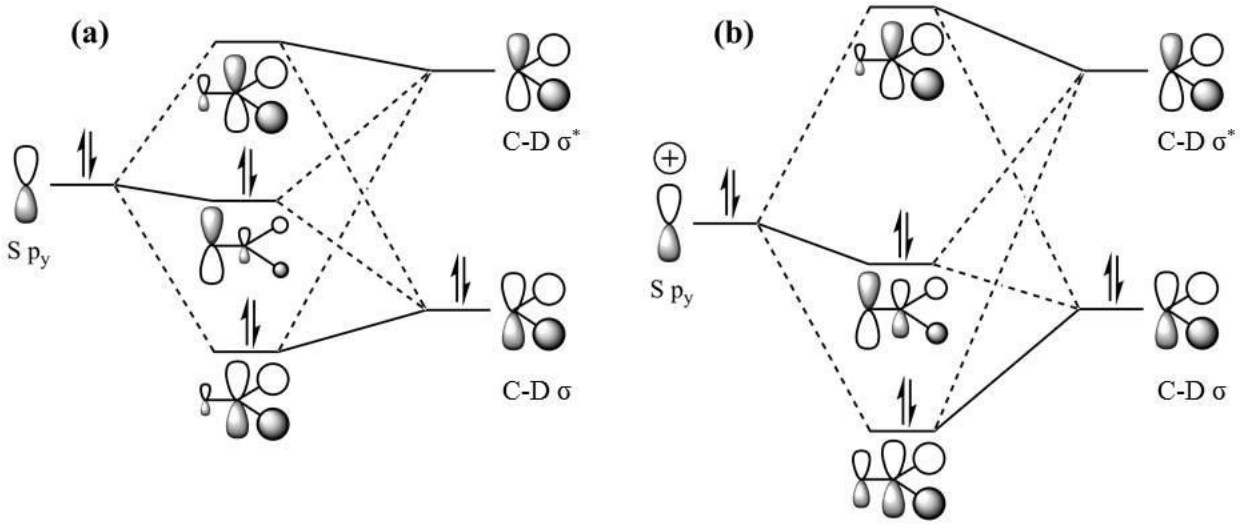

Fig. S5. Schematic molecular orbital diagram for interaction of C-D orbitals with adjacent sulfur orbitals in the (a) absence and (b) presence of a positively charged ion.

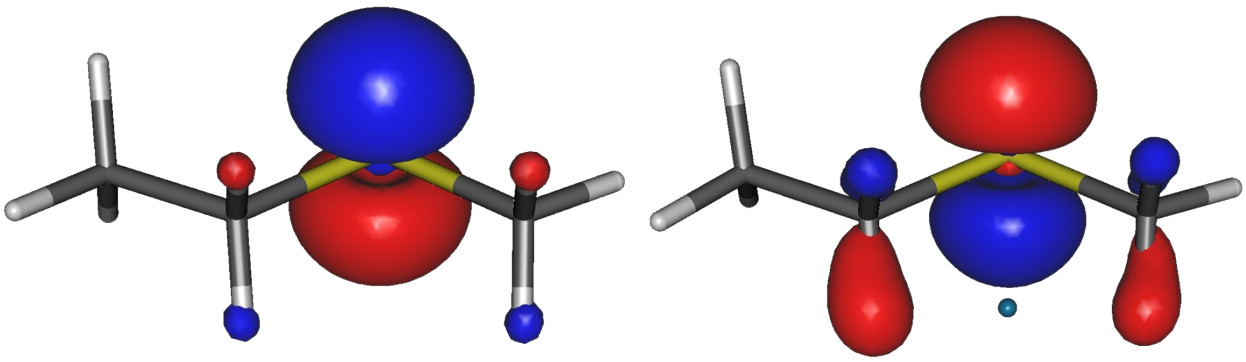

Fig. S6. Highest occupied molecular orbital (HOMO) of sulfur in the absence (left) and presence (right) of a point charge (1.00 a.u.) in methyl ethyl sulfide.

References

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