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COMMUNICATION

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

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Proteins tune the reactivity of metal sites; less understood is the impact of association with a redox partner. We demonstrate the utility of carbon-deuterium labels for selective analysis of delicate metal sites. Introduced into plastocyanin, they reveal substantial strengthening of the key Cu-Cys89 bond upon association with cytochrome *f*.

Blue copper proteins (BCPs) serve as an exemplar for illustrating how protein environments tune reactivity.[1–3] Extensive investigation has illuminated how the surrounding environment impacts redox centers in individual proteins.[4,5] An additional consideration for inter-protein electron transfer (ET) is that the reaction occurs after proteins associate into a complex where the redox center potentially experiences a different environment. Comparatively little is known about how complexation might alter a redox center and its properties toward electron transfer (ET).[6–11] Herein we assessed impact to the copper (Cu) site of the BCP plastocyanin (Pc) from the association with its redox partner cytochrome *f* (cyt *f*) by introducing carbon-deuterium (CD) bonds to serve as a vibrational reporter at the key cysteine ligand Cys89 (d_2Cys89) (Fig. 1). Massive increase in the CD frequencies upon association of Pc with cyt *f* indicates substantial strengthening of the interaction between the cysteine ligand and the Cu ion. The changes to the Cu site are predicted to promote rapid ET in the complex with the partner cyt *f*.

The Cu site of Pc and other BCPs has ET properties distinct from small molecular Cu complexes. The Cu ion is coordinated by ligands (His39, Cys89, His92, Met97 for *Nostoc* Pc) in a distorted tetrahedral geometry (Fig. 1a,b).[12,13] The distorted geometry arises from abnormally short Cu-Cys89 thiolate and long Cu-Met97 thioether bonds attributed to constraints imposed by the protein scaffold. The distortion creates an entatic state, in which the geometry of the Cu(I) protein is held near that preferred for Cu(II), lowering the reorganization energy.[14,15] The coordination environment of Cu sites in BCPs also leads to high midpoint potentials (~360 mV for Pc).[15–17] Additionally, the strong bond to Cys89 promotes a long-range ET pathway proceeding through Cys89 to a remote Tyr.[13,18–20] Any perturbation to the Cu coordination thus could modulate the reactivity of BCPs.

To directly probe the Cys89 ligand (d_2Cys89) of *Nostoc* Pc, we introduced d_2-C_β-cysteine (Supporting Information, Fig. 1c). The CD bonds absorb in a "transparent window" of the infrared (IR) spectrum free from intrinsic protein vibrations. Their use overcomes the massive spectral congestion of protein IR spectra and enables selective characterization of the vibrations because their absorptions can be individually detected and analyzed.[21,22] First demonstrated as probes of redox state in cytochrome *c*,[22] a number of CD-labeled amino acids have since

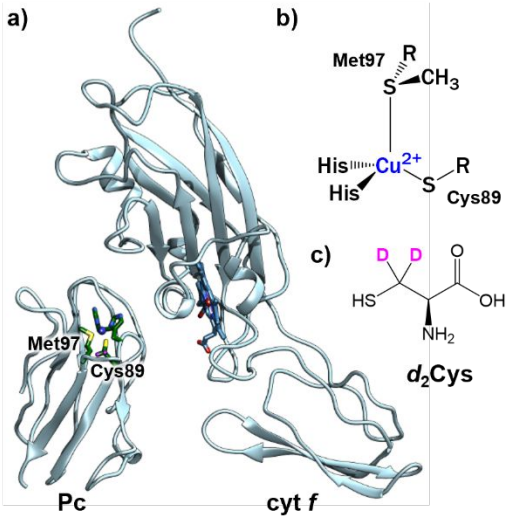

Fig. 1 a) Structural model of the complex of Pc with cyt *f* (PDB:1TU2). b) Structure of the Cu site of Pc. c) Structure of d_2Cys. CD bonds are highlighted in magenta.

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been applied to study protein molecular recognition, folding, and catalysis.[23–27] An important advantage for this study is that CD bonds are non-perturbative, enabling their incorporation at the delicate metal site. Additionally, unlike most electronic spectroscopy methods, IR spectroscopy can be applied to investigate the closed shell reduced state of Pc and thus analyze both the reactant and product of the redox reaction.

We previously introduced (methyl-d_3)-methionine at the axial Met97 ligand of Pc (d_3Met97) and found the frequency of the CD vibrations to be sensitive to interaction of the adjacent sulfur *p* orbital with the metal center.[28] Density functional calculations of a minimal model of the Cu site indicate that the stabilization of the sulfur p_y orbital from interaction with the positively charged metal center results in higher CD frequencies (Figs. S5 and S6). While the CD probes at Met97 report significant impact to the bonding due to changes in the metal center, they indicate only minor perturbation upon Pc-cyt *f* binding (Table 1). The low sensitivity to protein association could be due the weak interaction between Met97 and the Cu ion. We reasoned the compensatory strong interaction of the Cu ion with Cys89 might make CD vibrations at the Cys89 ligand a more sensitive probe of the Cu site.

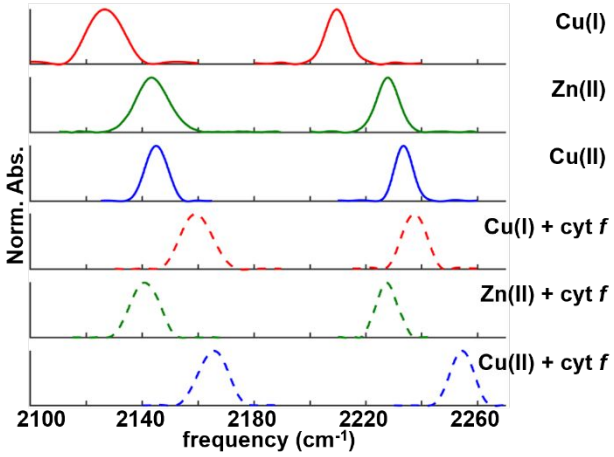

Fig. 2 FT IR absorption bands of CD bonds of d_2Cys89 Pc and the complex with cyt *f*.

FTIR spectra of d_2Cys89 Pc show absorptions at approximately 2125-2145 and 2210-2230 cm^{-1} corresponding to the symmetric and asymmetric stretching modes of the CD vibrations (Fig. 2). Upon oxidation of Cu(I) to Cu(II) Pc, the vibrational frequencies of the d_2Cys89 symmetric and asymmetric stretches respectively increase by ~ 18 and 24 cm^{-1}. The higher frequencies for d_2Cys89 in oxidized Cu(II) than reduced Cu(I) Pc reflect the stronger metal-ligand interaction.

To deconvolute the impact from the change in the charge of the metal ion and in electron configuration, we also characterized Zn(II)-substituted Pc. Zn(II) has the same nominal charge as Cu(II) but same electron configuration as Cu(I). The frequencies of the CD vibrations for Zn(II)-substituted Pc are ~17 cm^{-1} higher than found for the reduced Cu(I) protein, while they are 1.4/5.7 cm^{-1} lower than found for oxidized Cu(II) Pc (Fig. 2, Table 1). The increase in CD frequencies upon oxidation thus is primarily due to the approximate doubling of metal charge. The CD bonds at Cys89 are ~3-fold more sensitive to the metal charge than at Met97 (Table 1), which we attribute to the stronger interaction of Cys89 with the metal ion. In addition, changing the electron configuration oppositely affects the CD frequencies for Cys89 and Met97. Cu(II) Pc has an unoccupied $d_{x^2-y^2}$ Cu orbital that mixes via a π-bonding interaction with the p_z orbital of Cys89.[29,30] The CD vibrations indicate that this covalent interaction is associated with stronger bonding with Cys89 and weaker bonding with Met97, consistent with the known compensatory interaction of the ligands with the metal center.[31,32]

Table 1 Frequencies of CD bond vibrations in Pc[a]

	ν_s (d_2Cys) (cm^{-1})	ν_{as} (d_2Cys) (cm^{-1})	ν_s (d_3Met) (cm^{-1})[b]
Cu(I)	2126.8 ± 0.3	2209.6 ± 0.1	2123.3 ± 0.1
Zn(II)	2143.6 ± 0.2	2227.9 ± 0.2	2127.71 ± 0.04
Cu(II)	2145.0 ± 0.2	2233.56 ± 0.01	2125.7 ± 0.1
Cu(I)/Fe(II) cyt *f*	2159.4 ± 0.1	2237.3 ± 0.3	2123.6 ± 0.1
Zn(II)/Fe(II) cyt *f*	2141.0 ± 0.4	2227.5 ± 0.3	
Cu(II)/Fe(III) cyt *f*	2165.5 ± 0.6	2254.4 ± 0.1	2126.12 ± 0.03

[a]Values and standard deviations come from replicate measurements of three distinct samples, reported frequencies are the center (maximum) of a Gaussian fit to each absorption; [b]Data taken from ref[28]

After calibrating the response of the CD probes for d_2Cys89 Pc individually, we used them to analyze how binding cyt *f* might impact the metal site. FT IR spectra were acquired for d_2Cys89 Pc in the presence of the soluble domain of cyt *f* (Supporting Information). For the oxidized proteins, association into a complex leads to an increase in the CD frequencies by ~20 cm^{-1}. An even larger increase of ~30 cm^{-1} occurs upon formation of the complex of the reduced proteins. These huge increases in the CD frequency at Cys89 reflect a stronger Cu-Cys89 interaction upon association of Pc with cyt *f*. The impact is equal to or more than doubling the metal charge. In comparison, the CD frequencies at Met97 increase only slightly (0.3-0.4 cm^{-1}) upon protein association.[28]

Analysis of d_2Cys89 of Zn(II)-substituted Pc finds no effect upon addition of Fe(III) or Fe(II) cyt *f* (Fig. S2). We note that the metal substitution should not hamper binding of the redox partners because the affinity of the Zn(II)-substituted Pc for cyt *f* is greater than the native protein.[33] While the result is unexpected, the insensitivity of the CD probes of Zn(II)-substituted Pc to cyt *f* binding implies the perturbation to the Cu site observed for the native protein is not due to reduced permittivity of the local environment in the complex or sensitivity to charges on cyt *f*. This is consistent with the growing understanding of ET partner complexes as a loosely associated ensemble that lacks a tightly packed interface.[34–37] Nonetheless, the IR data show that protein association perturbs the Cu site. We hypothesize that subtle structural differences between the Zn(II)-substituted and native Pc may disrupt the cyt *f*-induced change found for the Cu site. Structures of native and Zn(II)-substituted BCP azurin show that the Zn(II) ion is displaced

relative to the Cu(II) ion.[38] Additionally, the Zn(II)-substituted and apoprotein of spinach Pc have increased absorbance at <260 nm attributed to structural perturbation.[33]

The current model of the Pc-cyt *f* complex is based on constraints provided by NMR paramagnetic relaxation of spin probes, experiments that are not able to resolve small-scale, sub-Å changes in the structure of the metal site and, moreover, are often performed with Zn(II)- or Cd(II)-substituted Pc.[36,39–44] Structural analysis with high resolution by extended X-ray absorption fine structure (EXAFS) spectroscopy detects small perturbation to both Cu(II) and Cu(I) sites of Pc due to complexation with cyt *f*.[9] The study finds no significant change in the Cu-Cys89 bonding but suggests lengthening of the bonds with the His ligands and shortening of that with Met97, resulting in more equal bond lengths among the ligands and a coordination geometry that is more tetragonal. In comparison to these findings, IR spectroscopy of the CD probes indicates that complexation only slightly affects the bonding by Met97, while bonding with the Cys89 ligand is substantially stronger. Together the CD probes at the two ligands imply the Cu ion coordination in the complex is more anisotropic. While EXAFS and IR spectroscopy appear inconsistent, we note that EXAFS spectroscopy probes structure, while IR spectroscopy probes bonding. Small changes in bond lengths of ≤ 0.1 Å may not be resolved but could substantially affect the electronic structure and thereby the vibrations of the CD probes.[13] Consistent with this possibility, shifts are observed in the K-edge spectra that indicate change to the electronic distribution of the Cu(II) site upon Pc-cyt *f* association.[9]

Just as the coordination geometry imposed by the protein scaffold tunes the ET properties of the Cu center in the individual protein, the altered bonding induced upon complexation likewise is predicted to have functional implications. For example, the increased anisotropy from stronger metal bonding to Cys89 compared to Met97 is expected to reduce the reorganization energy. Oxidation of the Cu site leads to an unoccupied orbital that engenders a Jahn-Teller force to adopt a distorted geometry that eliminates orbital degeneracy.[13] In BCPs, structural rearrangement is minimized by pre-distorting the reduced site through the elongated Met and shortened Cys bonds. The CD probes reveal that complexation of Pc with cyt *f* further lowers of the coordination symmetry, and to a greater extent for the reduced than oxidized protein. The net change reduces the difference in coordination between redox states and thereby is predicted to lower the reorganization energy. Future efforts to experimentally test these predictions could utilize ruthenium-based photoreductants to compare the ET kinetics to the Cu site of Pc individually and within the associated complex.[45,46]

Another property attributed to the distorted geometry of the Cu site in Pc is the high midpoint potential.[47–50] The long Met97 axial ligand is thought to destabilize the oxidized relative to the reduced state, so the stronger Cu-Cys bonding in the complex could likewise alter the midpoint potential. There are varying reports of the sensitivity of Pc midpoint potential to complexation with cyt *f*.[16,51] Our analysis of concentrated solutions of *Nostoc* Pc and cyt *f* however does not detect any change due to complexation (Fig. S3). We hypothesize that the perturbation to each redox state might be counteracting and thus lead to no net effect to the thermodynamics. The CD probes indicate that the impact from association of Pc with cyt *f* is ~50% greater for reduced than oxidized Pc; selective stabilization of the reduced state would predict higher midpoint potential. On the other hand, the stronger metal-ligand interaction would be more stabilizing for the more highly charged oxidized state and opposingly decrease the midpoint potential.

The altered metal-ligand bonding in the complex also could impact ET via promotion of specific superexchange pathways in Pc. A longer pathway that proceeds via the Cu-Cys89 bond to a remote Tyr residue competes with a shorter route to an adjacent site.[18–20] The ET rate via the route to the remote site is similar to that for the adjacent site because of the strong covalent Cu-Cys89 bonding that leads to a large electronic coupling.[13] The stronger Cu-Cys89 bond in the complex with cyt *f* would engender even stronger electronic coupling that would make the ET pathway to the remote site even more greatly favored.

Conclusions

In summary, use of CD bonds as vibrational probes introduced at the key Cys89 ligand of the Cu site of Pc has directly uncovered large changes in bonding due to association with the redox partner cyt *f*. Serving as selective reporters at specific residues in both redox states of Pc, the CD probes reveal that association of Pc with cyt *f* impacts the Cys89 much more so than Met97 and the reduced more than the oxidized state. The changes are predicted to facilitate rapid reduction of Pc by cyt *f* by reducing reorganization energy and to modulate the efficiency of ET pathways between them. Importantly, CD vibrational spectroscopy uncovers the potentially large impact of protein-protein association to redox centers and suggests the possibility similar mechanisms could be in play for other ET partners. Such binding-induced changes in redox centers that promote rapid inter-protein ET kinetics could serve to fine-tune the specificity of ET in a physiological context. Furthermore, this work demonstrates the sensitivity of C-D vibrations at cysteine for probing metal sites in any redox state and their potential utility for analysis of other metalloprotein active sites.

Author Contributions

CCM: investigation, validation, visualization, writing – original draft, review, & editing; REA: investigation; NMG: investigation; YW: investigation; MCT: conceptualization, methodology, project administration, funding acquisition, writing – original draft, review, & editing, supervision.

Conflicts of interest

There are no conflicts to declare.

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