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Protein Dynamics and Cytochrome *c*: Correlations between Ligand Vibrations and Redox Activity

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The cytochromes are an important class of redox active proteins, in which the polypeptide chain plays a key role in establishing the properties of the heme cofactor.[1] For example, cytochrome *c* (cyt *c*) selectively stabilizes the neutral, reduced Fe^{2+} species relative to the cationic, oxidized Fe^{3+} species, thus increasing the heme's redox potential. Cyt *c* is thought to stabilize the Fe^{2+} state of the cofactor by coordinating the iron with the "soft" methionine sulfur atom from Met80, which favors the "soft" Fe^{2+} state, and also by providing a hydrophobic environment that destabilizes the charged Fe^{3+} state. Characterization of the structural and dynamic properties of the Met80 ligand in oxidized and reduced cyt *c*, as well as in mutants with altered redox potentials would provide a unique opportunity to examine the relationship between protein vibrations and redox activity. Here, we apply a recently developed technique to observe the molecular vibrations of cyt *c*.[2]

The spectral complexity inherent to proteins prevents direct observation of the constituent vibrations using conventional techniques. To circumvent this problem, we incorporate deuterium atoms at nonexchangeable positions of cyt *c*.[2] Deuteration is nonperturbative, but shifts the bond stretching frequency to a region of the infrared spectrum ($\sim$2100 cm^{-1}) that is unobscured by other protein vibrations. The C−D stretching vibration is predominantly local-mode in character due to the large (adiabatic) separation of the C−D stretching frequency from the frequencies of other molecular vibrations.[3] Therefore, the motion corresponds largely to the bond vibration. This significantly simplifies interpretation of the vibration's frequency and line width, where the frequency is sensitive to the environmental polarity and the strength of the bond, and the line width is sensitive to local motions of the protein or cofactor on the vibrational time scale.

We recently reported that deuteration of the methyl group of Met80 in horse heart cyt *c* results in easily observable C−D absorption bands (Figure 1).[2] Characterization of this C−D stretching frequency in the oxidized, reduced, and CN^- or NO-bound forms indicated that the strength of the C−D bond is primarily sensitive to the electron density centered at the sulfur atom. Such sensitivity may arise from hyperconjugation of the C−D σ-orbitals with sulfur-centered orbitals. Increased orbital overlap of the Fe^{2+}−S bond (soft metal−soft ligand) relative to the Fe^{3+}−S bond (hard metal−soft ligand) results in increased σ-donation from the sulfur atom to the iron center in the reduced state relative to the oxidized state.[4] Thus, the sulfur atom in the reduced protein has less electron density and withdraws more density from the C−D σ-orbitals, which reduces the stretching frequency of the C−D bond in the reduced protein relative to the oxidized protein. The line width of the C−D absorption was not sensitive to the oxidation state of the protein; however, an increase in both frequency and line width was observed upon cleavage of the Fe−S bond with either CN^- or NO. Therefore, both the C−D bond strength and line width are sensitive to the Met80 ligation environment.

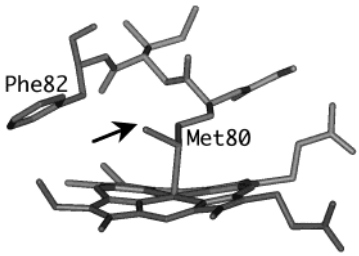

Figure 1. Heme, Met80, and Phe82 of cyt *c*.[6] The arrow indicates the site of deuteration.

Detailed structural and functional studies have examined the role of Phe82 in the redox activity of cyt *c*.[5] The aromatic ring of the Phe82 side-chain packs on the heme and the methylene is in van der Waals contact with the methyl group of Met80 (Figure 1).[5a,6] Crystal structures of the F82G, F82S, and F82Y yeast iso-1- cyt *c* mutants have shown that residue 82 tolerates a wide variety of substitutions with only local structural readjustments[5] and in vivo studies have demonstrated that mutants with any of the twenty amino acids at this position will fold and be sufficiently active to support yeast growth.[7] Phe82 mutants have also been characterized in vitro.[7] Mutation has been found to have a large effect on the protein's electron-transfer properties, which has been discussed in terms of binding site hydrophobicity, solvent or substrate accessibility, and protein dynamics. However, it has been difficult to disentangle the relative importance of each factor. Herein, we report the characterization of the stretching frequency and line width of the Met80 methyl group C−D bonds of the wild-type, and six mutant proteins where Phe82 has been mutated to His, Ser, Leu, Val, Ala, and Gly. Redox potentials were also determined for each mutant (see Supporting Information). The data are shown in Figure 2 and in Supporting Information.

As with the wild-type protein, the C−D bond is stronger in the oxidized state of each mutant than in the reduced state, as indicated by the higher C−D stretching frequency for each oxidized protein (Figure 2A). The strength of the C−D bond in the reduced protein is weakly dependent on the reduction potential; however, a stronger dependence is observed in the oxidized protein. The correlation between C−D frequency and redox potential is lost upon cleavage of the Met80 S−Fe bond with the cyanide ligand. The F82H cyt *c* mutant is unique in that upon oxidation, the imidazole of His82 intramolecularly displaces the Met80 ligand.[8] For the reduced protein, with the native Met80-Fe bond, the C−D absorption frequency is consistent with the trend observed with the other mutant proteins. However, for the F82H oxidized protein, the frequency is indistinguishable from those of the cyanide-bound

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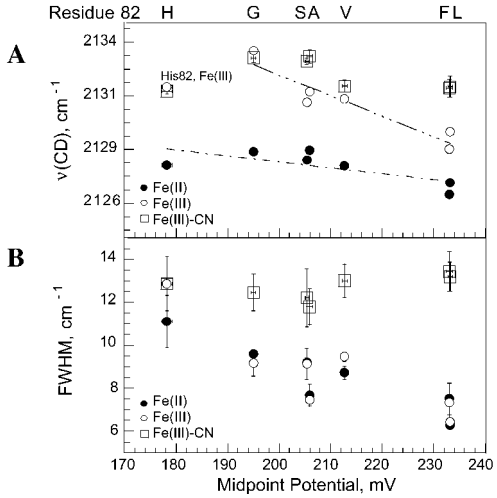

Figure 2. Redox dependence of the (A) C−D stretching frequency and (B) C−D line width (Full Width Half Maximum) of (d_3-methyl)Met80 in wt and Phe82 mutants of cyt *c*.[12] Linear correlations are shown for ν(CD) of the reduced and oxidized Fe forms, but the His82 Fe^{3+} data is excluded from the oxidized fit (see text.) The identity of residue 82 is indicated at the top of the figure.

proteins. Thus, the correlation is dependent on dynamic or structural properties that are unique to the Met80 ligated state.

Mutation-dependent effects that may contribute to the observed shift in C−D bond frequency include both through-space polarity changes and through-bond effects. The mutants have altered redox potentials, which implies a variable environmental polarity. The C−D stretching frequency of (d_3-methyl) methionine is known to be sensitive to polarity effects, shifting to higher frequency in more polar solvent.[2] Therefore, the observed shifts of the protein vibrations to higher frequency are consistent with a mutation-induced increase in polarity, but the dependence of the shift on the protein's oxidation state is not consistent with solely polarity changes contributing to the observed frequency shift. To fully account for the observed shifts, differences between the oxidized and reduced proteins must be considered. Through-bond interactions between the S−Fe^{3+} or S−Fe^{2+} centers and the C−D σ-bonds may be important. For example, the S−Fe^{3+} bond may be more polarizable, due to its less covalent character, and may therefore be more susceptible to polarity changes. A more polarizable bond would respond more strongly to changes in the polarity, leading to an increased charge centered at sulfur, and decreasing the electron delocalization from the CD_3 bonds in the oxidized protein, relative to the reduced protein. A similar model, based on better overlap between the more diffuse Fe^{2+} orbitals, relative to Fe^{3+}, with porphyrin π* orbitals, has been used to explain the observed decrease of heme Raman frequencies upon oxidation.[9]

A strong correlation is observed between C−D line width and redox potential of the mutant proteins (Figure 2B). Again, the correlation is lost upon cleavage of the Fe-Met80 sulfur bond. The methyl group vibrations are apparently sensitive to a mutation-dependent broadening mechanism when Met80 is ligated to the metal center. Such broadening mechanisms may include both intra- and intermolecular components. Intramolecular contributions may result from anharmonic couplings as well as environmental heterogeneity. The heterogeneity contributes to line broadening if the absorption frequency is sensitive to, and experiences, multiple environments. In cyt *c*, the heme is expected to produce a highly anisotropic electrostatic environment that strongly influences the absorption frequency of the proximal Met80 methyl group. Heterogeneity in the Met80 environment, resulting from thermal motion of the ligand relative to the anisotropic electrostatic field at the heme, may be the origin of the observed line broadening. Thus, mutation at Phe82, which is known to result in large changes in the protein's redox properties, is correlated with increased protein fluctionality, at least in proximity to Met80. Despite the demonstrated environmental sensitivity of the protein vibration, each mutant protein has an identical line width in the oxidized and reduced states (Figure 2B).

The oxidation-state independence of the observed line widths in each protein is of particular interest because redox-dependent changes in protein flexibility have been widely debated in the cyt *c* literature and are often invoked to explain the distinct behaviors of the protein in the two oxidation states.[5a,6] The line width data described above rule out any redox-dependent changes in the motions of the Met80 methyl group. Because Met80 vibrations are sensitive to the environment, and Met80 is both bound to the heme and critical for establishing the structural and electronic properties of the cofactor, it seems likely that the Met80 ligand is sensitive to at least local redox-linked environmental changes. This implies that the protein's flexibility in the Met80 region of the polypeptide is not strongly dependent on the redox state of the cofactor and that the redox-dependent changes, at least at Met80, are apparently thermodynamic and restricted to polarity and through-bond interactions, as described above. The direct demonstration that the protein's flexibility is the same in the oxidized and reduced proteins contrasts with previous, less direct crystallographic or NMR studies, which are unable to directly address the protein's vibrational motions.

The picture that emerges is that mutation of the protein at position 82 causes a redox state-independent increase in the protein's fluctionality. The less rigid protein may allow increased water (or substrate) access to the buried heme[1,10] or an increase in the ability of the protein to reorient and accommodate the increased charge of the oxidized cofactor.[11] Thus, cyt *c* may have evolved to maximize structural ridgidity in order to provide the hydrophobic environment required to tune the Fe^{2+}−Fe^{3+} redox couple appropriately for biological function.

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Supporting Information Available: Tabulated data and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) The data result from multiple protein preparations and error bars are included for all data points, though in some cases are smaller than the symbols. All FT-IR samples were ~10 mM cyt c in 100 mM sodium acetate, pH 5, with the addition of 100 mM ascorbic acid or 200 mM $K^{13}C^{15}N$ (Cambridge Isotopes Laboratories) to generate the Fe^{2+} or Fe^{3+}−CN forms, respectively.

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