In summary, we have found that DA reactions involving neutral apolar reactants and a remarkably solvent-adaptable activated complex exhibit anomalous solvent effects in highly aqueous solvents. The "hydrophobic acceleration" is due to marked destabilization of the reactants as well as to hydrogen-bonding stabilization of the polarizable activated complex.

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Redox-Dependent Molecular Recognition in Proteins: Site-Directed Mutagenesis Suggests That Cytochrome *c* Oxidation State Governs Binding and Recognition to Cytochrome *c* Peroxidase

R. Hake,[†] G. McLendon,*[,†,‡] A. Corin,*[,‡,§] and D. Holzschu[§]

Department of Chemistry and NSF Center for Photoinduced Charge Transfer, University of Rochester Rochester, New York 14627-0216
Eastman Kodak Company Corporate Research Laboratories Rochester, New York 14650

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Understanding the way in which redox proteins interact is central to understanding their function.[1] Molecular recognition is crucial to insuring the physiological specificity of redox proteins. In addition, studies of redox protein interactions may lead to some understanding of the coupling of conformation and redox state. Such coupling plays a central role in biology energy transduction. To these ends, we and others have pursued detailed studies of recognition, binding, and reactions between redox proteins (for recent reviews, see refs 1-5). The cytochrome *c* (cyt *c*):cytochrome *c* peroxidase (Ccp) system is particularly attractive, since both proteins are readily isolated in pure form, high-resolution structures are known for both proteins,[6,7] and detailed static and dynamic models of the interaction have been proposed.[8-10] Finally, both proteins have been cloned,[11,12] opening the possibility for site-directed mutagenesis as a tool to probe specific interactions.

A variety of approaches have been used previously to probe the interaction of cyt *c* and Ccp including UV spectroscopy, magnetic resonance, and fluorescence energy transfer.[13-17] Such methods have demonstrated, for example, the dependence of binding on ionic strength. Recent thermodynamic and kinetic data have shown that Ccp binding by yeast cyt *c* is far less dependent on ionic strength than for the homologous horse cyt *c*.[17c] Thus, qualtitative and quantitative comparisons of binding and recognition of these proteins require comparison over a range of ionic strengths. In the present work, an approach based on affinity chromatography[17b,19] is used to map the binding of cyt *c* to several site-specific Ccp mutants, over a range of solution conditions. In essence, binding yeast iso-1 cyt *c* to thiosepharose (via cyt *c* Cys 107)[19b,c] creates an affinity column to which Ccp (and single-site Ccp mutants) are strongly bound. Ccp is then eluted with a salt gradient. Those derivatives that bind more strongly elute at a higher salt concentration.

In this way, the relative binding of several site-specific mutants of Ccp has been assessed. The mutations chosen for study (D37K, D217K, D79K) include the acidic residues which are thought to provide the central electrostatic interactions in the models of cyt *c*:Ccp binding.[17-19] An example of the (relative) binding patterns for Ccp and these mutants is shown in Figure 1. The implication is that D37 is central to the binding of cyt *c* to Ccp, while D217 and D79 are less strongly involved. Surprisingly, the substitution D79K actually improves binding to Fe(III) cyt *c*. These observations accord well with independent binding measurements made by fluorescence energy transfer at a single ionic strength.[17] Note that the binding constants are defined by the vertical line(s) at a given salt concentration. Thus (for a given oxidation state), the apparent differences in binding at an ionic strength of 10 mM, a condition commonly used for binding studies, are quite different from those at, e.g., 50 mM. Absorbance measurements suggest that the concentration of cyt *c* on the column is $\approx 10^{-4}$ M. Thus, when the fraction of Ccp eluted is equal to 0.5, then $K_{binding} \approx 10^4$ M^{-1}.[24]

The primary focus of this study is the comparison of Fe(III) cyt *c* and Fe(II) cyt *c*. Note that the reactant, Fe(II) cyt *c*, remains bound at higher ionic strengths than does the product, Fe(III) cyt *c*. Such differences arise from known redox-state and species-dependent differences in (competitive) anion binding.[22,23] While such differences are known, they had not been measured up to physiological [Cl^-] concentrations, where these differences can become particularly dramatic. Such differences can be quantitated, using the approach to quantitative affinity chromatography outlined by Chaiken.[24] At 0.2 M KCl (pH 6.2), Ccp elutes from the 1-mL Fe(III) cyt *c* affinity column in a 5-mL elution volume, whereas 200 mL is the volume required to elute Ccp from an identical Fe(II) cyt *c* column. This difference in elution volume corresponds to a 50-fold difference in binding affinity between Fe(II) cyt *c*/Ccp and Fe(III) cyt *c*/CCp, under these conditions.[24] These differences were confirmed under the present experimental conditions, by a gel permeation chromatography experiment which shows that while Fe(II) cyt *c* remains bound to Ccp throughout elution with 0.2 M NaCl (pH 6), Fe(III) cyt *c* is not bound, under the same conditions, and so migrates

* To whom correspondence should be addressed.
† University of Rochester.
‡ NSF Center for Photoinduced Charge Transfer.
§ Eastman Kodak Company.

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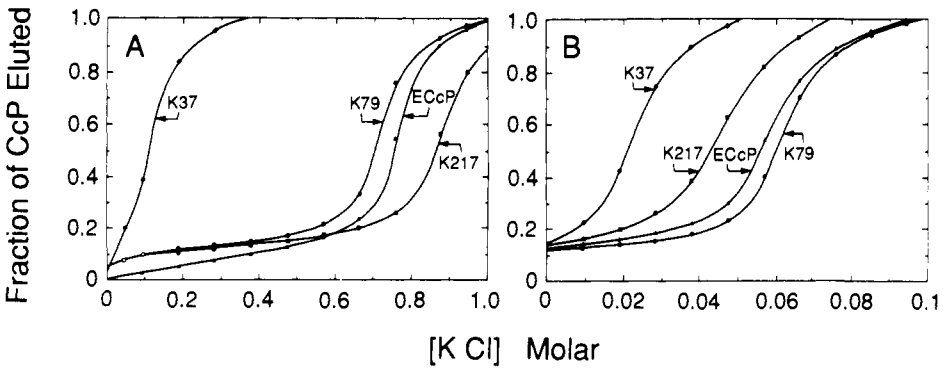

Figure 1. Panels A and B show binding curves to the cyt *c* affinity column as a function of potassium chloride concentration. Wild-type Ccp, obtained from yeast, is similar to the native recombinant Ccp (ECcp). The reduced column material was produced as previously described,[19a,b] the oxidized material was prepared according to the method of Azzi et al.,[20] and the oxidation state of the column material was determined spectroscopically.[19b] Several control experiments indicate that the derivatized cyt *c* binds Ccp and has binding and redox behavior identical to that of the cys 107 free cyt *c*.[19b,21] Elutions were conducted using the methods described in ref 19b. We obtained data in panel A using reduced cyt *c* column material and 25 mM sodium acetate pH 6.0 buffer. We obtained the data in panel B using oxidized cyt *c* column material and 5 mM sodium acetate pH 6.0 buffer. Note the difference in the horizontal axis [KCl] scale for the oxidized and reduced affinity materials. Experiments at pH 7 using TrisHCl equilibration buffer with either a TrisHCl or a KCl gradient for elution gave similar results.

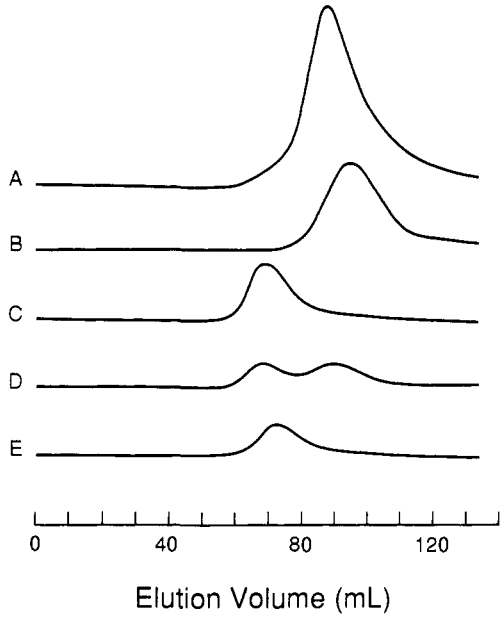

Figure 2. Gel permeation chromatograms of cyt *c*:Ccp elution profiles [using a Pharmacia FPLC apparatus with a 2.5 × 32.5 cm precision bore column, containing Sephadex G50 material, sodium acetate pH 6.0, 0.2 M ionic strength (KCl)]: (A) Reduced cyt *c* only, 78 mL; (B) oxidized cyt *c* only, 94 mL; (C) Ccp only, 69 mL; (D) Ccp plus oxidized cyt *c*, 70 and 87 mL, respectively; (E) Ccp plus reduced cyt *c*, 69.5 mL. The elution volumes listed correspond to the peak. Elution profiles were monitored at 405 nm. Note that, in chromatogram D, the peaks elute independently, showing *no* binding interaction, while in chromatogram E, only a single peak eluted, which contains both Ccp and cyt *c*, bound in a complex (as confirmed spectroscopically).

independently of Ccp (Figure 2).

The second observation is more surprising. The pattern of Ccp mutational effects at positions 79 and 217 observed in binding Fe(III) cyt *c* is *reversed* for binding to Fe(II) cyt *c*. For binding to Fe(III) cyt *c*, the observed pattern is D217K < Ccp < D79K, while for Fe(II) cyt *c*, the observed pattern is D79K < Ccp < D217K. At physiological ionic strengths, this reversal corresponds to a large change in relative affinity.

The simplest explanation for such a large change is that Fe(II) cyt *c* binds to a location centered about D37 and D79 on Ccp, which is different from the binding site for Fe(III) cyt *c*, which is centered around D37 and D217.[10] This difference might be viewed as analogous to the known differences in the binding of small anions to Fe(III) cyt *c* vs Fe(II) cyt *c*.[16] Indeed, the chromatographic technique works, in part, by the competition of Ccp and smaller anions for cyt *c*.[23] The present results show redox-dependent differences in binding, even with a large protein like Ccp, and roughly localizes the binding site for each oxidation state of cyt *c*. If the reactant, Fe(II) cyt *c*, and product, Fe(III) cyt *c*, binding sites indeed differ, as the present results suggest, then during oxidation or reduction, cyt *c* must move along the Ccp surface from the (reactant) Fe(II) cyt *c* site to the (product) Fe(III) cyt *c* site.

In summary, affinity chromatography has been used to study the binding to cyt *c* of single-site mutants of Ccp. The order of binding constants for the Ccp variants is different for Fe(II) cyt *c* and Fe(III) cyt *c*. This suggests that Ccp can sense the oxidation state of cyt *c*, so that the different redox states of cyt *c* may bind to different subdomains of the Ccp binding domain.

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Registry No. Ccp, 9029-53-2; cyt *c*, 9007-43-6; aspartic acid, 56-84-8.

Tuning the Redox Potential of Cytochrome *c* through Synergistic Site Replacements

Sonja Komar-Panicucci,[†] John Bixler,[‡] Gerald Bakker,[§] Fred Sherman,[∥] and George McLendon*[,†]

Department of Chemistry, University of Rochester Rochester, New York 14627-0216

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The free energy release along the electron transport chain of proteins in the inner mitochondrial membrane is sufficient to generate a proton-motive force that is used to drive the synthesis of the biological energy source, ATP. The penultimate reductant in this chain, cytochrome *c*, is positioned at an appropriate energy level between cytochrome reductase and cytochrome oxidase such that the electron transfer for each successive reaction is exergonic. Accordingly, eukaryotic cytochromes *c* have a high and essentially invariant redox potential (±20 mV). This invariance *presumably* reflects a strong coupling between the driving force for the reaction

* Author to whom correspondence should be addressed.
† Department of Chemistry, University of Rochester.
‡ On sabbatical leave at the Department of Chemistry, University of Rochester, from SUNY Brockport, Brockport, NY 14420.
§ On sabbatical leave at the Department of Chemistry, University of Rochester, from Earlham College, Richmond, IN 47374.
∥ Departments of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester.

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