Cytochrome *c*/Cytochrome *c* Peroxidase Complex: Effect of Binding-Site Mutations on the Thermodynamics of Complex Formation†

James E. Erman,*,‡ Gordon C. Kresheck,‡ Lidia B. Vitello,‡ and Mark A. Miller§

Department of Chemistry, Northern Illinois University, DeKalb, Illinois 60115, and Department of Chemistry, University of California, San Diego, La Jolla, California 92093

Received October 21, 1996; Revised Manuscript Received January 27, 1997⊗

ABSTRACT: The cytochrome *c*/cytochrome *c* peroxidase system has been extensively investigated as a model for long-range electron transfer in biology. Two models for the structure of the one-to-one cytochrome *c*/cytochrome *c* peroxidase complex in solution exist: one is based upon computer docking of the two proteins and the second is based upon the structure of the complex in the crystalline state. Titration calorimetry is used to investigate the interaction of horse ferricytochrome *c* with baker's yeast cytochrome *c* peroxidase and with six cytochrome *c* peroxidase mutants. Five of the six peroxidase mutants eliminate a negative charge in the cytochrome *c* binding site by replacing a side-chain carboxylate with an amide. The sixth mutation replaces a surface alanine residue with phenylalanine. The binding affinity between cytochrome *c* and the cytochrome *c* peroxidase mutants varies from no significant change in comparison to the wild-type enzyme to a 4-fold decrease in the equilibrium association constant. The pattern of decreasing cytochrome *c* binding affinity for the cytochrome *c* peroxidase mutants is consistent with the cytochrome *c* binding domain defined by X-ray crystallography [Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748−1755]. For those mutants which have lower affinity for cytochrome *c*, the lower affinity is due to a decrease in the entropy change upon complex formation, consistent with the difference in hydration of carboxylate and amide groups.

Electron transfer between redox proteins is an important process in biological systems. Electron transfer occurs both within static protein complexes such as the four multisubunit, membrane-bound complexes which form the mitochondrial electron transport chain and via dynamic protein complexes such as those between cytochrome *c* and cytochrome *c* peroxidase (CcP).[1] In dynamic electron transfer complexes, the reaction partners form an association complex, electron transfer occurs within the complex, and the reaction partners dissociate. These dynamic complexes allow reducing equivalents to be transferred via diffusion of small redox-active proteins such as cytochrome *c*. Specificity in electron transfer is determined both by complex formation between reactants (protein−protein recognition) and by the rate of electron transfer within the protein complex.

The cytochrome *c*/cytochrome *c* peroxidase system has been extensively investigated as a model for electron transfer within dynamic protein complexes (McLendon, 1988). The cytochrome *c*/CcP system has a number of properties which make it a useful model for electron transfer. Both proteins are water soluble and easy to isolate (Brautigan et al., 1978; Vitello et al., 1990). Both proteins are small; horse cytochrome *c* has a molecular weight of 12 400 (Bushnell et al., 1990) and yeast CcP has a molecular weight of 34 200 (Poulos et al., 1980). Each protein consists of a single polypeptide chain and a single heme; the crystallographic structures of the two proteins are known (Bushnell et al., 1990; Poulos et al., 1980). Horse cytochrome *c* is a basic protein, pI = 10.2 (Marini et al., 1967), with its net charge dominated by 19 lysine residues. CcP is an acidic protein, pI = 5.25 (Yonetani, 1967), with 45 glutamate and aspartate residues. The binding affinity between cytochrome *c* and CcP decreases with increasing ionic strength consistent with the interaction between oppositely charged proteins (Vitello & Erman, 1987).

In the late 1970s, Margoliash and co-workers (Kang et al., 1978) were able to define the CcP-binding domain on the surface of horse cytochrome *c* by using derivatives of horse cytochrome *c* modified at individual lysine residues. Two types of derivatives were developed: trinitrophenyl derivatives which neutralized the positive charge on the lysine residue and carboxydinitrophenyl derivatives which introduced a negatively charged carboxylate at the lysine location. The CcP-catalyzed oxidation of the modified cytochromes *c* was slower than or equal to unmodified horse cytochrome *c* at pH 6.0, 0.05 M ionic strength. The inhibition was in the order of modification at residues 72 > 86 > 87 > 13 = 27 > 8, leading to the conclusion that the CcP-interaction domain of horse cytochrome *c* included lysine residues 72, 86, 87, 13, and 27 with lysine 8 being near the periphery. These residues surround the solvent-exposed edge of the heme group in cytochrome *c*.

With the elucidation of the crystal structure of CcP, Poulos and Kraut (1980) made the first attempt at defining the

† This work was supported in part by grants from the NSF, MCB 91-21414 (J.E.E. and L.B.V.), CHE 92-06158 (G.C.K. and J.E.E.), and MCB 94-29845 (M.A.M.), and from the NIH, R15 GM54328-01 (J.E.E. and L.B.V.).

* Author to whom correspondence should be addressed.

‡ Northern Illinois University.

§ University of California, San Diego.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.

[1] Abbreviations: CcP, cytochrome *c* peroxidase; CcP(E32Q), CcP mutant in which the glutamate (E) at position 32 in the wild-type enzyme has been replaced by glutamine (Q) (the same naming convention is used for all of the CcP mutants in this study); Ru27-hCc, modified horse cytochrome *c* in which a ruthenium complex is covalently attached to Lys-27.

S0006-2960(96)02632-3 CCC: $14.00

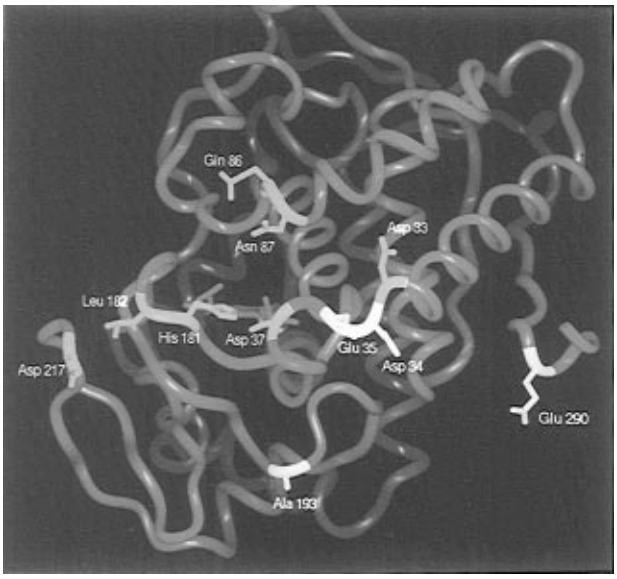

FIGURE 1: Surface of cytochrome *c* peroxidase showing two possible cytochrome *c* binding sites. The residues in yellow represent the cytochrome *c* binding site originally determined by visual docking of cytochrome *c* peroxidase and cytochrome *c* using computer graphic techniques (Poulos & Kraut, 1980; Poulos & Finzel, 1984). This binding site is referred to as the Poulos/Finzel/Kraut binding site in the text. The residues in white define the cytochrome *c* binding site, determined by X-ray analysis of two cytochrome *c*/CcP complexes (Pelletier & Kraut, 1992). The binding site in white is referred to as the Pelletier/Kraut binding site in the text.

structure of the cytochrome *c*/CcP complex. A hypothetical model of the complex was proposed on the basis of the visual docking of the two proteins with the aid of a computer graphics display system. The model was determined by optimizing interactions between carboxylate groups on the surface of CcP with the lysine residues surrounding the solvent-exposed heme edge on cytochrome *c* identified by Kang et al. (1978) as being within the CcP-binding domain on cytochrome *c*. The model paired aspartates 37, 79, and 217 of CcP with lysines 13, 27, and 72 on cytochrome *c*, respectively. Use of the refined crystal structures of CcP and tuna cytochrome *c* altered the model slightly (Poulos & Finzel, 1984). In the refined model, strong interactions were predicted between aspartates 33, 37, and 217 on CcP and lysines 8, 13, and 72 on cytochrome *c*, respectively.

Pelletier and Kraut (1992) reported crystal structures of a horse cytochrome *c*/CcP complex and a yeast iso-1 cytochrome *c*/CcP complex. The two complexes were crystallized under different salt concentrations, and there are small differences in the relative orientation of cytochrome *c* to CcP in the two complexes. In the crystal structure of the horse cytochrome *c*/CcP complex, Glu-35, Asn-38, and Glu-290 on CcP interact with lysines 87, 8, and 72 on cytochrome *c*, respectively. The CcP-binding domain on the surface of horse cytochrome *c* is nearly identical to that predicted by Kang et al. (1978). However, the cytochrome *c* binding domain on CcP is quite different from that predicted by Poulos and Kraut (1980) and Poulos and Finzel (1984). A comparison of the cytochrome *c* binding sites on CcP determined by the computer graphics docking model (Poulos/Finzel/Kraut site) and by X-ray crystallography (Pelletier/Kraut site) is shown in Figure 1. The two binding sites approach one another near the center of the figure, where five of the six residues between positions 32 and 37 are negatively charged. However, the two sites do not overlap, and it is possible to simultaneously bind a cytochrome *c* at each site without steric constraint.

The conditions used to determine the structure of the horse cytochrome *c*/CcP complex were intricate (Pelletier & Kraut, 1992). The mole ratio of CcP to horse cytochrome *c* in the crystals was consistently greater than one. Two CcP molecules and a maximum of one and a half horse cytochrome *c* molecules were observed in each asymmetric unit of the crystal. One CcP and one horse cytochrome *c* within the asymmetric unit form a well-ordered structure, and this cytochrome *c*/CcP pair is the basis for the one-to-one horse cytochrome *c*/CcP structure. The structure of the second CcP molecule within the asymmetric unit could be solved although the structure of the second cytochrome *c* could not. The best approximation for the interaction of the second cytochrome *c* and CcP is that cytochrome *c* is located near Leu-294 of CcP. In addition, there are CcP/CcP interactions within the crystal. Lysine residues on the back side of the first CcP molecule interact with the putative high-affinity cytochrome *c* binding site on the second CcP molecule, specifically Asp-34, Glu-35, and Glu-290. These interactions cause some concern about the impact of crystal forces in determining the structure of the CcP/horse cytochrome *c* complex and whether the horse cytochrome *c*/CcP complex in the crystal is the same as the complex in solution. In this paper we report on calorimetric studies of the interaction between horse cytochrome *c* and CcP mutants in solution designed to probe the high-affinity cytochrome *c* binding site on CcP. Our conclusion is that the structure of the one-to-one horse cytochrome *c*/CcP complex in solution is consistent with that found in the crystal (Pelletier & Kraut, 1992).

Binding of cytochrome *c* to wild-type CcP is characterized by a small unfavorable enthalpy change and dominated by a large positive entropy change (Kresheck et al., 1995). These thermodynamic parameters are consistent with the release of solvent upon complex formation. In this study we have also determined the effect of eliminating negatively charged sites within the cytochrome *c* binding domain on the surface of CcP in terms of the thermodynamic parameters for complex formation. Conversion of binding-site carboxylates to amides has no significant effect on the enthalpy change for complex formation. The decrease in affinity is due to a decrease in the entropy change, consistent with weaker hydration of the amides in comparison to the carboxylate residues.

MATERIALS AND METHODS

Wild-type yeast CcP was isolated as previously described (Vitello et al., 1990). CcP solutions were prepared by dissolving the crystalline enzyme into 0.050 M ionic strength buffers, pH 6.0 (0.010 M potassium phosphate with added KNO_3 to adjust ionic strength). Samples were dialyzed against four changes of buffer at a buffer/sample ratio of >100. After dialysis, samples were centrifuged at 17 000*g* to remove any insoluble material. CcP concentrations were determined spectroscopically using an absorptivity of 98 mM^{-1} cm^{-1} at 408 nm (Vitello et al., 1990). PZ values for the three preparations used in this study were 1.25, 1.29, and 1.30. Absorbance ratios at 408/380 and 620/647 nm were 1.50 ± 0.01 and 0.74 ± 0.01, respectively, indicating

that the CcP preparations were five-coordinate, high-spin Fe(III) forms (Vitello et al., 1990).

Surface mutants of CcP were constructed, expressed in *E. coli*, and isolated as previously described (Fishel et al., 1987; Liu et al., 1995). Solutions of the mutant enzymes were prepared from crystalline samples as described above for the wild-type enzyme.

Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Cytochrome *c* solutions were prepared by dissolving the lyophilized protein in 0.050 M ionic strength buffer, pH 6.0. At the high concentrations of cytochrome *c* used in these studies (1.67−2.46 mM) the pH of the resulting solution had to be readjusted to pH 6.0 with dilute HNO_3. Samples were then dialyzed in the same manner as described for CcP. Cytochrome *c* concentrations were determined spectrophotometrically using an absorptivity of 106.1 mM^{-1} cm^{-1} at 410 nm (Margoliash & Frohwirt, 1959).

Isothermal titration calorimetry experiments were carried out with a MicroCal ITC titration calorimeter (MicroCal, Inc., Northhampton, MA) using procedures previously described (Kresheck et al., 1995). The reaction cell volume of the instrument is 1.34 mL, and the titrant was added in 25 10-μL increments. The time between injections was 480 s, and the injection syringe was rotated at 400 rpm for the duration of each experiment. Cytochrome *c* was treated as the ligand and placed in the injection syringe with CcP in the cell. Following the procedure recommended by MicroCal, each titration was integrated manually using the data before and after each peak to establish a base line. The heat changes that accompanied the addition of buffer to solutions of CcP in the cell were negligible. However, corrections were necessary to account for the heat change that resulted when cytochrome *c* was placed in the syringe and diluted into the cell containing just buffer.

Data analysis to determine binding parameters was conducted using a nonlinear iterative procedure utilizing the Marquardt algorithm (Bevington, 1969). The data were fit to a model describing one-to-one complex formation as described in detail by Wiseman et al. (1989).

RESULTS

Significant absorption of heat occurs upon injecting 10 μL aliquots of horse ferricytochrome *c* (1.67−2.46 mM) into the calorimeter cell (1.34 mL) containing either CcP or one of the CcP mutants (42−109 μM). The absorption of heat indicates that complex formation is endothermic. Figure 2 shows calorimetric titrations of the reaction between horse ferricytochrome *c* and wild-type yeast CcP and between horse ferricytochrome *c* and a mutant CcP in which the negatively charged Glu-290 is replaced by a neutral asparagine residue, CcP(E290N). Duplicate titrations are shown to give an indication of the reproducibility of the data. Experimental conditions are given in the figure legend.

The titration curves can be analyzed on the basis of a model assuming simple binding between the two proteins to give a one-to-one complex (Wiseman et al., 1989; Kresheck et al., 1995). The analysis gives both the equilibrium association constant and the enthalpy change for complex formation. Equilibrium constants and enthalpy changes for the titrations shown in Figure 2 are collected in Table 1 along with those for the five other CcP mutants used in this study. Entropy changes, calculated from the equilibrium constants and enthalpy changes, are included in Table 1.

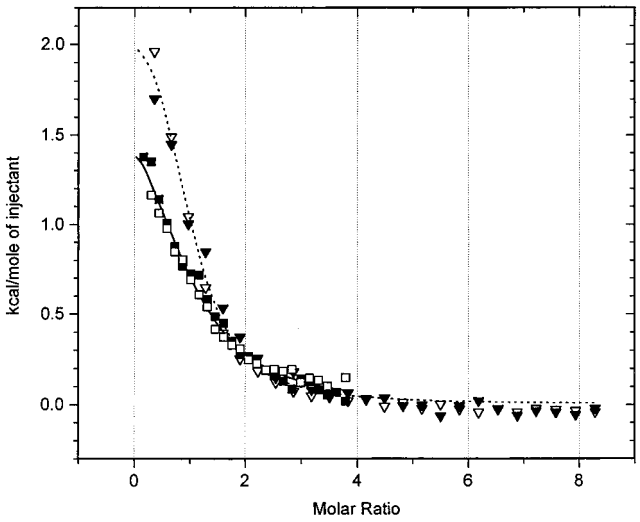

FIGURE 2: Calorimetric titrations for the binding of horse ferricytochrome *c* to CcP. Symbols: open and filled triangles, duplicate titrations of 49.0 μM yeast CcP (1.34 mL) using 10 μL injections of 1.97 mM cytochrome *c*; open and filled squares, duplicate titrations of 90.9 μM CcP(E290N) (1.34 mL) using 10 μL injections of 1.67 mM cytochrome *c*. Experimental conditions: pH 6.0, 0.010 M potassium phosphate buffer with added KNO_3 to adjust ionic strength to 0.050 M, 25 ± 1 °C. The lines represent the best fit to the average of the duplicate titrations.

Table 1: Equilibrium Constants and Thermodynamic Parameters for the Binding of Horse Ferricytochrome *c* to Wild-Type and Mutant Cytochrome *c* Peroxidase[a]

enzyme	K_A[b] (10^4 M^{-1})	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
CcP	10 ± 4 (11)	9.4 ± 0.8	129 ± 3
CcP(E291Q)	11 ± 3 (2)	8.9 ± 0.3	126 ± 3
CcP(E32Q)	3.6 ± 1.6 (4)	9.4 ± 0.1	122 ± 1
CcP(E35Q)	3.2 ± 0.8 (3)	4.4 ± 0.3	102 ± 2
CcP(A193F)	2.9 ± 1.5 (2)	8.0 ± 2.9	112 ± 10
CcP(D34N)	2.5 ± 1.5 (2)	10.5 ± 2.7	119 ± 10
CcP(E290N)	2.3 ± 0.4 (2)	8.6 ± 0.3	113 ± 2

[a] pH 6.0, 0.010 M potassium phosphate buffer, with added KNO_3 to adjust the ionic strength to 0.050 M, 25 ± 1 °C. [b] Number in parentheses indicates the number of titrations performed.

The equilibrium association constant, K_A, is reproducible to about 35%, estimated from the standard deviation of multiple titrations. The enthalpy and entropy changes are reproducible to about ±1.5 kJ mol^{-1} (∼17% of the mean value) and ±6 J mol^{-1} K^{-1} (∼5% of the mean), respectively.

DISCUSSION

Thermodynamic Parameters. The strong ionic strength dependence of the interaction between CcP and cytochrome *c* indicates that the binding is largely electrostatic in nature (Vitello & Erman, 1987; Kresheck et al., 1995). Previous studies show that, for native CcP and horse ferricytochrome *c*, formation of the horse cytochrome *c*/CcP complex is endothermic with a small, positive enthalpy change of 9 ± 1 kJ mol^{-1}, independent of ionic strength between 0.01 and 0.20 M (Kresheck et al., 1995). The thermodynamics of complex formation, including the strong ionic strength dependence of the reaction, is dominated by the positive entropy change which increases from 92 to 155 J mol^{-1} K^{-1}

between 0.20 and 0.010 M ionic strength (Kresheck et al., 1995). The large positive entropy change strongly suggests that the thermodynamics of complex formation are dominated by solvation effects. The mechanism for CcP/cytochrome *c* binding is that electrostatic forces bring the two macroions together, squeezing the solvent from the protein−protein interface. The released water of hydration provides a substantial increase in entropy for the reaction. The enthalpy change for the reaction is small, indicating that the enthalpic contributions of the protein−protein and water−water interactions in the product essentially cancel the enthalpic contribution of the protein−hydration layer interactions in the uncomplexed proteins.

The CcP mutations used in this study are relatively benign, and their effect on the binding of horse cytochrome *c* is small. The CcP mutants segregate into two groups on the basis of their affinity for cytochrome *c*. The binding of cytochrome *c* to CcP(E291Q) is identical to that of wild-type enzyme within experimental error. The cytochrome *c* binding affinity for the other five CcP mutants is three to four times weaker than that of wild-type CcP. The effect of the mutations can be interpreted in the context of the thermodynamic parameters for binding of wild-type CcP and horse cytochrome *c* (Kresheck et al., 1995). For all of the mutants, except CcP(A193F), the charge on specific glutamates or aspartates is removed by substitution of amides, and for one mutation, E290N, a methylene bridge is eliminated from the side chain. Substitution of amides in the mutants for carboxylate groups in wild-type CcP still permits the mutants to form hydrogen bonds with appropriate lysine residues on cytochrome *c*. The major difference in the thermodynamic parameters of the mutants relative to the wild-type enzyme will be due to differences in releasing water of hydration from a neutral asparagine or glutamine residue rather than from a charged aspartate or glutamate as the two proteins interact. As can be seen from Table 1, the enthalpy change upon complex formation for all of the mutants, except CcP(E35Q), is identical to that to the wild-type enzyme (within experimental error), suggesting that the hydrogen bonding between the CcP mutants and cytochrome *c* is similar to that of wild-type CcP and cytochrome *c*. The major decrease in binding affinity is an entropic effect with smaller increases in the entropy of interaction in the mutants. This is consistent with weaker hydration of the amide groups in the mutants relative to the charged carboxylate groups in wild-type CcP and a smaller increase in entropy as the water of hydration is released from the mutants upon complex formation.

Similarity of the Cytochrome c/CcP Complex in Solution and Crystal. One purpose of this study is to determine if the crystal and solution structures of the cytochrome *c*/CcP complex are the same. Figure 1 shows the surface of CcP and indicates the cytochrome *c* binding site determined by crystallography (Pelletier & Kraut, 1992). Figure 1 also indicates the cytochrome *c* binding site identified by computer docking (Poulos & Kraut, 1980; Poulos & Finzel, 1984).

Three regions of the CcP molecule make contact with horse cytochrome *c* in the crystalline CcP/horse cytochrome *c* complex. Two of the three regions are composed of acidic amino acid residues: the first contact point includes residues 32−35 and the second includes residues 290 and 291. Mutations at residues 32, 34, 35, 290, and 291 were created to assess the contribution of individual carboxylate residues

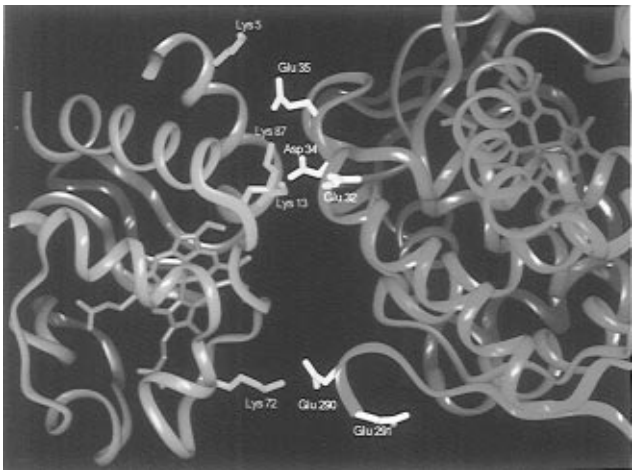

FIGURE 3: Interface between cytochrome *c* peroxidase and horse cytochrome *c* at the Pelletier/Kraut binding site (Pelletier & Kraut, 1992). CcP is shown in blue on the right and horse cytochrome *c* is shown in yellow on the left. Five of the six residues which were mutated in CcP and used in this study are shown in white (Ala-193 is not shown but would be in the background toward the center of the interface).

to formation of the CcP/horse cytochrome *c* complex in solution. Since each of these mutations converts a solvent-exposed carboxylate residue to an amide, the effect of each mutation on the surrounding solvent should be approximately the same. The results in Table 1 show that all three mutants in the 32−35 cluster (E32Q, D34N, and E35Q) have similar effects on K_A, decreasing the affinity of CcP for horse cytochrome *c* by 3−4-fold. On the other hand, although CcP(E290N) also causes a 4-fold decrease in K_A, the E291Q mutation has no effect on the binding of cytochrome *c*.

The results are consistent with the crystal structure of the CcP/horse cytochrome *c* structure complex (Figures 1 and 3). Figure 3 gives a perpendicular view of the interface between horse cytochrome *c* and CcP as determined from the crystal structure. In Figure 3, the CcP molecule is on the right with Glu-32 toward the top and in the foreground of the figure. Glu-291 in CcP is toward the bottom foreground of the figure. Ala-193 of CcP is not shown but would be in the background, toward the center of the interface. The interactions between horse cytochrome *c* and CcP shown in Figure 3 can be used to rationalize the binding affinities of the CcP mutants given in Table 1. The crystal structure of the complex shows that the carboxylate side chain of CcP Asp-34 is within hydrogen-bonding distance of the side-chain nitrogen of Lys-13 of horse cytochrome *c* and that the carboxylate oxygen atoms of CcP Glu-35 are 4.5 Å from the respective side-chain nitrogen atoms of Lys-5 and Lys-87 in horse cytochrome *c*. The carboxylate of Glu-32 bends away from the binding interface in the crystal structure (Figure 3) but is still close enough to influence the binding of cytochrome *c*.

Particularly interesting are the relative binding affinities of mutations at Glu-290 and Glu-291 in CcP. Conversion of Glu-290 to an asparagine causes a 4-fold decrease in binding affinity while conversion of Glu-291 to a glutamine has no effect even though this two residues are adjacent. This is easily reconciled by Figure 3 where it is seen that Glu-290 makes a strong hydrogen bond to Lys-72 of horse cytochrome *c* while Glu-291 projects away from the cytochrome *c* binding interface.

The third region of interaction between CcP and horse cytochrome *c* is near Ala-193 and Ala-194 of CcP and Gln-12 of cytochrome *c*. The A193F mutation was created to decrease the affinity of CcP for horse cytochrome *c* by introducing steric conflict to formation of the CcP/horse cytochrome *c* complex seen in the crystal. In the crystalline CcP/horse cytochrome *c* complex, Ala-193 is only 4.8 Å from the side chain of Gln 16 of horse cytochrome *c*. Computer simulations showed that the Phe side chain at position 193 will have very restricted movement about the bond between the α and β carbons and will exist predominantly in the *trans* conformation (i.e., with a dihedral angle of approximately 180°). This orientation will make the distance between the phenyl ring and the side chain of Gln-16 less than the sum of the standard van der Waals radii in the CcP/horse cytochrome *c* complex and was expected to decrease the affinity for horse cytochrome *c* by increasing the enthalpy of complex formation. Our results provide no evidence for this type of effect, however. Instead, the decreased affinity for complex formation for the A193F mutant is caused by entropic effects, much like the other mutants examined here. The results suggest that the effect of the A193F mutation on complex formation is caused by a change in the solvation of the side chain at residue 193 instead of a steric effect.

In summary, these binding studies are consistent with the horse cytochrome *c*/CcP interface as determined from X-ray crystallography (Pelletier & Kraut, 1992) and shown in Figures 1 and 3.

Previous Studies. The Poulos/Finzel/Kraut model of the cytochrome *c*/CcP complex (Poulos & Kraut, 1980; Poulos & Finzel, 1984) has been important in promoting the cytochrome *c*/CcP system as a model for detailed studies of electron transfer within dynamic protein complexes. It was the first model for an electron transfer complex between physiologically related proteins and prompted a number of investigations into the binding and electron transfer properties of this system. Previous studies designed to test the Poulos/Finzel/Kraut model did show discrepancies from predictions, and these can now be understood in light of the crystallographically defined binding site.

Bechtold and Bosshard (1985) found that acidic residues 33, 34, 35, 37, 221, and 224 and at least one of the three carboxylates between residues 290 and 294 in CcP became less reactive when bound to cytochrome *c*. The negative cluster between residues 33−37 is consistent with either binding site, but the protection of a residue (or residues) between 290 and 294 is only consistent with the binding site determined by crystallography. The altered reactivity at residues 221 and 224 cannot be explained by binding of cytochrome *c* at either the Pelletier/Kraut or the Poulos/Finzel/Kraut sites. Residues 221 and 224 are too far from either binding site to be blocked by a single cytochrome *c* molecule bound to either site. There must be some other explanation for the altered reactivity at residues 221 and 224.

Waldmeyer and Bosshard (1985) cross-linked CcP and horse cytochrome *c* with a carbodiimide under conditions where one-to-one complex formation is favored. Two major cross-links were localized: one between Lys-13 of cytochrome *c* to an acidic residue within the cluster of acidic residues between positions 32 and 37 on CcP and the second between Lys-86 of cytochrome *c* and a carboxyl group in the same cluster of acidic residues in CcP. These two cross-links are consistent with the crystallographic data (Figure 3). No cross-links involving the region of CcP including residues 221 or 224 were detected.

Das et al. (1988) replaced Lys-32 of yeast iso-1 cytochrome *c* with several different amino acid residues and found these mutations had little influence on the interaction between yeast iso-1 cytochrome *c* and CcP. Lys-32 in yeast iso-1 cytochrome *c* is analogous to Lys-27 in horse cytochrome *c* and was proposed to interact with Asp-79 in CcP in the original Poulos/Kraut model of the complex (Poulos & Kraut, 1980). This interaction does not occur in the crystal structures of either the yeast iso-1 cytochrome *c*/CcP or the horse cytochrome *c*/CcP complexes.

Corin et al. (1991) made point mutations in CcP at residues 37, 79, and 217, converting the aspartate residues in wild-type CcP to lysine residues. The mutations at positions 79 and 217 had no significant effect on the binding of yeast iso-1 cytochrome *c* or the steady-state kinetics of horse ferrocytochrome *c*. The results reported for residues 79 and 217 are consistent with the crystal structure in that these residues are far from the CcP/horse cytochrome *c* interaction site. Although Asp-37 is not at the interface, it would not be surprising to find that mutation of this residue to lysine has a significant effect on the local structure of CcP near the interaction site. Asp-37 forms a charge-mediated hydrogen bond with the imidazolium side chain of His-181 and is known to stabilize the native conformation of the enzyme (Miller et al., 1988). Replacing Asp-37 with lysine would introduce charge−charge repulsion between the imidazolium of His-181 and the ϵ-amino group of lysine. This in turn would decrease the pK_a of His-181 and destabilize the native conformation of the enzyme. The decreased affinity of the Lys-37 mutant enzyme may therefore represent a perturbation of the binding region by a more global effect on enzyme structure.

The observation that modification of Asp-217 does not influence the binding and kinetic properties of CcP with cytochrome *c* and that modification of Glu-290 causes significant changes provides strong evidence that the Pelletier/Kraut binding site is the correct site and that the Poulos/Finzel/Kraut site is not (Figure 1).

Electron Transfer within the Horse Cytochrome c/CcP Complex. Miller et al. (1994) and Liu et al. (1995) have studied the kinetics of electron transfer between cytochrome *c* and CcP using many of the same CcP mutants used in this calorimetric study. Liu et al. (1995) have used a ruthenium-modified horse cytochrome *c* to explore electron transfer between cytochrome *c* and CcP. A ruthenium(II) complex is covalently attached to Lys-27 of horse ferricytochrome *c* (Ru27-hCc). At low ionic strength, stoichiometric mixtures of Ru27-hCc and CcP form a one-to-one complex. Photoexcitation of the Ru(II) ion produces Ru(II*), a strong reductant. Ru(II*) rapidly reduces the heme iron of the covalently attached ferricytochrome *c* ($k = 2.3 \times 10^7$ s^{-1}) to generate ferrocytochrome *c* and Ru(III). Ru(III) is a strong oxidizing agent and, in the one-to-one complex with CcP, rapidly ($k = 7 \times 10^6$ s^{-1}) oxidizes Trp-191 in CcP to a radical state regenerating ground-state Ru(II). The photoinitiated redox cycle is completed by electron transfer from the heme iron of cytochrome *c* to the Trp-191 radical in CcP with a rate constant of 6.1×10^4 s^{-1}. As the ionic strength is increased, dissociation of the Ru27-hCc/CcP complex occurs and the amplitude of the intramolecular reactions between

Table 2: Comparison of K_A for Binding of Ruthenium-Modified and Unmodified Horse Cytochrome *c* to Wild-Type and Mutant Cytochrome *c* Peroxidase[a]

	horse cytochrome *c*			ruthenium-27 horse cytochrome *c*[b]		
enzyme	K_A (10^4 M^{-1})	relative affinity	$\Delta\Delta G$ (kJ mol^{-1})	K_A (10^4 M^{-1})	relative affinity	$\Delta\Delta G$ (kJ mol^{-1})
CcP	10	100		98	100	
CcP(E291Q)	11	110	−0.4	89	91	0.2
CcP(E32Q)	3.6	36	2.5	49	50	1.7
CcP(E35Q)	3.2	32	2.8			
CcP(A193F)	2.9	29	3.0	18	18	4.2
CcP(D34N)	2.5	25	3.4			
CcP(E290N)	2.3	23	3.6	10	10	5.7

[a] pH 6.0, 0.050 M ionic strength. [b] Ruthenium complex covalently attached to Lys-27 of horse cytochrome *c*. Data from Liu et al. (1995).

sites on Ru27-hCc and CcP decreases. From the decrease in amplitude the fraction of complexed and uncomplexed proteins can be estimated and an estimated equilibrium constant for complex formation determined. These kinetically determined equilibrium association constants determined by Liu et al. at pH 6.0, 0.050 M ionic strength are shown in Table 2 and compared with the calorimetric results.

The values of the kinetically determined equilibrium constants may be subject to systematic errors. These values depend upon interpretation of kinetic data, the assumed kinetic mechanism for the reaction, and how well rapid-equilibrium conditions are met. In spite of the possible systematic errors, the trend in the kinetically determined equilibrium constants for the Ru27-hCc derivative and the CcP mutants is almost identical to those for native horse cytochrome *c* and the CcP mutants. These data strongly support the conclusion that Ru27-hCc binds to the same surface domain on CcP as horse cytochrome *c*.

Miller et al. (1994) investigated the reaction between CcP compound I and both horse and yeast ferrocytochrome *c*. The redox reactions were studied under somewhat different conditions than our calorimetry experiments: 0.11 M ionic strength sodium phosphate/NaCl buffer at pH 7.0, 25 °C. Under these high ionic strength conditions, the reaction between CcP compound I and ferrocytochrome *c* is second order. Assuming that the redox mechanism involves rapid binding of CcP compound I and ferrocytochrome *c*, followed by intramolecular electron transfer within the complex, k_{et}, the observed second-order rate constant is given by $K_A k_{et}$. The observed second-order rate constant for the reaction was 1.3×10^8 M^{-1} s^{-1} for the parental form of CcP expressed in *E. coli*, CcP(MI). Miller et al. (1994) found very similar effects due to mutations in the cytochrome *c* binding domain on CcP as we have found in direct binding studies. The observed bimolecular rate constants for CcP(MI) and its mutants decrease in the following order: CcP(E32Q) ≈ CcP(MI) ≈ CcP(E291Q) > CcP(E35Q) > CcP(D34N) > CcP(A193F) = CcP(E290N). The only exception to the ordering listed in Table 1 is that of the CcP(E32Q) mutant. In the study of Miller et al. (1994) CcP(E32Q) had the same electron transfer rate as the native recombinant protein, CcP(MI), and CcP(E291Q). The observation that the apparent bimolecular rate constants for the reaction between horse ferrocytochrome *c* and CcP compound I for the CcP mutants correlate with the binding constants in this study suggests that the rate of intracomplex electron transfer is not disrupted by the surface mutations and that k_{et} is similar for CcP(MI) and the surface mutants shown in Tables 1 and 2.

Many physiological processes require the formation of specific protein/protein complexes. The structural basis for the specificity of protein/protein recognition can only be examined through the study of complexes where structure of the complex is known. In the present study we have begun the characterization of one such system, that of the complex formed between cytochrome *c* peroxidase and cytochrome *c*. The studies reported in this paper demonstrate that the structure of the CcP/horse cytochrome *c* complex in solution is consistent with the structure of the complex in the crystalline state. Moreover, the crystal structure shows few strong interactions between CcP and cytochrome *c*, a result consistent with the unfavorable enthalpy of complex formation. The binding of CcP and cytochrome *c* is dominated by a favorable entropy change as the protein/protein interface is desolvated upon complex formation. This type of interaction is beneficial for formation of dynamic protein complexes where the reacting partners must associate and dissociate rapidly. The rate of formation of the cytochrome *c*/CcP complex is extremely fast, probably diffusion controlled (Matthis et al., 1995), and the half-time of dissociation is on the millisecond time scale (Yi et al., 1994). It appears that the binding-site specificity in these types of dynamic, electrostatically stabilized protein/protein complexes is determined by the electrostatic potentials of the interacting partners rather than by multiple, strong complementary side-chain interactions at the binding surface. These studies provide a basis for further exploration into more fundamental questions of specificity, dynamics, and electron transfer pathways in the CcP/cytochrome *c* complex.

REFERENCES

Bechtold, R., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5191−5200.

Bevington, P. R. (1969) *Data Reduction and Error Analysis in the Physical Sciences*, McGraw-Hill, New York, NY.

Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1978) *Methods Enzymol. 53*, 128−164.

Bushnell, G. W., Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 585−595.

Corin, A. F., McLendon, G., Zhang, Q., Hake, R. A., Falvo, J., Lu, K. S., Ciccarelli, R. B., & Holzschu, D. (1991) *Biochemistry 30*, 11585−11595.

Das, G., Hickey, D. R., Principio, L., Conklin, K. T., Short, J., Miller, J. R., McLendon, G., & Sherman, F. (1988) *J. Biol. Chem. 263*, 18290−18297.

Fishel, L. A., Villafranca, J. E., Mauro, J. M., & Kraut, J. (1987) *Biochemistry 26*, 351−360.

Kang, C. H., Brautigan, D. L., Osheroff, N., & Margoliash, E. (1978) *J. Biol. Chem. 253*, 6502−6510.

Kresheck, G. C., Vitello, L. B., & Erman, J. E. (1995) *Biochemistry 34*, 8398−8405.

Liu, R. Q., Hahm, S., Miller, M., Durham, B., & Millett, F. (1995) *Biochemistry 34*, 973−983.

Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570−572.

Marini, M. A., Marti, G. E., Berger, R. L., & Martin, C. J. (1980) *Biopolymers 19*, 885−898.
Matthis, A. L., Vitello, L. B., & Erman, J. E. (1995) *Biochemistry 34*, 9991−9999.
McLendon, G. (1988) *Acc. Chem. Res. 21*, 160−167.
Miller, M. A., Hazzard, J. T., Mauro, J. M., Edwards, S. L., Simon, P. D., Tollin, G., & Kraut, J. (1988) *Biochemistry 27*, 9081−9088.
Miller, M. A., Liu, R. Q., Hahm, S., Geren, L., Hibdon, S., Kraut, J., Durham, B., & Millett, F. (1994) *Biochemistry 33*, 8686−8693.
Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748−1755.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322−10330.
Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115−171.
Poulos, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Eriksson, B., Xuong, N., Yonetani, T., & Kraut, J. (1980) *J. Biol. Chem. 254*, 3730−3735.
Satterlee, J. D., Moench, S. J., & Erman, J. E. (1987) *Biochim. Biophys. Acta 912*, 87−97.
Vitello, L. B., & Erman, J. E. (1987) *Arch. Biochem. Biophys. 258*, 621−629.
Vitello, L. B., Huang, M., & Erman, J. E. (1990) *Biochemistry 29*, 4283−4288.
Waldmeyer, B., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184−5109.
Yi, Q., Erman, J. E., & Satterlee, J. D. (1994) *J. Am. Chem. Soc. 116*, 1981−1987.
Yonetani, T. (1967) *J. Biol. Chem. 242*, 5008−5013.

BI962632X