Interaction Domain for the Reaction of Cytochrome *c* with the Radical and the Oxyferryl Heme in Cytochrome *c* Peroxidase Compound I†

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ABSTRACT: Site-directed mutants of cytochrome *c* peroxidase (CcP) were created to modify the interaction domain between CcP and yeast iso-1-cytochrome *c* (yCC) seen in the crystal structure of the CcP–yCC complex [Pelletier & Kraut (1992) *Science 258*, 1748–1755]. In the crystalline CcP–yCC complex, two acidic regions of CcP contact lysine residues on yCC. Mutants E32Q, D34N, E35Q, E290N, and E291Q were used to examine the effect of converting individual carboxylate side chains in the acidic regions to amides. The A193F mutant was used to test the effect of introducing a phenyl moiety at the point of closest contact between CcP and yCC in the crystal structure. Stopped-flow experiments carried out in 310 mM ionic strength buffer at pH 7 revealed that yCC initially reduced the indole radical on Trp-191 of the parent CcP compound I with a bimolecular rate constant $k_a = 2.5 \times 10^8$ M^{-1} s^{-1}. A second molecule of yCC subsequently reduced the oxyferryl heme of compound II with a rate constant $k_b = 5 \times 10^7$ M^{-1} s^{-1}. The bimolecular rate constants k_a and k_b were affected in parallel by each mutation examined. CcP mutants D34N and E290N that are closest to a complementary yCC lysine residue in the crystalline CcP–yCC complex gave the lowest values for k_a and k_b, which were 25–50% of the values of the CcP parent. Mutants E32Q and E291Q that are removed from the interaction domain gave the same k_a and k_b values as the CcP parent. The A193F mutant gave k_a and k_b values that were 25–45% of the values for the CcP parent. The effects of the mutations on the bimolecular reaction with horse CC were nearly the same as on the reaction with yCC, indicating that both cytochromes use the same interaction domain. The rate constants k_{et} for intracomplex electron transfer from the heme group of ruthenium derivatives of horse CC to the Trp-191 radical of CMPI were measured by flash photolysis at low ionic strength. The values of k_{et} varied with the mutant CcP enzymes, as well as the position of the ruthenium label on horse CC. The results of all the kinetic studies are consistent with the following conclusions: (1) the same interaction domain is used for the reactions of CC with the radical in compound I, and with the oxyferryl heme in compound II; (2) the interaction domain used for both electron transfer reactions in solution resembles the interaction domain in the crystalline CcP–yCC complex.

The reaction between two redox proteins generally involves at least three distinct steps: (1) formation of a transient binary substrate complex between the two proteins, (2) electron transfer within the binary complex, and (3) dissociation of the product complex. It is important to identify the interaction domain between the two proteins since it is largely responsible for the specificity of the reaction and encloses the electron transfer pathway. The reaction between cytochrome *c* and cytochrome *c* peroxidase has been used extensively to address fundamental questions about protein interaction domains and electron transfer pathways (Finzel et al., 1984; Edwards et al., 1987; Wang et al., 1990; Everest et al., 1991; Wallin et al., 1991; Northrup et al., 1987, 1988). Cytochrome *c* (CC) forms a stable product complex with cytochrome *c* peroxidase (CcP) at low ionic strength, suggesting that the electron transfer reaction proceeds through a transient substrate complex (Mochan, 1970; Mochan & Nichols, 1971; Leonard & Yonetani, 1974; Kang et al., 1977; Erman & Vitello, 1980; Dowe et al., 1984; Vitello & Erman, 1987; Corin et al., 1991; Moench et al., 1992). Both the stability of the product complex and the rate of the electron transfer reaction decrease significantly with increasing ionic strength, indicating the importance of electrostatic interactions (Yonetani & Ray, 1966; Kang et al., 1977). Poulos and Kraut (1980) proposed a hypothetical model for the 1:1 complex between tuna CC and CcP that was stabilized by charge-pair interactions between lysines 13, 27, 72, 86, and 87 surrounding the heme crevice of CC and the carboxylate groups on aspartates 34, 37, 79, and 217 of CcP. Extensive chemical modification studies demonstrated the involvement of CC lysines 13, 27, 72, 86, and 87 (Kang et al., 1978; Smith & Millett, 1980; Pettigrew, 1978) and CcP carboxylates at residues 33, 34, 35, 37, 221, 224, and 290 or 291 (Bechtold & Bosshard, 1985; Waldmeyer & Bosshard, 1985). These results supported the general features of the Poulos–Kraut model, except that Asp-79 of CcP did not appear to be involved in complex formation (Bechtold & Bosshard, 1985). More recently, Corin et al. (1991, 1993) and Hake et al. (1992) found that replacement of Asp-37 on CcP with a lysine residue greatly decreased both the binding affinity and the steady-state electron transfer activity, as predicted by the Poulos–Kraut model. However, replacement of Asp-79 or Asp-217 with a lysine residue had only very subtle effects on binding and electron transfer activity, suggesting that these residues are not directly involved in the interaction.

Pelletier and Kraut (1992) recently determined the three-dimensional structure of a 1:1 complex between yeast CcP

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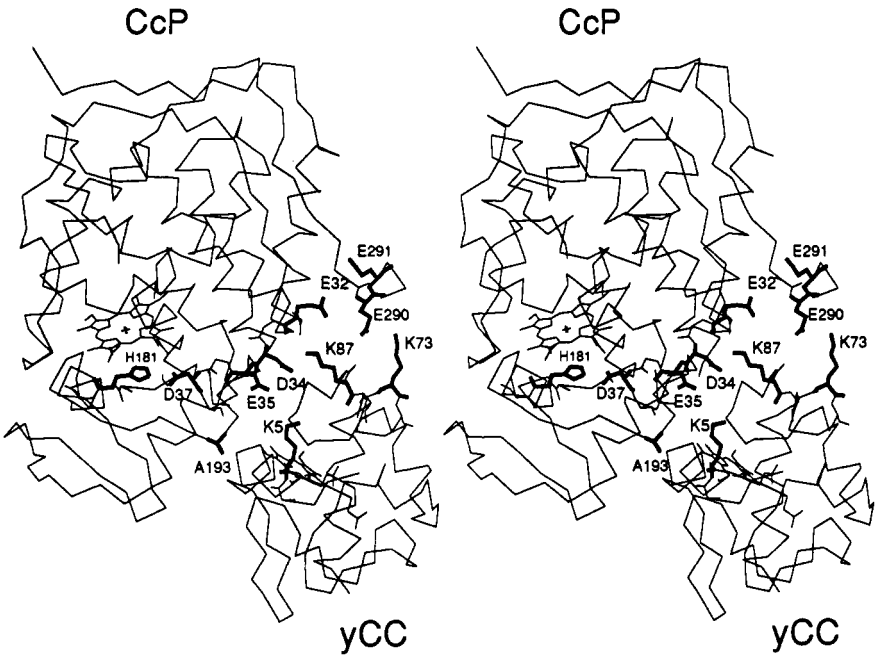

FIGURE 1: X-ray crystal structure of the complex between yeast cytochrome *c* peroxidase and yeast iso-1-cytochrome *c* (Pelletier & Kraut, 1992). The side chains of CcP residues E32, D34, E35, D37, H181, A193, E290, and E291, and of yCC residues K5, K73, and K87, are shown and labeled. The heme groups of both proteins are also shown.

and yeast iso-1-cytochrome *c* (yCC)[1] crystallized at high ionic strength (150 mM NaCl, pH 7). The binding domain is significantly different than that of the Poulos–Kraut model (Figure 1). Although there are no direct hydrogen bonds between the two proteins, the positive charges on CC lysines 73 and 87 are each about 3.8 Å from the negative charges on CcP Glu-290 and Asp-34, respectively. Electrostatic interactions involving these residues as well as others could help stabilize the complex. At the center of the binding domain, there are prominent van der Waals interactions between CcP residues Ala-193 and Ala-194 and the exposed heme CBC methyl group of CC (Figure 1). These interactions could provide a potential electron transfer pathway extending from the exposed heme methyl group of CC through CcP residues Ala-194, Ala-193, and Gly-192 to the indole group of Trp-191, which is in van der Waals contact with the heme group.

The reaction between cytochrome *c* and cytochrome *c* peroxidase is quite complicated, involving at least two different cycles of complex formation, electron transfer, and complex dissociation (Kim et al., 1990). The resting ferric state of the enzyme, CcP, is oxidized by hydrogen peroxide to CMPI, which contains an oxyferryl heme Fe^{IV}=O and a radical on the indole ring of Trp-191 (Mauro et al., 1988; Sivaraja et al., 1989; Erman et al., 1989; Fishel et al., 1991). CMPI is then reduced to CMPII by one molecule of ferrocytochrome *c* [CC(II)], and CMPII is reduced to CcP by a second molecule of CC(II) (Kim et al., 1990). Coulson et al. (1971) and Ho et al. (1983, 1984) identified two forms of the singly oxidized state, CMPII(IV,R), containing the Fe^{IV}=O center, and CMPII(III,R•), containing the radical.

Establishing the rates of reduction of the two oxidizing sites of CMPI by CC is a fundamental requirement for determining the mechanism of electron transfer. Studies from this laboratory have focused on examining the rates of electron transfer under the simplest kinetic conditions possible. Geren et al. (1991) and Hahm et al. (1992) found that ruthenium-labeled horse and yeast CC derivatives react first with the radical in CMPI and then with the Fe^{IV}=O center in CMPII as shown in Scheme 1. This reaction sequence is obeyed at low ionic strength, where intracomplex electron transfer is observed, and at high ionic strength, where bimolecular kinetics are observed. Scheme 1 is also observed in stopped-flow measurements of the second-order reactions between native horse CC (hCC) and CMPI at high ionic strength and pH 7–8 (Hahm et al., 1993, 1994).

Scheme 1

$$\text{CMPI(IV,R}^{\bullet}) + \text{CC(II)} \longrightarrow \text{CMPII(IV,R)} + \text{CC(III)} \quad (1)$$

$$\text{CMPII(IV,R)} + \text{CC(II)} \longrightarrow \text{CcP(III,R)} + \text{CC(III)} \quad (2)$$

Scheme 2

$$\text{CMPI(IV,R}^{\bullet}) + \text{CC(II)} \longrightarrow \text{CMPII(IV,R)} + \text{CC(III)} \quad (1)$$

$$\text{CMPII(IV,R)} \rightleftharpoons \text{CMPII(III,R}^{\bullet}) \quad (3)$$

$$\text{CMPII(III,R}^{\bullet}) + \text{CC(II)} \longrightarrow \text{CcP(III,R)} + \text{CC(III)} \quad (4)$$

In the accompanying paper (Liu et al., 1994), the reduction of the CMPI radical at Trp-191 is shown to be followed by intramolecular electron transfer from Trp-191 to the Fe^{IV}=O center of CMPII (reaction 3 of Scheme 2). Moreover, a strong correlation is seen between the rate of reduction of the Fe^{IV}=O center and the fraction of CMPII(IV,R) that is converted to CMPII(III,R•) after reaction 1. The results are consistent with the mechanism shown in Scheme 2, where the reduction of the Fe^{IV}=O center (reaction 2 of Scheme 1) actually requires two steps: intramolecular electron transfer from Trp-191 to Fe^{IV}=O (reaction 3) and intermolecular electron transfer from CC to the Trp-191 radical (reaction 4). The equilibrium constant *K* for reaction 3 is less than 0.15 at pH 7, accounting for the slow rate of reduction of the Fe^{IV}=O center (Liu et al., 1994). In Scheme 2, both interprotein electron transfer reactions proceed by reduction of the Trp-191 radical by CC. Thus, both reactions may utilize a common electron transfer pathway and a common interaction domain.

[1] Abbreviations: hCC, horse cytochrome *c*; yCC, yeast iso-1-cytochrome *c*; Ru-27-CC, bis(bipyridine)(4,4′-dicarboxybipyridine-Lys-27-cytochrome *c*)ruthenium(II).

To confirm the existence of such a mechanism, it is important to identify the interaction domain(s) for intermolecular electron transfer and to examine the effect of perturbations of this interaction domain on both electron transfer reactions. The present work describes CcP mutants that were created to establish the relevance of the interaction domain found in the crystalline CcP–yCC product complex to electron transfer reactions between these two proteins in solution. By characterizing the effect of these mutations on electron transfer reactions 1 and 2, it is also possible to determine whether or not the interaction domain is the same for both reactions. Mutations were introduced at CcP residues near the interaction domain of the crystalline CcP–yCC complex. The mutations E32Q, D34N, E35Q, E290N, and E291Q neutralized acidic groups that potentially interact with lysine residues on CC, while the A193F mutation added a bulky phenyl moiety at the point of closest contact between the heme of CC and Trp-191. Decreases in the rate of both electron transfer reactions relative to the parent enzyme were found to occur in proportion to the proximity of the mutation to the interaction domain of the crystal structure. The results are therefore consistent with an interaction domain similar to that seen in the crystalline complex for both electron transfer reactions.

EXPERIMENTAL PROCEDURES

Materials. Horse cytochrome *c* (type VI) and yeast iso-1-cytochrome *c* (type VIII-B) were purchased from Sigma Chemical Co. Horse CC derivatives labeled at single lysine amino groups with (dicarboxybipyridine)bis(bipyridine)-ruthenium(II) (Ru-CC) were prepared as described by Pan et al. (1988) and Durham et al. (1989). CcP(MI) and mutants CcP(MI,E32Q), CcP(MI,D34N), CcP(MI,E35N), CcP(MI,-E290N), CcP(MI,E291Q), and CcP(MI,A193F) were prepared as described by Fishel et al. (1987, 1991).

Stopped-Flow Kinetics. Transient kinetic studies were carried out on a Durrum Model D-110 stopped-flow spectrophotometer equipped with a 2-cm flow cell as described by Hahm et al. (1993, 1994). CC(II) and CMPI in buffers containing sodium phosphate and NaCl were placed in the syringes of the stopped-flow apparatus. The transients were fitted to the second-order equation:

$$\Delta A = 2\Delta\epsilon\left(b_0 - \frac{\exp[(b_0 - a_0)kt] - 1}{\exp[(b_0 - a_0)kt]/a_0 - 1/b_0}\right) \quad (5)$$

where b_0 = initial [CC(II)], a_0 = [CMPI], and $\Delta\epsilon$ is the appropriate difference extinction coefficient. The following difference coefficients were used: CC(II) → CC(III), $\Delta\epsilon_{416} = -40\ mM^{-1}\ cm^{-1}$, $\Delta\epsilon_{434} = 0$; CMPI → CMPII(III,R•), $\Delta\epsilon_{416} = -13\ mM^{-1}\ cm^{-1}$, $\Delta\epsilon_{434} = -27\ mM^{-1}\ cm^{-1}$; CMPI → CMPII(IV,R), $\Delta\epsilon < 3\ mM^{-1}\ cm^{-1}$ at both wavelengths (Hahm et al., 1994; Coulson et al., 1971; Ho et al., 1983).

The kinetics were studied using two different protocols. In protocol A, CC(II) was mixed with excess CMPI at high enough ionic strength to resolve the entire transient in the stopped-flow spectrophotometer, which had a deadtime of 2 ms. The second-order rate constant k_a measured from the 416-nm transients represents all reactions involving oxidation of CC(II) by CMPI. The typical range of concentrations was [CC(II)] = 0.3–1 μM and [CMPI] = 0.5–3 μM, with [CMPI] > [CC(II)]. In protocol B excess CC(II) was mixed with CMPI at high ionic strength. The second-order rate constant k_b measured from the 434-nm transients represents all reactions involving reduction of the oxyferryl heme Fe(IV) by CC(II). Typical concentrations were [CC(II)] = 1–10 μM, and [CMPI] = 0.5 μM. The 434-nm transients were fitted to the full second-order eq 5.

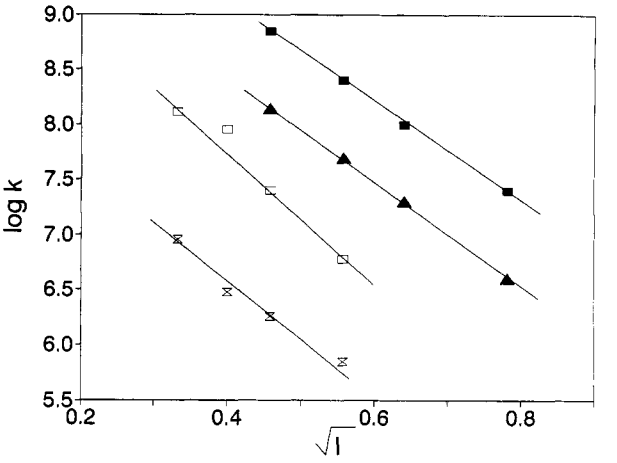

FIGURE 2: Ionic strength dependence of the rate constants k_a and k_b for the reaction between yeast and horse CC(II) and CcP(MI) CMPI. k_a and k_b were measured at 25 °C as described in the text in buffers containing 5 mM sodium phosphate, pH 7, with NaCl added to the indicated ionic strength. The errors in the rate constants are ±20%. yCC: k_a (■), k_b (▲). hCC: k_a (□), k_b (×). The data are plotted as log k vs square root of ionic strength (M).

Flash Photolysis Studies of Ru-CC Derivatives. Laser flash photolysis studies of the reactions between Ru-CC derivatives and the CMPI form of the cytochrome *c* peroxidase mutants were carried out as described by Hahm et al. (1992). The buffer contained 2 mM sodium phosphate, pH 6, 1–10 mM aniline, and 0–500 mM sodium chloride. Aniline was present to reduce Ru(III) to Ru(II) and prevent the back-reaction. The kinetic transients were fitted to a biexponential equation (Hahm et al., 1992).

RESULTS

Stopped-Flow Spectroscopy. The reaction between yeast iso-1-cytochrome *c* and recombinant CcP(MI) CMPI was studied using the stopped-flow protocols of Hahm et al. (1993, 1994). It was necessary to use much higher ionic strength in order to resolve the entire extent of the reaction in the stopped-flow instrument than was the case for hCC. The first electron transfer step in the reaction was studied by mixing yCC(II) with excess CMPI (protocol A). It was found that yCC(II) reduced the radical in CMPI(IV,R•) with a second-order rate constant of $k_a = (2.5 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$ in 310 mM ionic strength buffer (5 mM sodium phosphate, pH 7, 300 mM NaCl). No significant absorbance change was observed at 434 nm (a CC isobestic), indicating that reduction of the Fe^{IV}=O center was less than 15% that of the radical under these conditions. The complete reduction of CMPI to CcP was studied by mixing CMPI with excess yCC(II) (protocol B). The 416-nm transient was biphasic, with the fast phase due to reduction of the radical in CMPI(IV,R•) by yCC(II), and the slow phase due to reduction of the Fe^{IV}=O center in CMPII(IV,R) by a second molecule of yCC(II). The 434-nm transient was monophasic and is due to reduction of Fe^{IV}=O in CMPII(IV,R). It had the same rate constant as the slow phase of the 416-nm transient. The second-order rate constant k_b was measured to be $(5.0 \pm 0.7) \times 10^7\ M^{-1}\ s^{-1}$ from 434-nm transients obtained by mixing 0.5 μM CMPI with 1–5 μM yCC(II) in the pH 7, 310 mM ionic strength buffer.

The second-order rate constants k_a and k_b for the reaction between yCC(II) and CMPI both increased as the ionic strength was decreased at pH 7 (Figure 2). This behavior is

Table 1: Effect of Charge Mutants on the Reaction between Ferrocytochrome c and Cytochrome c Peroxidase CMPI[a]

mutant	k_a, horse	k_b, horse	k_a, yeast	k_b, yeast
CcP(MI)	1.3×10^8	9.0×10^6	2.5×10^8	5.0×10^7
mutant	% control	% control	% control	% control
E32Q	105	95	91	100
E34N	45	40	40	17
E35Q	80	67	80	50
E290N	25	40	50	40
E291Q	96	100	105	100
A193F	25	37	45	40

[a] The second-order rate constants k_a and k_b were measured using stopped-flow spectroscopy protocols A and B, respectively. For the reaction with hCC, the buffer was 5 mM sodium phosphate, pH 7, and 100 mM NaCl at 25 °C. For the reaction with yCC, the buffer was 5 mM sodium phosphate, pH 7, and 300 mM NaCl. The rate constants are given in units of M^{-1} s^{-1}, and the errors are ±20%. The rate constants of the mutants are given as a percentage of the rate constant of the CcP(MI) control.

expected for the reaction between oppositely charged proteins. The value of k_a was 7.0×10^8 M^{-1} s^{-1} at 200 mM ionic strength, which is at the upper limit of the time resolution of the Durrum stopped-flow spectrophotometer. At lower ionic strength, less than 50% of the theoretical absorbance change was resolved in the 416-nm transients obtained using protocol A. The value of k_b was lower than that of k_a at all ionic strengths, but the slope of the ionic strength dependence plot was nearly the same (Figure 2). The k_a and k_b values for yCC(II) were much larger than those for hCC(II) at a given ionic strength, but the slopes of the ionic strength dependence plots were similar (Figure 2).

The second-order rate constants k_a and k_b for the reactions of hCC(II) with mutants of CcP(MI) were measured in 110 mM ionic strength buffer at pH 7, while the rate constants for the reactions of yCC(II) were measured in 310 mM ionic strength buffer at pH 7 (Table 1). Two of the charge mutants, E32Q and E291Q, had the same k_a and k_b values as CcP(MI) for the reactions with both hCC(II) and yCC(II), while the rate constants of the E35Q mutant were slightly smaller. The rate constants for the D34N and E290N mutants ranged from 17% to 50% of the values for wild-type CcP(MI). The rate constants for the A193F mutant ranged from 25% to 45% of the control CcP(MI).

Flash Photolysis Studies Using Ru-CC Derivatives. The reactions between the Ru-CC derivatives and mutants of CcP(MI) were studied at low ionic strength (2 mM sodium phosphate, pH 6) using the methods described by Hahm et al. (1992). The transients measured at 550 nm were generally biphasic, and both rate constants were independent of protein concentration above 5 μM, consistent with intracomplex electron transfer. No absorbance changes were observed at 434 nm (a CC isobestic) with rate constants corresponding to either the fast or the slow phase of the 550-nm transient. This indicates that both of these phases are due to electron transfer to the radical site in CMPI, as determined previously for the reaction with wild-type CcP (Hahm et al., 1992). The fast and slow phase rate constants of the mutants are generally comparable to those of CcP(MI) as shown in Table 2. The fast phase rate constants for the reactions of the E290N and A193F mutants with Ru-27-CC were about half the value for CcP(MI), while the other mutants had rate constants that were more similar to that of CcP(MI). The charge mutants E32Q, E34N, E35Q, and E290N displayed monophasic kinetics for the reaction with Ru-13-CC, compared to biphasic kinetics for the reaction with CcP(MI).

Table 2: Intracomplex Electron Transfer between Ru-CC Derivatives and the CMPI Form of CcP(MI) Mutants[a]

	Ru-13-CC			Ru-27-CC			Ru-72-CC		
mutant	k_f	k_s	f	k_f	k_s	f	k_f	k_s	f
CcP(MI)	6.6	2.3	0.6	48	4.1	0.8	14	3.0	0.6
E32Q	3.4		1.0	56	3.0	0.6	16	3.9	0.4
D34N	3.2		1.0	39	3.2	0.5	10	2.4	0.4
E35Q	2.1		1.0	32	2.0	0.7	15	2.9	0.4
E290N	3.5		1.0	23	1.5	0.6	11	3.5	0.3
E291Q	7.1	1.6	0.6	40	1.2	0.8	14	2.2	0.5
A193F	10.9	1.9	0.3	22	1.9	0.6	12	3.9	0.6

[a] The solutions contained 5 μM Ru-CC and 6–15 μM CMPI mutant in 2 mM sodium phosphate, pH 6, and 1–10 mM aniline at 25 °C. The rate constants were measured from biexponential fits of the 550-nm transients following a single laser flash. The rate constants are in units of 10^3 s^{-1}. The errors are ±20%.

DISCUSSION

The identification of interaction domains used in the reactions between redox proteins presents a number of difficult problems. Equilibrium binding studies are inherently limited to the measurement of binding constants of product complexes, such as the complex between CC(III) and CcP(III,R). Transient substrate complexes, such as the complex between CC(II) and CMPI(IV,R•), are generally too short-lived to characterize directly. It is quite possible that both the interaction domain and binding constant of the substrate complex are different from those of the product complex (Hake et al., 1992). On the other hand, kinetic studies generally yield rate constants that are complicated functions of binding, electron transfer, and dissociation steps. The recent determination of the crystal structure of the complex between yCC(III) and CcP(III,R) revealed both an unexpected interaction domain and a highly efficient potential pathway for electron transfer (Pelletier & Kraut, 1992). An important question is whether the interaction domain for the crystalline product complex is used for the transient substrate complex during the electron transfer reaction. A related question is whether the same interaction domain and electron transfer pathway are used for reduction of both the radical on Trp-191 and the oxyferryl heme in reactions 1 and 2 of Scheme 1.

A number of different techniques have been used to characterize the reaction between cytochrome c and cytochrome c peroxidase (Yonetani et al., 1966; Yonetani & Ray, 1966; Kang et al., 1977; Kim et al., 1990; Summers & Erman, 1988; Hazzard et al., 1987, 1988a–c; Hazzard & Tollin, 1991; Nuevo et al., 1993). The kinetics measured under steady-state conditions are particularly complicated, since at least two complete cycles of complex formation, electron transfer, and product dissociation are involved (Kim et al., 1990; Erman et al., 1991). In addition, the large excess of cytochrome c used in these studies could allow more than one molecule of cytochrome c to bind to cytochrome c peroxidase at a time, raising the possibility of multiple binding domains and electron transfer pathways (Kang et al., 1977, 1978; Erman & Vitello, 1980; Kornblatt & English, 1986; Cheung & English, 1988; Hoffman & Ratner, 1987; Wallin et al., 1991; Everest et al., 1991; Vitello & Erman, 1987; Stemp & Hoffman, 1993; Zhou & Hoffman, 1993). The stopped-flow protocols recently developed by Hahm et al. (1993, 1994) were designed to characterize the reactions of cytochrome c with cytochrome c peroxidase under the simplest conditions compatible with the stopped-flow technique. In protocol A, the first step in the reduction of CMPI is isolated by using an excess of CMPI. The second-order rate constant k_a represents two steps: (a) complex formation between CC(II) and CMPI and (b)

intracomplex electron transfer to the radical. In protocol B, excess CC(II) is used so that both reactions 1 and 2 of Scheme 1 take place. In the mechanism of Scheme 1, the rate constant k_b represents two steps: (a) complex formation between CC(II) and CMPII(IV,R) and (b) intracomplex electron transfer to the oxyferryl heme. In the alternative mechanism of Scheme 2, k_b represents three steps: (a) conversion of CMPII(IV,R) to CMII(III,R$^\bullet$), (b) complex formation between CC(II) and CMPII(III,R$^\bullet$), and (c) intracomplex electron transfer to the radical. At the high ionic strengths used in these studies, it is unlikely that product dissociation of CC(III) from CMPII limits the rate of reaction or that more than one molecule of CC binds to cytochrome *c* peroxidase at a time. Rapid equilibrium conditions are likely to apply, in which case the observed second-order rate constant k_a will be given by $k_a = (k_{form.}/k_{diss})k_{et}$, where $k_{form.}$ and k_{diss} are the rate constants for formation and dissociation of the transient substrate complex and k_{et} is the rate constant for intracomplex electron transfer. For the mechanism of Scheme 2, k_b will be given approximately by $k_b = K(k_{form.}/k_{diss})k_{et}$, where K is the equilibrium constant for reaction 3. It is important to note that a mutation or other perturbation could lead to a change in the observed second-order rate constant by altering any of the constants for the individual steps in the reaction.

Ionic strength dependence studies have been used extensively to characterize electrostatic interactions between redox proteins (Smith & Millett, 1980; Smith et al., 1981; Eltis et al., 1991). Although a number of different approaches have been developed to interpret the ionic strength dependence of second-order rate constants, it is generally agreed that the slope of a plot of log k vs square root of ionic strength is proportional to the strength of the electrostatic interaction (Smith et al., 1981; Eltis et al., 1991; Northrup et al., 1987, 1988). yCC(III) binds much more strongly to CcP than hCC (Kang et al., 1977; Geren et al., 1991; Hahm et al., 1992; Moench et al., 1992). It is therefore not surprising that much higher ionic strength is needed to slow down the reaction of yCC(II) with CMPI enough to measure k_a and k_b by stopped-flow spectroscopy (Figure 2). The fact that the slopes of the ionic strength dependence plots for k_a and k_b are nearly the same indicates that the strength of the electrostatic interactions for these two reactions is nearly the same. This result suggests that the interaction domains used for reactions 1 and 2 in Scheme 1 (or reactions 1 and 4 in Scheme 2) could be the same. Although the values of k_a and k_b for yCC are much larger than those for hCC at a given ionic strength, the slopes in the ionic strength dependence plots are similar. This suggests that the electrostatic component of the interaction energy for yCC is about the same as for hCC, but that the van der Waals and hydrophobic interactions are much larger.

The interaction domain between CcP and CC can be identified more precisely through the effect of point mutations on the electron transfer rates. The charge → amide mutants D34N and E290N have the lowest values of k_a for the reaction between CMPI and yCC. The E35Q mutant has an intermediate value of k_a, while the E32Q and E291Q mutants have the same value as the CcP parent (Table 1). In the crystal structure, the terminal oxygen atoms of CcP Asp-34 and Glu-290 are 3.8 and 3.2 Å from the amino groups of yCC lysines 87 and 73, respectively. The distances between these residues are close enough for significant electrostatic interactions. The terminal carboxyl oxygens of Glu-35 are also located within the predicted interaction domain, but are more distant (4–5 Å) from complementary yCC lysine residues 5 and 87. The charged residues Glu-32 and Glu-291 are only 5 and 8 Å from the nearest complementary lysine resides of yCC, but are not within the interaction domain of the crystalline complex. The absence of an effect of the E32Q and E291Q mutations on k_a is consistent with the fact that the electrostatic interaction falls off very rapidly with increasing distance at the high ionic strengths used in the stopped-flow studies (Smith et al., 1981). The effects of the charge → amide mutations on the bimolecular electron transfer rate k_a are therefore entirely consistent with the interaction domain predicted by the crystalline CcP–yCC complex.

The effects of the charge substitutions on k_a for the reaction with hCC are nearly the same as those seen for yCC (Table 1). These results suggest that hCC uses the same interaction domain for electron transfer as yCC. The crystal structures of the CcP–hCC complex at low ionic strength and the CcP–yCC complex at high ionic strength (Pelletier & Kraut, 1992) also support similar interaction domains for yCC and hCC in solution. Converting the hCC complex to the productive yCC complex would require a rotation of the hCC molecule to bring the hCC heme CBC methyl group into contact with Ala-193 of CcP. Pelletier and Kraut (1992) suggested that this rotation would require the breaking of charge-pair hydrogen bonds between hCC Lys-72 and Lys-87, and CcP Glu-290 and Glu-35, respectively. The strength of these charge-mediated interactions will decrease with increasing ionic strength, allowing hCC to achieve the productive complex more readily. The present results are consistent with this hypothesis.

The results obtained with the A193F mutant are also consistent with the interaction domain predicted by the crystalline CcP–yCC complex. The van der Waals interaction between Ala-193 on CcP and the heme CBC methyl group of yCC is at the center of the interaction domain in the crystalline complex. The A193F mutation adds a phenyl group to CB of Ala-193. Although rotation about CB is technically possible, rotation of the phenyl group out of the interaction domain will be prevented by contact with the neighboring Tyr-229. The introduction of this bulky group produced a 2–4-fold decrease in k_a for both yCC and hCC; this effect may represent a change in the intracomplex electron transfer rate and/or a decrease in the binding constant for the CcP–CC complex.

All of the mutations examined had similar effects on both k_a and k_b (Table 1). This fact provides evidence that the same interaction domain is used for both reactions 1 and 2 of Scheme 1 under the present conditions. In contrast, mutation of Met-230 near the Trp-191 radical decreased the rate of reaction 2, but did not affect reaction 1 (Liu et al., 1994). The effect of both types of mutations can be readily interpreted in the context of the mechanism shown in Scheme 2 described above. Mutations at the interaction domain will affect the common pathway for both reactions 1 and 4, while those near Trp-191 will inhibit reaction 3, the redistribution of the oxidizing equivalent from the Fe^{IV}=O center to Trp-191. The requirement for oxidation of Trp-191 by the Fe^{IV}=O center would also explain the very slow rate of electron transfer from hCC to the Fe^{IV}=O center of the W191F mutant (Mauro et al., 1988).

To examine the changes in electron transfer in more detail, laser flash photolysis was used to measure the rates of intracomplex electron transfer from Ru-hCC derivatives to CcP mutants (Table 2). The reactions measured at low ionic strength were typically biphasic, and both phases represented intracomplex electron transfer from RuCC(II) to the Trp-191 radical in CMPI (Hahm et al., 1992, 1994). The greatest

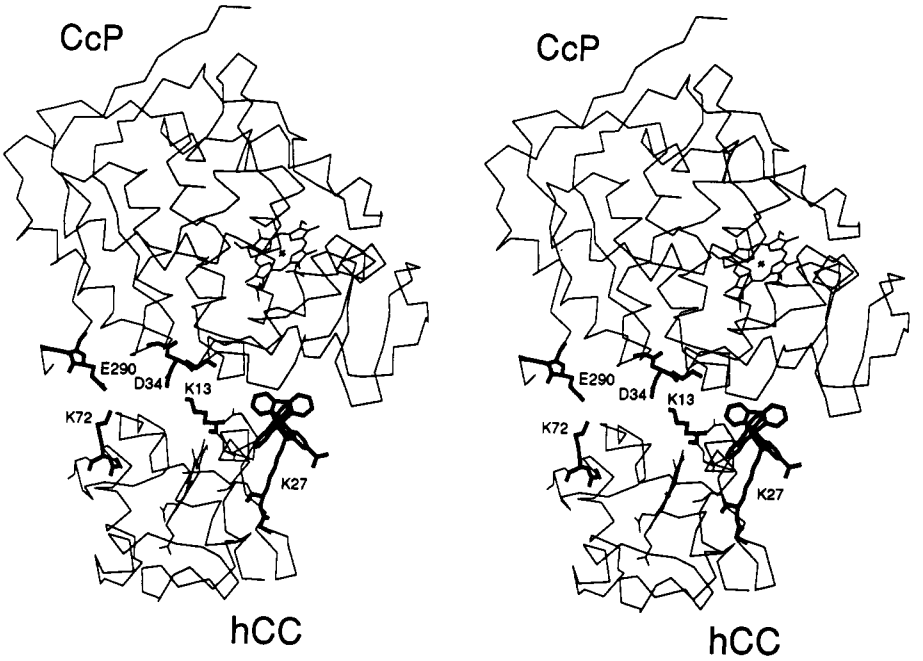

FIGURE 3: X-ray crystal structure of the CcP–hCC complex (Pelletier & Kraut, 1992). A ruthenium label was attached to the amino group of hCC lysine 27 without any steric problems at the complex interface. The side chains of hCC residues K13, K27, and K72, and of CcP residues D34 and E290, are shown and labeled.

changes in the intracomplex electron transfer rate were observed for the E290N and A193F mutants, which had k_{etf} values that were approximately 2-fold less than that of the CcP parent when the Ru-27-CC derivative was used. Two observations indicate that these effects must be interpreted with caution: (1) corresponding decreases in k_{etf} were not seen when Ru-13-CC and Ru-72-CC were employed, and (2) the rate constant k_{etf} for the CcP parent varied by 7-fold between Ru-27-CC and Ru-13-CC (Table 2). Both observations indicate that the rate of intracomplex electron transfer is quite sensitive to the location of the Ru label. A probable basis for this sensitivity is illustrated in Figure 3, which shows a model structure of the Ru label attached to hCC-Lys-27 in the crystal structure of the hCC–CcP complex. It is clear from Figure 3 that the introduction of the Ru label at either Lys-13 or Lys-72 would interfere with the formation of the crystalline CcP–CC complex. Because the Ru label can fit within the predicted complex at Lys-27, the Ru-27-CC derivative should resemble the parent CcP–CC complex more closely than the other derivatives. A ruthenium label can also be attached to Lys-27 in the crystalline yCC–CcP structure without any steric repulsion. The decrease in the rate constant k_{etf} may therefore represent a true effect of the E290N and A193F mutations on intracomplex electron transfer in the native complex, but additional evidence will be required to establish this fact.

The combined influence of CcP mutations and the position of the Ru label on CC is indicated by the disappearance of the slow phase in the reactions between Ru-13-CC and the E32Q, D34N, E35Q, and E290N mutants (Table 2). The slow phase represents the movement of Ru-hCC from a nonproduction orientation to the orientation of the productive electron transfer complex (Hahm et al., 1992). Previous work has shown that as the complex is weakened by increasing ionic strength, the interconversion between productive and nonproductive orientations becomes faster than the rate of electron transfer, and a single phase is seen. The present results suggest that a bulky Ru label at hCC Lys-13, together with the CcP charge mutations, may weaken the CcP–hCC interaction sufficiently to allow rapid exchange between the productive and nonproductive complexes at low ionic strength. The combined influence of the Ru label position and the CcP mutations may be taken as further evidence in favor of an interaction domain that is similar to that observed in the crystal.

The results obtained in the present work are in general agreement with previous experimental work designed to identify the interaction domain between CC and CcP. In the classic experiments of Bechtold and Bosshard (1985), the carboxyl groups on CcP residues 33, 34, 35, 37, 221, 224, and 290 or 291 were shielded from chemical modification by complex formation with 1 equiv of hCC(III). This method of course provides information on the product complex between CC(III) and CcP rather than the transient substrate complex. The shielding effect on residues 34, 35, and 290 is consistent with the Pelletier–Kraut interaction domain for hCC(III), but residues 33 and 37 are a little outside this domain. It does not appear possible for a single molecule of CC that is bound to the acidic patches at residues 33–37 and 290 to also bind to residues 221 and 224. The shielding effect on residues 221 and 224 must therefore arise from multiple binding sites, or long-range conformational effects. Corin et al. (1991, 1993) have found that substitution of Asp-79 or Asp-217 with Lys led to only relatively subtle effects on binding, steady-state electron transfer activity, and intracomplex electron transfer, indicating that these residues are probably not directly involved in the interaction domain. However, substitution of Asp-37 with Lys reduced both the binding strength and the steady-state electron transfer activity more than 10-fold. This is significantly larger than the effects observed for the mutations D34N, E35Q, and E32Q in the present studies, even though Asp-37 is further from the Pelletier–Kraut interaction domain. It is important to note that Asp-37 is not a surface residue, but is instead a very important structural residue. Asp-37 is part of a four-residue stretch of left-handed helical turn, where the carboxylate side chain of Asp-37 forms charge-mediated hydrogen bonds with His-181 of CcP. It is well-known that His-181 is of critical importance in maintaining the structural integrity of the enzyme [cf. Miller et al. (1988, 1990)]. The

Asp-37 → Lys mutation will place a charged ϵ-amino group approximately 2.8 Å from His-181. This will cause substantial steric and charge repulsion problems, since His-181 has an apparent $pK_a > 8.5$ (Gross & Erman, 1985; Dhaliwal & Erman, 1985). The charge repulsion created by this mutation may therefore cause significant structural changes in the interaction domain near Asp-37, or in the 175–191 loop that is anchored by His-181 (Finzel et al., 1984). The observed functional changes in this mutant could thus arise indirectly as a result of these structural changes rather than directly from the change in charge.

CONCLUSIONS

The cytochrome *c* peroxidase mutants E32Q, D34N, E35Q, E290N, E291Q, and A193F were studied to characterize the interaction domain used in the electron transfer reaction with cytochrome *c*. Substitution of Ala-193 at the center of the Pelletier–Kraut interaction domain with phenylalanine led to 2–4-fold decreases in the second-order rate constants for reduction of the radical in CMPI and the oxyferryl heme in CMPII at high ionic strength. Among the charge mutants, D34N and E290N exhibited the largest decreases in the rate constants. The magnitude of the decreases in the rate constants for reduction of the radical and the oxyferryl heme were similar, suggesting that both reactions use the same interaction domain. The effects of the mutants on the reaction with hCC(II) were about the same as those on the reaction with yCC(II), indicating that both cytochromes use the same interaction domain. All of the mutants were able to form stable complexes with Ru-CC derivatives at low ionic strength, allowing the measurement of rate constants for intracomplex electron transfer between the heme group of Ru-CC and the radical in CMPI. The rate constants of the mutants were comparable to those for wild-type CcP(MI), indicating that the mutations caused relatively subtle changes in the orientation of the complex.

These studies indicate that the interaction domain identified in the X-ray crystal structure of the product complex between yCC(III) and CcP(III,R) (Pelletier & Kraut, 1992) is used for the reaction of CC(II) with both the radical in CMPI and the oxyferryl heme in CMPII. These conclusions are valid under the conditions used in the present studies where CMPI interacts with one molecule of CC(II) at a time. There is evidence from other laboratories that, under conditions of excess cytochrome *c* and low ionic strength, a second low-affinity interaction site might be involved in electron transfer or product dissociation (Stemp & Hoffman, 1993; Zhou & Hoffman, 1993; Yi et al., 1994; Erman et al., 1991).

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