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Electron Transfer Between Cytochrome *c* and Cytochrome *c* Peroxidase

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The reaction between cytochrome *c* (CC) and cytochrome *c* peroxidase (CcP) is a very attractive system for investigating the fundamental mechanism of biological electron transfer. The resting ferric state of CcP is oxidized by hydrogen peroxide to compound I (CMPI) containing an oxyferryl heme and an indolyl radical cation on Trp-191. CMPI is sequentially reduced to CMPII and then to the resting state CcP by two molecules of CC. In this review we discuss the use of a new ruthenium photoreduction technique and other rapid kinetic techniques to address the following important questions: (1) What is the initial electron acceptor in CMPI? (2) What are the true rates of electron transfer from CC to the radical cation and to the oxyferryl heme? (3) What are the binding domains and pathways for electron transfer from CC to the radical cation and the oxyferryl heme? (4) What is the mechanism for the complete reaction under physiological conditions?

KEY WORDS: Electron transfer; cytochrome *c*; cytochrome *c* peroxidase; ruthenium.

1. INTRODUCTION

Electron transfer reactions between metalloproteins play critical roles in many important biological processes. However, these reactions are not well understood because they are dependent on so many different factors, including the kinetics of complex formation and dissociation, the electronic properties of the redox centers, the distance and pathway for electron transfer, and the driving force and reorganization energy. The reaction between cytochrome *c* (CC)[3] and cytochrome *c* peroxidase (CcP) has become one of the most widely used testing grounds for investigating the importance of each of these factors. The individual proteins have known X-ray crystal structures (Finzel *et al.*, 1984; Edwards *et al.*, 1987; Louie and Brayer, 1990) and have been characterized extensively by numerous spectroscopic techniques. Most significantly, Pelletier and Kraut (1992) have determined the X-ray crystal structure of a 1:1 complex between yeast iso-1-CC (yCC) and CcP at a physiological ionic strength of 150 mM (Figs. 1, 2). The binding domain includes hydrophobic van der Waals interactions between the two proteins, as well as electrostatic interactions between CC lysine amino groups and CcP carboxylate groups. At the center of the binding domain, the CC heme methyl CBC group is in van der Waals contact with CcP residues Ala-193 and Ala-194. On the basis of the crystal structure, Pelletier and Kraut proposed an electron transfer pathway that extends from the CC heme methyl group through CcP residues Ala-194, Ala-193, and Gly-192 to the indolyl radical on Trp-191, which is in van der Waals contact with the heme group (Figs. 1, 2).

The resting ferric state of CcP is oxidized by hydrogen peroxide to compound I, CMPI(IV,R$^{\bullet}$), which contains an oxyferryl heme Fe(IV)=O and a radical

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[3] Abbreviations: CC, cytochrome *c*; hCC, horse CC; yCC, yeast iso-1-CC; CcP, cytochrome *c* peroxidase; CMPI, CcP compound I; Ru-27-hCC, Ru(bipyridine)$_2$(4-methyl, 4′-carboxybipyridine-lysine-27-cytochrome *c*); Ru-39-yCC, Ru(bpy)$_2$(4,4′-dimethylbipyridine-Cys-39-yeast-iso-1-cytochrome *c*).

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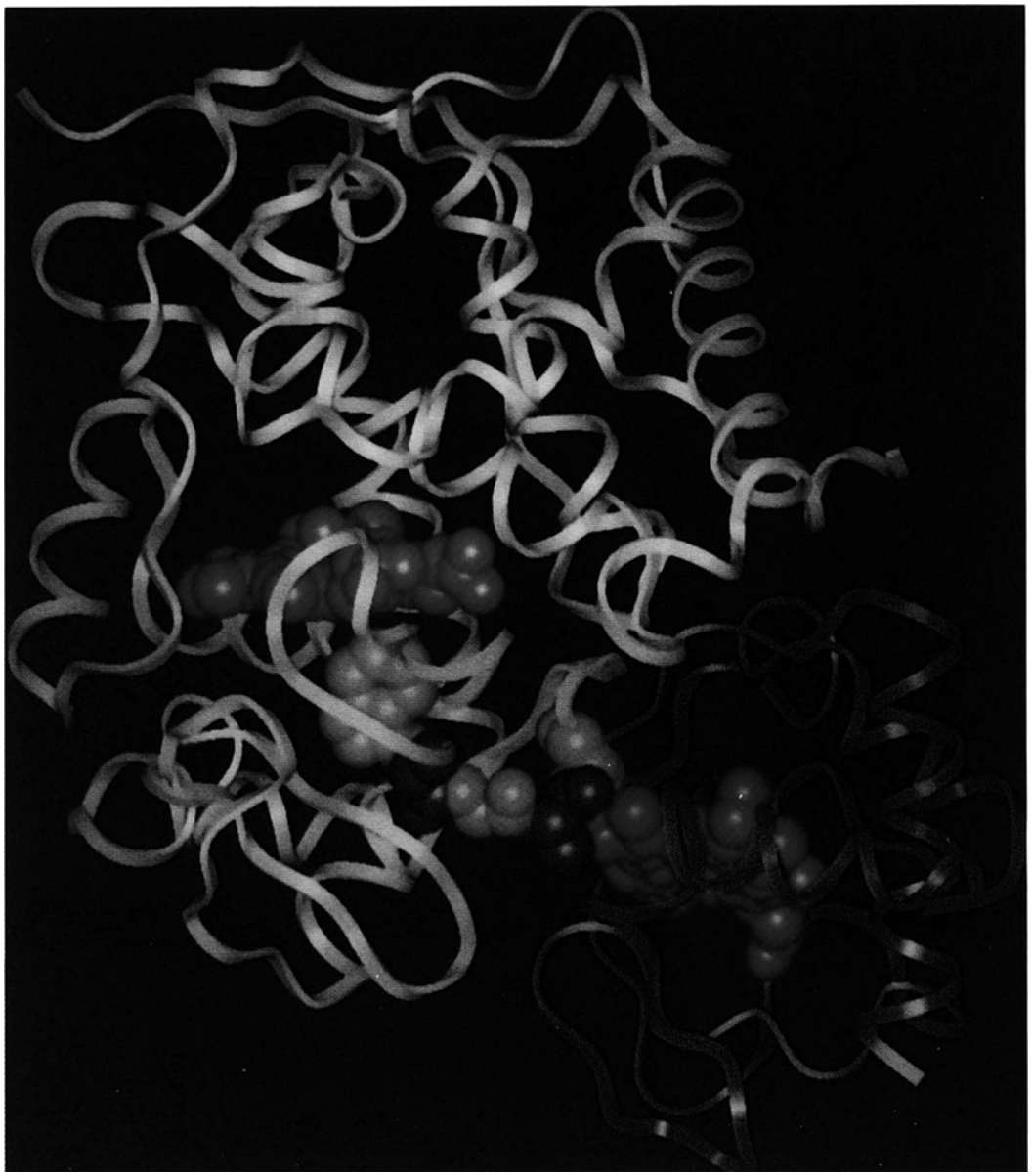


Fig. 1. X-ray crystal structure of the CcP:yCC complex (Pelletier and Kraut, 1992). The heme groups on the two proteins are shown in red CPK models, and the Trp-191 indole group is shown in purple. The proposed electron transfer pathway from the CBC heme methyl group of CC through CcP residues Ala-194, Ala-193, Gly-192 to Trp-191 are shown in CPK models. Ala-194 and Gly-192 are yellow and Ala-193 and the peptide backbone of Trp-191 are green.

located on the indole group of Trp-191 (Mauro *et al.*, 1988; Scholes *et al.*, 1989; Erman *et al.*, 1989; Sivaraja *et al.*, 1989; Fishel *et al.*, 1991). Mutagenesis and crystallographic studies have shown that Arg-48 and His-52 on the distal side of the heme facilitate the reaction of hydrogen peroxide with the ferric heme and stabilize the resulting oxyferryl heme (Fig. 3; Erman *et al.*, 1993; Vitello *et al.*, 1993). The orientation of Trp-191 is maintained by a hydrogen bond network linking Trp-191, Asp-235, and His-175, the heme iron ligand (Fig. 3; Wang *et al.*, 1990). The hydrogen bonding interaction between Asp-235 and His-175 stabilizes the ferric heme iron and probably the oxyferryl heme iron (Wang *et al.*, 1990; Goodin and McRee, 1993; Houseman *et al.*, 1993; Ferrer *et al.*, 1994). Purcell and Erman (1976) have estimated that the redox potential of the oxyferryl heme is about 1.0 V. Miller *et al.* (1994a) have identified a strong cation binding site in

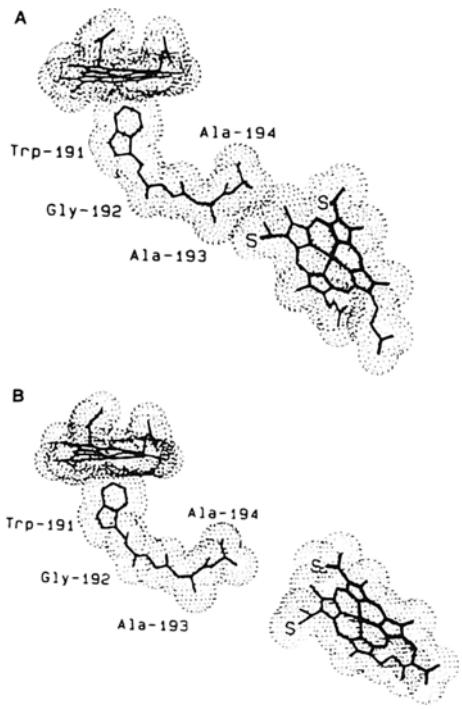


Fig. 2. X-ray crystal structures of the CcP:yCC (A) and CcP:hCC (B) complexes showing the proposed electron transfer pathway from heme *c* (right) to Trp-191 and the CcP heme (left). Reprinted with permission from Pelletier and Kraut (1992).

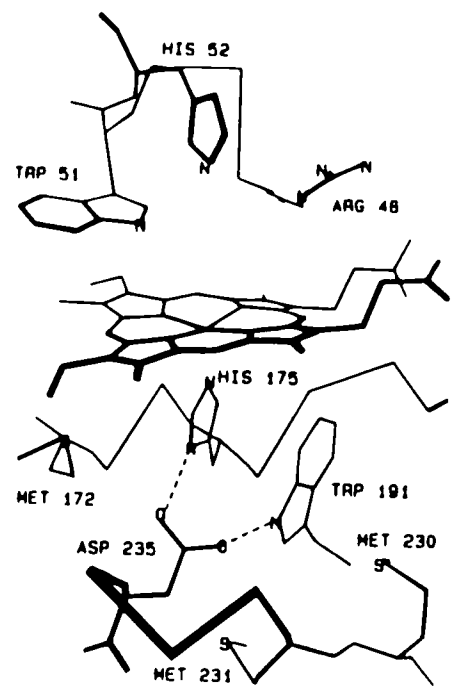


Fig. 3. X-ray crystal structure of CcP showing the heme crevice domain. Reprinted with permission from Wang *et al.* (1990).

the cavity formed when Trp-191 is replaced with Gly. They proposed that the radical on Trp-191 is an indolyl radical cation which is stabilized by the backbone carbonyl oxygen atoms of residues 175 and 177, the carboxylate side chain of Asp-235, and the heme propionates. Miller *et al.* (1994a) estimated the redox potential of the Trp-191 indolyl radical cation to be 0.65 V, compared to 1.0 V for a Trp radical in solution. Fitzgerald *et al.* (1994) found that a series of positively charged imidazolium ions preferentially bind to the cavity in the W191G mutant, and proposed that both steric interactions and positive charge are important factors in the specificity of binding.

A minimal mechanism for the enzyme involves oxidation of CcP to CMPI by hydrogen peroxide, followed by sequential reduction to CMPII and CcP by two molecules of CC(II) as shown in Scheme 1 (Yonetani *et al.*, 1966; Kim *et al.*, 1990):

$$\text{CcP} + \text{H}_2\text{O}_2 \rightarrow \text{CMPI} + \text{H}_2\text{O} \quad (1)$$

$$\text{CMPI} + \text{CC(II)} \rightarrow \text{CMPII} + \text{CC(III)} \quad (2)$$

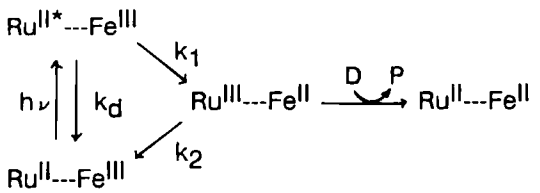
$$\text{CMPII} + \text{CC(II)} + 2\text{H}^+ \rightarrow \text{CcP} + \text{CC(III)} + \text{H}_2\text{O} \quad (3)$$

Scheme 1

Each of the electron transfer reactions (2) and (3) in Scheme 1 involves at least three distinct steps: (a) formation of a transient substrate complex between CC(II) and CMPI (or CMPII), (b) electron transfer within the substrate complex to form the product complex, and (c) dissociation of the product complex. A major goal of kinetics studies is to accurately measure the rate constants for each step in these reactions. The measurement of intracomplex electron transfer has been a particularly difficult problem since the reactions appear to be quite fast.

We have introduced a new method to study interprotein electron transfer that utilizes a photoredox-active tris(bipyridine)ruthenium complex [Ru(II)] covalently attached to one of the proteins (Geren *et al.*, 1991; Hahm *et al.*, 1992). Methods have been developed to specifically attach ruthenium polypyridine complexes to lysine amino groups (Pan *et al.*, 1988; Durham *et al.*, 1989), cysteine sulfhydryl groups (Geren *et al.*, 1991; Willie *et al.*, 1992; Geren *et al.*, 1995), and histidine imidazole groups (Durham *et al.*, 1990). Photoexcitation of a Ru-CC derivative leads to formation of the metal-to-ligand charge-transfer state,

Ru(II*), a strong reducing agent which rapidly transfers an electron to the ferric heme (Scheme 2). A



Scheme 2

sacrificial electron donor D is used to reduce Ru(III) to Ru(II), leaving Fe(II) poised for electron transfer to another protein (Scheme 2). Two important criteria must be satisfied in the design of Ru-CC derivatives optimized for the measurement of interprotein electron transfer (Willie *et al.*, 1992; Geren *et al.*, 1995). The ruthenium group should not interfere with the reaction with the other protein, and the rate of electron transfer from Ru(II*) to heme *c* should be fast compared to the rate of electron transfer from heme *c* to the initial acceptor in the other protein. This requires optimum redox properties for Ru(II*) and an efficient pathway for electron transfer from Ru(II*) to heme *c*. This new technique has been used to measure intracomplex electron transfer between cytochrome *c* and its physiological partners: cytochrome *c* oxidase (Geren *et al.*, 1995; Pan *et al.*, 1993), cytochrome c_1 (Heacock *et al.*, 1993), cytochrome *c* peroxidase (Geren *et al.*, 1991; Hahm *et al.*, 1992; 1994; Miller *et al.*, 1994b; Liu *et al.*, 1994, 1995), and cytochrome b_5 (Willie *et al.*, 1992, 1993; Scott *et al.*, 1994).

In the present review we address several important questions concerning the mechanism of electron transfer between CC and CcP.

2. WHAT IS THE INITIAL ELECTRON ACCEPTOR IN CMPI?

Two forms of the singly oxidized state CMPII have been identified, CMPII(IV,R) containing the oxyferryl heme and CMPII(III,R$^{\bullet}$) containing the radical cation (Coulson *et al.*, 1971; Ho *et al.*, 1983). An important question is whether CC(II) first reduces the oxyferryl heme in CMPI to form CMPII(III,R$^{\bullet}$), or instead reduces the radical cation to form CMPI(IV,R). Coulson *et al.* (1971) originally found that addition of one equivalent of CC(II) to CMPI at pH 7 or above resulted in formation of CMPII(IV,R) as a stable species, indicating initial reduction of the radical cation. In contrast, Summers and Erman (1988) reported that addition of excess horse CC(II) to CMPI at low ionic strength in a stopped-flow spectrophotometer resulted in reduction of the oxyferryl heme to form CMPII (III,R$^{\bullet}$) with a rate constant of 450 s^{-1}, followed by a slow conversion to CMPII(IV,R) before reaction with a second molecule of CC(II) could occur. Hazzard *et al.* (1987) also reported that a substoichiometric amount of CC(II) generated by a flash photolysis technique initially reduced the oxyferryl heme in CMPI at low ionic strength, but did not observe conversion of CMPII(III,R$^{\bullet}$) to CMPII(IV,R) (Hazzard and Tollin, 1991).

The ruthenium photoreduction technique is well suited to study electron transfer in this system, since the CC heme can be reduced very rapidly by Ru(II*), leaving it poised for electron transfer to CMPI. As an example, photoexcitation of a 1:1 complex between horse Ru-27-CC and CMPI at low ionic strength resulted in electron transfer from Ru(II*) to the heme *c* Fe(III) with rate constant $k_1 = 2 \times 10^7$ s^{-1}, followed by electron transfer from heme *c* Fe(II) to CMPI with rate constant $k_{et1} = 6 \times 10^4$ s^{-1} (Fig. 4). Aniline was used as a sacrificial electron donor D to reduce Ru(III) and prevent the back reaction k_2 (Scheme 2). The transient absorption spectrum in the Soret region demonstrated conclusively that the reaction involved reduction of the Trp-191 radical cation rather than the oxyferryl heme (Hahm *et al.*, 1992). Excitation with a second laser pulse resulted in electron transfer from heme *c* Fe(II) to the oxyferryl heme with rate constant $k_{et2} = 350$ s^{-1}. These results demonstrate that Ru-27-CC first transfers an electron to the Trp-191 radical

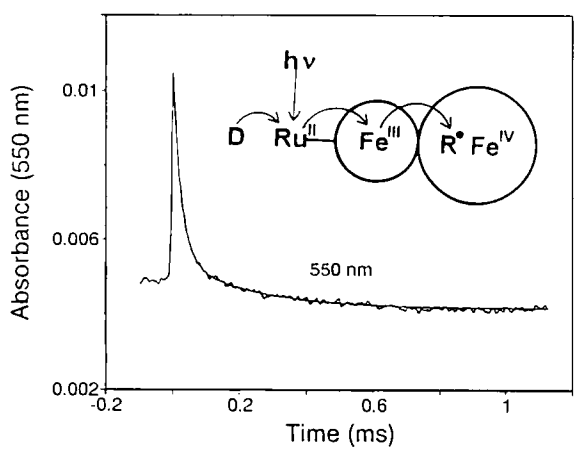


Fig. 4. Photoinduced electron transfer from Ru-27-CC to CMPI. The solution contained 5 μM Ru-27-CC and 5 μM CMPI in 2 mM sodium phosphate, pH 6, and 10 mM aniline at 25°C.

cation in CMPI(IV,R$^{\bullet}$), and then transfers a second electron to the oxyferryl heme in CMPII(IV,R) as shown in Scheme 3:

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

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

Scheme 3

Increasing the ionic strength above 100 mM leads to second-order kinetics as the complex is dissociated. The second-order rate constant of Ru-27-CC is twofold greater than that of native horse CC at high ionic strength, indicating that the ruthenium complex does not interfere in the reaction (Hahm *et al.*, 1992). Similar kinetic results were observed for more than 12 other horse Ru-CC derivatives labeled at different lysine amino groups.

Yeast CcP binds much more tightly to its physiological partner yeast iso-1-CC than to horse CC (Kang *et al.*, 1977). Therefore, yeast Ru-102-CC labeled at Cys-102 on the backside of the molecule was prepared (Geren *et al.*, 1991). The intracomplex rate constant k_{et1} for reduction of the radical in CMPI was found to be greater than 10^5 s^{-1}, while the rate constant k_{et2} for reduction of the oxyferryl heme in CMPII was about 5000 s^{-1} at low ionic strength. Higher ionic strength was needed to dissociate the yeast Ru-102-CC:CMPI complex than the horse Ru-CC:CMPI complexes, consistent with the stronger affinity of yeast CC for CcP. At high ionic strength the second-order rate constant for electron transfer from Ru-102-CC to the radical in CMPI was the same as that of native yeast iso-1-CC, indicating that the ruthenium label did not interfere in the interaction between the two proteins. All of the horse and yeast Ru-CC derivatives obey Scheme 3 at both low ionic strength where intracomplex electron transfer occurs, and at high ionic strength where the reactions are bimolecular.

In order to study the reactions of native horse and yeast CC with CMPI, we developed new stopped-flow protocols to measure the second-order rate constants for each of the one-electron reduction steps in Scheme 1 (Hahm *et al.*, 1993, 1994). High ionic strength was needed to resolve the entire reaction in the stopped-flow instrument. The first step in the reaction was measured by mixing excess CMPI with CC(II), while both steps were measured by mixing excess CC(II) with CMPI. The results demonstrated that native yCC(II) first reduced the Trp-191 radical cation in CMPI with a second-order rate constant

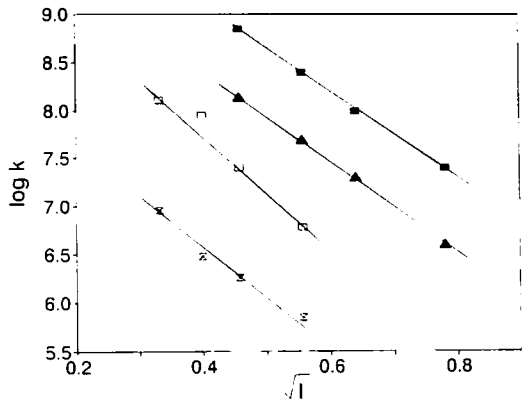


Fig. 5. Ionic strength dependence of the rate constants k_a and k_b for the reaction between yeast and horse CC(II) and CMPI. The buffers contain 5 mM sodium phosphate, pH 7, and 0–400 mM NaCl. yCC:k_a (■), k_b (▲). hCC:k_a (□), k_b (⧖). Reprinted with permission from Miller *et al.* (1994b).

of $k_a = 2.5 \times 10^8$ M^{-1}s^{-1} at 310 mM ionic strength, and then a second molecule of yCC(II) reduced the oxyferryl heme in CMPII with a rate constant of $k_b = 5 \times 10^7$ M^{-1}s^{-1} (Miller *et al.*, 1994b). Both second-order rate constants increased with decreasing ionic strength, as expected for a reaction between oppositely charged proteins (Fig. 5; Miller *et al.*, 1994b). The rate constants for the reaction of hCC with CMPI were smaller than those of yCC at a given ionic strength, but the dependence on ionic strength was nearly the same (Fig. 5). This suggests that the electrostatic contribution to the binding interaction is similar for hCC and yCC, but that the hydrophobic contribution is larger for yCC. Nuevo *et al.* (1993) have also found that hCC reduces CMPI according to Scheme 3 at ionic strengths above 100 mM. The reaction between excess CMPI and CC(II) at low ionic strength was found to be too fast to resolve in the stopped-flow experiments, but spectral analysis of the product formed within 1 ms indicated that the radical cation was reduced rather than the oxyferryl heme (Hahm *et al.*, 1993, 1994; Summers and Erman, 1988; Erman *et al.*, 1993). The results of stopped-flow experiments involving mixing excess CC(II) with CMPI at low ionic strength are more ambiguous, however. Summers and Erman (1988) and Nuevo *et al.* (1993) report initial reduction of oxyferryl heme, whereas experiments in our laboratory indicate initial reduction of the radical cation (Hahm *et al.* 1993, 1994).

3. WHAT IS THE BINDING DOMAIN AND ELECTRON TRANSFER PATHWAY FOR THE REACTION OF CC WITH THE RADICAL AND THE OXYFERRYL HEME IN CMPI?

It is important to independently determine the binding domain used for the reaction of CC(II) with CMPI in solution, since it might be different from that of the crystalline yCC:CcP product complex. Pelletier and Kraut (1992) found that two acidic regions of CcP are close to lysine residues on yCC in the binding domain of the crystalline yCC:CcP complex (Fig. 6). Miller *et al.* (1994b) created the CcP surface mutants E32Q, D34N, E35Q, E290N, and E291Q in order to study the involvement of individual carboxylate side chains in the proposed binding domain by converting them to amides. The A193F mutant was prepared to test the effect of introducing a bulky phenyl group at the center of the Pelletier–Kraut binding domain (Figs. 1, 2). The reactions of hCC and yCC with these mutants were studied using the stopped-flow protocols discussed above (Miller *et al.*, 1994b). Mutations at D34 and E290, which provide carboxylate side chains that are closest to a positively charged yCC lysine residue

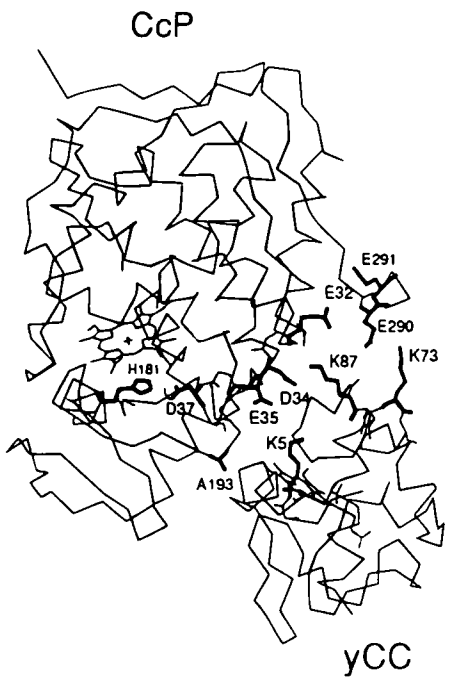


Fig. 6. X-ray crystal structure of CcP:yCC complex (Pelletier and Kraut, 1992). The side chains of CcP residues E32, D34, E35, D37, H181, A193, E290, and E291, and yCC residues K5, K73, and K87 are shown and labeled. Reprinted with permission from Miller *et al.* (1994b).

in the crystalline yCC:CcP complex, caused the largest decreases in the rate constant k_a for reduction of the radical in CMPI (Table I). The k_a values for the mutants E32Q and E291Q that are just outside the binding domain were the same as the CcP parent, indicating that the electrostatic interaction is short-range at high ionic strength (Table I). The effects of the mutations on the rate constant k_b for electron transfer to the oxyferryl heme in CMPII(IV,R) were nearly the same as the effect on k_a. These results indicate that the same binding domain is used for the solution phase electron transfer reactions of yCC(II) with both the radical in CMPI and the oxyferryl heme in CMPII, and closely resembles the binding domain in the crystalline yCC:CcP product complex (Fig. 1,6). The introduction of a bulky phenyl group at Ala-193 in the center of the binding domain decreased both k_a and k_b to 40–45% of the values for the CcP parent. This provides the first experimental evidence that the van der Waals contact between the CBC heme methyl group of CC and Ala-193 may provide the first step in the electron transfer pathway to both the indolyl radical cation on Trp-191 and the oxyferryl heme, as proposed by Pelletier and Kraut (1992). The effects of the mutations on k_a and k_b for hCC were nearly the same as for yCC (Table I), indicating that similar binding domains are used by the two cytochromes. The equilibrium binding constant K_B for the complex between horse Ru-27-CC and CcP was decreased 10-fold by the E290N mutant and more than 10-fold by the E34N mutant at 50 mM ionic strength (Table I; Liu *et al.*, 1994). The X-ray crystal structure of the hCC:CcP complex at low ionic strength is similar to that of yCC:CcP at high ionic strength, except that hCC is rotated slightly to a position where there is a 7 Å gap between the hCC heme methyl CBC group and the Ala-193, Ala-194 loop on CcP (Fig. 2). There are also charge-pair hydrogen bonds between hCC Lys-72 and Lys-87, and CcP Glu-290 and Glu-35, respectively. Pelletier and Kraut (1992) suggested that at high ionic strength these charge-pair hydrogen bonds could break, allowing hCC to rotate to the same orientation observed in the yCC:CcP complex with direct contact between the heme methyl group and Ala-193.

4. WHAT IS THE MECHANISM FOR INTRAMOLECULAR ELECTRON TRANSFER BETWEEN THE OXYFERRYL HEME AND TRP-191 IN CMPII?

Liu *et al.* (1994) investigated the mechanism of reduction of the oxyferryl heme using the ruthenium

Table I. Effect of Charge Mutants on the Reaction Between CC(II) and CMPI[a]

CC	hCC	hCC	yCC	yCC	Ru-27-hCC
Parameter	k_a ($M^{-1}s^{-1}$)	k_b ($M^{-1}s^{-1}$)	k_a ($M^{-1}s^{-1}$)	k_b ($M^{-1}s^{-1}$)	K_B (M^{-1})
CcP(MI)	1.3×10^8	9.0×10^6	2.5×10^8	5.0×10^7	9.5×10^5
Mutant	% control	% control	% control	% control	% control
E32Q	105%	95%	91%	100%	48%
E34N	45%	40%	40%	17%	<5%
E35Q	80%	67%	80%	50%	—
E290N	25%	40%	50%	40%	11%
E291Q	96%	100%	105%	100%	89%
A193F	25%	37%	45%	40%	18%

[a] The second-order rate constants k_a and k_b were measured using stopped-flow spectroscopy in 5 mM sodium phosphate, pH 7, 100 mM NaCl for the reaction with hCC, and 5 mM sodium phosphate, pH 7, 300 mM NaCl for the reaction with yCC (Miller *et al.*, 1994). The rate constants of the CcP mutants are given as a percentage of the rate constant of the CcP(MI) control. The binding constant K_B for complex formation between Ru-27-hCC and the CcP mutant in 2 mM sodium phosphate, pH 6, 50 mM NaCl is given as a percentage of the K_B for the CcP(MI) control (Liu *et al.*, 1995).

photoexcitation technique. Ru-27-CC initially reduced the Trp-191 indolyl radical cation in CMPI(IV,R$^{\bullet}$) with a rate constant of 4.3×10^4 s^{-1}, followed by internal electron transfer from Trp-191 to the oxyferryl heme in CMPII(IV,R) to form CMPII(III,R$^{\bullet}$) with a rate constant of 1100 s^{-1} at pH 5. The extent of conversion of CMPII(IV,R) to CMPII(III,R$^{\bullet}$) decreased from 56% at pH 5 to less than 10% at pH 7 and above. Substitution of Met-230 with Ile did not affect the initial reduction of the radical in CMPI(IV,R$^{\bullet}$), but eliminated the conversion of CMPI-I(IV,R) to CMPII(III,R$^{\bullet}$). Crystallographic studies demonstrated that the position of Trp-191 was unchanged in the M230I mutant (Liu *et al.*, 1994). It was therefore suggested that the sulfur atom of Met-230 stabilizes the indolyl radical cation on Trp-191 and favors conversion of CMPII(IV,R) to CMPII-(III,R$^{\bullet}$) (Fig. 3). Both pH and the M230I mutation affected the extent of the internal electron transfer reaction in parallel with the rate of bimolecular electron transfer from CC(II) to the oxyferryl heme in CMPII-(IV,R) (Liu *et al.*, 1994; Hahm *et al.*, 1994). These results suggest that internal electron transfer from Trp-191 to the oxyferryl heme is a required step in the complete reduction of CMPI to CcP, as shown in Scheme 4. In this mechanism both one-electron interprotein reactions involve reduction of the Trp-191 indolyl radical cation by CC(II) using the Pelletier–Kraut electron transfer pathway.

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

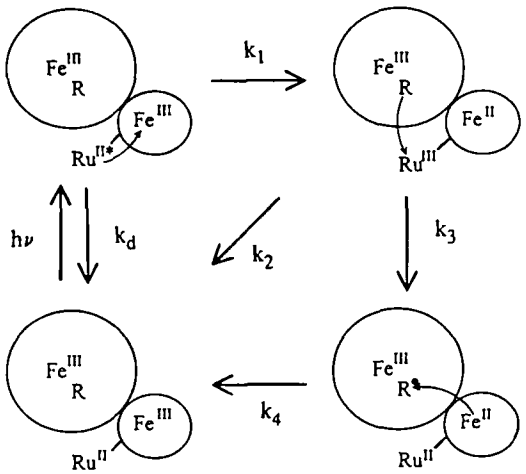
$$\text{CMPII(IV,R)} \rightleftarrows \text{CMPII(III,R}^{\bullet}\text{)} \quad (2)$$

$$\text{CMPII(III,R}^{\bullet}\text{)} + \text{CC(II)} \rightarrow \text{CcP(III,R)} + \text{CC(III)} \quad (3)$$

Scheme 4

5. DOES ELECTRON TRANSFER INVOLVE INTERCONVERSION BETWEEN DIFFERENT BINDING ORIENTATIONS OR DOMAINS?

Liu *et al.* (1995) recently discovered a novel photoinduced electron transfer cycle in the 1:1 complex between horse Ru-27-CC and resting state CcP. The photoexcited Ru(II*) transfers an electron to heme *c* Fe(III) with rate constant $k_1 = 2.3 \times 10^7$ s^{-1} (Scheme 5). The resulting Ru(III), a strong oxidant with a redox potential of 1.3 V, then oxidizes the indole ring of Trp-191 with rate constant $k_3 = 7 \times 10^6$ s^{-1}. The cycle is completed by electron transfer from heme *c* Fe(II) to the Trp-191 indolyl radical cation in CMPII(III,R$^{\bullet}$) with rate constant $k_4 = 6 \times 10^4$ s^{-1}. Molecular modeling indicated that the Ru group attached to Lys-27 can be placed in van der Waals contact with the methyl side chain of Ala-193 without perturbing the crystalline hCC:CcP complex (Fig. 7). This would provide a very efficient pathway for electron transfer between Ru(III) and the indole group of Trp-191, accounting for the



Scheme 5

large value of k_3. The value of k_4 is the same as the value of k_{et1} for electron transfer from heme *c* Fe(II) to the radical in CMPI(IV,R•), the first step in the normal mechanism of the enzyme. Scheme 5 thus provides a unique method to study the electron transfer reaction between CC(II) and the Trp-191 indolyl radical cation in the absence of a sacrificial donor and without the need to use H_2O_2 to prepare CMPI. This

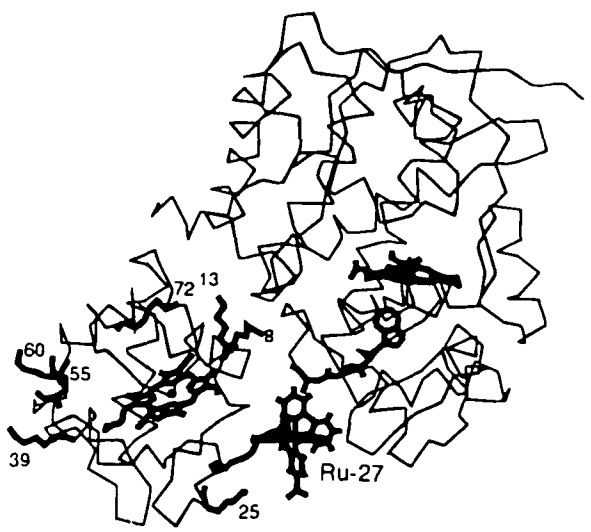


Fig. 7. X-ray crystal structure of the hCC:CcP complex (Pelletier and Kraut, 1992) with an Ru label attached to Nε of CC Lys-27. The conformation of the Ru-Lys-27 group was adjusted to place the Ru label in van der Waals contact with the methyl side chain of Ala-193 without leading to any steric problems at the complex interface. The CC lysine side chains, the CcP residues 191–193, and the hemes are shown in bold. Reprinted with permission from Liu *et al.* (1995).

could potentially allow the study of electron transfer in crystalline complexes of CcP and CC.

It is possible that the CC:CMPI complex formed at low ionic strength is not static, but undergo rapid interconversion between several conformational forms. In this case, the rate of electron transfer could depend on the rates of interconversion as well as the rate of electron transfer in each of the forms. This process, called "conformational gating" by Hoffman and Ratner (1987), may involve a change in the binding orientation of CC at the complex interface such as the difference between the hCC:CcP and yCC:CcP crystalline complexes (Fig. 2). We have recently obtained direct evidence for conformational gating in the horse Ru-27-CC:CcP complex using Scheme 5 to initiate electron transfer. The rate constant k_3 for oxidation of Trp-191 by Ru(III) was nearly independent of both temperature and viscosity. However, the rate constant k_4 for electron transfer from heme *c* to the Trp-191 radical was dramatically decreased by lowering the temperature or increasing the viscosity. These results are consistent with a mechanism in which the dominant form of the complex has the same structure as the crystalline hCC:CcP complex with a 7 Å gap between heme *c* and Ala-193, and with the ruthenium group in contact with the methyl group of Ala-193 as shown in Fig. 7. This form is in rapid equilibrium with a minor form that may have a structure resembling that of the crystalline yCC:CcP complex in which the gap between heme *c* and Ala-193 is closed. The rate constant k_3 will be determined by the rate of electron transfer between Trp-191 and Ru(III) in the dominant form of the complex. In contrast, the rate constant k_4 will be determined by the rate of conversion to the minor form of the complex that allows direct contact between heme *c* and Ala-193, and hence rapid electron transfer. Since k_4 is determined by the rate of reorientation of the complex, it will be highly dependent on both temperature and viscosity. Analysis of the data has indicated that the true rate constant for electron transfer between heme *c* and the Trp-191 radical is substantially greater than 10^5 s^{-1} in the minor form of the complex and less than 10^3 s^{-1} in the major form.

Kang *et al.* (1977) first reported the presence of a second low-affinity binding site for CC on CcP based on gel filtration studies. Stemp and Hoffman (1993) and Zhou and Hoffman (1993, 1994) have recently observed photoinduced electron transfer in 2:1 complexes between yCC and Zn-porphyrin CcP and between Zn-porphyrin-hCC and ferric CcP. However, it was not possible to study the oxidized CMPI state

in either of these studies, and the Trp-191 indole was not in the radical cation form. Under these conditions the normal Pelletier–Kraut electron transfer pathway would be inactivated. It is, therefore, not known whether the second low-affinity CC binding site is active in electron transfer to either the oxyferryl heme or the Trp-191 indolyl radical cation in the native CMPI state. The low-affinity site is most likely to contribute to electron transfer at low ionic strength where dissociation of product CC(III) from the high-affinity site is slow and potentially rate-limiting (Erman *et al.*, 1991). Yi *et al.* (1994) have measured the dissociation rate constant k_d of the 1:1 yCC:CcP complex to be 180 s^{-1} at 10 mM ionic strength using an NMR exchange method. The dissociation rate increased at very high yCC concentrations, leading them to propose a "substrate assisted" product dissociation mechanism, which may involve the low-affinity site. However, it appears unlikely that the low-affinity binding site makes a significant contribution to electron transfer at physiological ionic strength (about 150 mM) where product dissociation from the high-affinity site is rapid. The binding constant for yCC to this site is extremely small at 150 mM ionic strength, $K_B \ll 10^3$ M^{-1} compared to 4.4×10^4 M^{-1} for the high-affinity site (Mauk *et al.*, 1994). Electron transfer through the high-affinity binding site will therefore predominate because of its much larger binding constant and larger rates of electron transfer to both the radical cation and the oxyferryl heme.

6. WHAT ARE THE "TRUE" RATES OF ELECTRON TRANSFER FROM yCC TO THE TRP-191 RADICAL CATION AND THE OXYFERRYL HEME IN CMPI?

Our studies have shown that the rate constant for electron transfer from horse Ru-27-CC to the radical in CMPI is very large, 7×10^4 s^{-1}. However, the rate constant for intracomplex electron transfer from native yCC to the radical in CMPI is expected to be much larger than this value, since the 7 Å gap between the edge of heme *c* and Ala-193 in the hCC:CcP crystal structure is closed in the yCC:CcP complex (Fig. 2). We could only estimate a lower limit of 5×10^4 s^{-1} for the rate constant of yeast Ru-102-CC because the rate of electron transfer from Ru(II*) to heme *c* was slower than the rate of electron transfer from heme *c* to the radical (Geren *et al.*, 1991). We have recently prepared a new yeast Ru-39-CC derivative that appears to satisfy both design requirements discussed in the Introduction (Geren *et al.*, 1995). There is an efficient pathway for electron transfer from the ruthenium complex to heme *c* which involves 13 covalent bonds and one hydrogen bond (Fig. 8). The rate constants for electron transfer from Ru(II*) to heme *c* Fe(III) and for the back reaction from Fe(II) to Ru(III) are $k_1 = 6 \times 10^5$ s^{-1} and $k_2 = 1.0 \times 10^6$ s^{-1} (Geren *et al.*, 1995). The second-order rate constant for electron transfer to CMPI at high ionic strength is the same as that of native yCC, indicating that the Ru complex does not interfere with the reaction. We have recently estimated that the rate constant for electron transfer from Ru-39-yCC to the radical in CMPI is approximately 2×10^6 s^{-1} (Geren *et al.*, unpublished observations). This value is in good agreement with a theoretical estimate for the rate of electron transfer via the Pelletier–Kraut pathway using the dominant pathway model developed by Beratan *et al.* (1992). A comprehensive understanding of electron transfer in the yCC:CcP complex will require accurate measurement of the reorganization energy as well as the rate constant. In addition, it will be important to determine whether conformational gating occurs in the yeast Ru-39-CC:CcP complex.

Fig. 8. Structure of Ru-39-yCC based on the X-ray crystal structure of yCC (Louie and Brayer, 1990). The proposed pathway for electron transfer extends from Ru-39 through the peptide backbone of Ser-40 and Gly-41 where there is a hydrogen bond from the amide nitrogen of Gly-41 to the heme propionate group. Reprinted with permission from Geren *et al.* (1995).

The binding constant of the yCC:CcP complex is considerably larger than that of the hCC:CcP complex, and the structure of the yCC:CcP crystalline complex indicates that it is optimized for efficient electron transfer from heme *c* to the Trp-191 radical. We, therefore, expect that conformational gating is less likely to be a factor in the yCC:CcP complex. The large value of the rate constant in the Ru-39-CC:CMPI complex, 2×10^6 s^{-1}, supports this prediction.

The very much slower rate of reduction of the oxyferryl heme, approximately 5000 s^{-1}, is difficult to explain given the proximity of Trp-191 to the heme. Roe and Goodin (1993) have suggested that the transition state for reduction of the oxyferryl heme is controlled by proton transfer to the oxygen atom on Fe(IV)=O to form Fe(III)–OH, or release of the oxygen atom as H_2O. They propose that the indole group of Trp-51 may form a hydrogen bond with the ferryl oxygen, thus stabilizing the oxyferryl heme toward reduction.

7. WHAT IS THE MECHANISM FOR THE ELECTRON TRANSFER REACTION BETWEEN CC AND CMPI UNDER PHYSIOLOGICAL CONDITIONS?

The reactions of CC with its redox partners in intact mitochondria occur under physiological conditions of about 150 mM ionic strength, and CC concentrations of 100 to 700 μM (Gupte and Hackenbrock, 1988). Under these conditions, the reaction between yCC and CMPI can be described by the minimal mechanism shown in Scheme 6:

$$\text{CMPI(IV,R}^{\bullet}\text{)} + \text{CC(II)} \underset{k_d}{\overset{k_f}{\rightleftarrows}} \text{CMPI(IV,R}^{\bullet}\text{):CC(II)} \xrightarrow{k_{et1}} \text{CMPII(IV,R):CC(III)} \underset{k_f}{\overset{k_d}{\rightleftarrows}} \text{CMPII(IV,R)} + \text{CC(III)}$$

$$\text{CMPII(IV,R)} + \text{CC(II)} \underset{k_d}{\overset{k_f}{\rightleftarrows}} \text{CMPII(IV,R):CC(II)} \xrightarrow{k_{et2}} \text{CcP(III,R):CC(III)} \underset{k_f}{\overset{k_d}{\rightleftarrows}} \text{CcP(III,R)} + \text{CC(III)}$$

Scheme 6

Each one-electron reduction reaction requires formation of a substrate complex, electron transfer within the substrate complex to form the product complex, and dissociation of the product complex. For simplicity we shall assume that the formation and dissociation rate constants k_f and k_d are approximately independent of the redox states of CC and CcP. This appears reasonable, since the equilibrium binding constant K_B for the complexes of yCC with CcP or MgCcP is not significantly affected by the redox state of yCC (Mauk *et al.*, 1994; McLendon *et al.*, 1993). In addition, X-ray crystallography has shown that the surface structure of CMPI is the same as that of CcP, indicating that k_d and k_f are unlikely to be affected by the redox state of CcP.

Reasonable estimates can be made for each of the rate constants in Scheme 3 using information from a wide range of experimental techniques. Geren *et al.* (1991) found that the intracomplex rate constant k_{et1} for electron transfer from Ru-102-yCC to the radical in CMPI was greater than 10^5 s^{-1} throughout the ionic strength range from 2 mM to 150 mM. Although we have not yet measured k_{et1} for Ru-39-CC as a function of ionic strength, it is reasonable to assume that k_{et1} remains constant at 2×10^6 s^{-1} from low ionic strength up to physiological ionic strength. The value of k_{et2} for electron transfer from Ru-102-yCC to the oxyferryl heme in CMPII is approximately 5000 s^{-1} at low ionic strength, pH 7, and we assume that it also remains constant up to physiological ionic strength. The second-order rate constant for the reaction of native yCC(II) with the radical in CMPI was found to be $k_a = 1 \times 10^9$ M^{-1} s^{-1} at 200 mM ionic strength, and to increase with further decreases in ionic strength (Fig. 5; Miller *et al.*, 1994b). The formation rate constant k_f must be greater than or equal to k_a, so a reasonable estimate for k_f at a physiological ionic strength of 150 mM is about 2×10^9 M^{-1} s^{-1}, which is close to the diffusion limit. From the relation $K_B = k_f/k_d$, k_d can be estimated to be about 4×10^4 s^{-1} at 150 mM ionic strength using $k_f = 2 \times 10^9$ M^{-1} s^{-1}, and $K_B = 4.4 \times 10^4$ M^{-1} (Mauk *et al.* 1994). It is clear from these estimates that neither reduction of the radical cation nor product dissociation is rate limiting under physiological conditions. Instead, the rate-limiting step is likely to be intracomplex electron transfer from yCC(II) to the oxyferryl heme, k_{et2}. This conclusion is consistent with steady-state experiments at high ionic strength (Erman *et al.*, 1991).

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