Oxidation of Yeast Iso-1 Ferrocytochrome *c* by Yeast Cytochrome *c* Peroxidase Compounds **I** and **II**. Dependence upon Ionic Strength†

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Received February 23, 1995; Revised Manuscript Received May 18, 1995®

ABSTRACT: The reduction of cytochrome *c* peroxidase compound **I** by excess yeast iso-1 ferrocytochrome *c* is biphasic. Two pseudo-first-order rate constants can be measured by stopped-flow techniques. The fastest rate process is the reduction of cytochrome *c* peroxidase compound **I** to compound **II**, and the slower process is the reduction of **II** to the native enzyme. The yeast iso-1 ferrocytochrome *c* concentration dependence of the reduction of cytochrome *c* peroxidase compound **I** to compound **II** is consistent with a mechanism involving two binding sites for cytochrome *c* on cytochrome *c* peroxidase. Electron transfer from cytochrome *c* bound at the high-affinity binding site to the Fe(IV) site in cytochrome *c* peroxidase compound **I** is dependent upon ionic strength, increasing from 15 ± 6 to 2000 ± 100 s^{-1} over the ionic strength range 0.01−0.20 M. The reduction rate of the Fe(IV) site in the 2:1 yeast iso-1 ferrocytochrome *c*/cytochrome *c* peroxidase compound **I** complex is essentially independent of ionic strength with a value of 3800 ± 300 s^{-1}. The Fe(IV) site in cytochrome *c* peroxidase compound **I** is preferentially reduced by yeast ferrocytochrome *c* between 0.01 and 0.20 M ionic strength while the Trp-191 radical is preferentially reduced above 0.30 M ionic strength. The association rate constant for the binding of yeast iso-1 ferrocytochrome *c* to cytochrome *c* peroxidase compound **I** can be evaluated and varies from a remarkable 1×10^{10} M^{-1} s^{-1} at 0.01 M ionic strength to 1.2×10^5 M^{-1} s^{-1} at 1.0 M ionic strength. Between 0.01 and 0.20 M ionic strength, the reduction of cytochrome *c* peroxidase compound **II** to the native enzyme is anomalous. The reaction is independent of the cytochrome *c* concentration and directly proportional to the initial cytochrome *c* peroxidase compound **I** concentration.

In the preceding paper (Matthis & Erman, 1995), we have shown that steady-state kinetic studies of the cytochrome *c* peroxidase-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide (eq 1) are consistent with a

$$H_2O_2 + 2H^+ + 2c^{2+} \xrightarrow[CcP]{} 2c^{3+} + 2H_2O \qquad (1)$$

mechanism involving two cytochrome *c*-binding sites on cytochrome *c* peroxidase (CcP).[1]

As in the classical peroxidase mechanism (Bosshard et al., 1991), CcP reacts with hydrogen peroxide to generate a higher-oxidation-state intermediate, cytochrome *c* peroxidase compound **I** (CcP-**I**) (eq 2). The reaction shown in eq 2 is

$$CcP + H_2O_2 \rightarrow CcP\text{-}\mathbf{I} + H_2O \qquad (2)$$

a two-equivalent oxidation of the native Fe(III) state of CcP and is essentially irreversible. CcP-**I** is remarkably stable with a half-life of over 6 h at pH 6 (Erman & Yonetani, 1975). Because of the ability to form CcP-**I** stoichiometrically and its extreme stability, the properties of CcP-**I** have been well characterized. CcP-**I** is an oxyferryl, Fe(IV) species with an oxidized amino acid radical localized on Trp-191 (Erman et al., 1989; Sivaraja et al., 1989). The crystal structure of CcP-**I** has been determined (Edwards et al., 1987; Fulhop et al., 1994).

Excess ferrocytochrome *c* reduces CcP-**I** by two successive one-electron transfers, generating a transient intermediate, CcP compound II (CcP-**II**) (eqs 3 and 4). CcP-**II** is produced

$$CcP\text{-}\mathbf{I} + c^{2+} + H^+ \rightarrow CcP\text{-}\mathbf{II} + c^{3+} + H_2O \qquad (3)$$

$$CcP\text{-}\mathbf{II} + c^{2+} + H^+ \rightarrow CcP + c^{3+} \qquad (4)$$

by the one-equivalent reduction of CcP-**I** (eq 3) and can exist in either of two forms depending upon whether the Fe(IV) heme or the Trp-191 radical is reduced (Coulson et al., 1971). We have designated the form that retains the Fe(IV) site as CcP-$\mathbf{II}_F$ and the form that retains the Trp-191 radical as CcP-$\mathbf{II}_R$. CcP-$\mathbf{II}_F$ is ~50 times more stable than CcP-$\mathbf{II}_R$ between pH 7 and 7.5, and the two species equilibrate with a rate of ~5 s^{-1} at room temperature (Ho et al., 1983, 1984; Summers & Erman, 1988).

Reduction of CcP-**I** to either CcP-$\mathbf{II}_F$ or CcP-$\mathbf{II}_R$ depends upon the experimental conditions and the nature of the cytochrome *c*. Horse cytochrome *c* reduces CcP-**I** to CcP-$\mathbf{II}_R$ at low ionic strength ($I \leq 0.05$ M) while CcP-$\mathbf{II}_F$ is the product at high ionic strength ($I \geq 0.10$ M) (Nuevo et al. 1993). In this paper, we report that reduction of CcP-**I** by yeast iso-1 ferrocytochrome *c* produces CcP-$\mathbf{II}_R$ between 0.01 and 0.2 M ionic strength and CcP-$\mathbf{II}_F$ above 0.3 M ionic strength. Horse ferrocytochrome *c*, rapidly generated by flash photolysis of ferricytochrome *c* in the presence of flavins, reduces the Fe(IV) site in CcP-**I** prior to the radical

† This work was supported in part by Research Grant NSF MCB-9121414.

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® Abstract published in *Advance ACS Abstracts,* July 15, 1995.

[1] Abbreviations: CcP, cytochrome *c* peroxidase; CcP-I, cytochrome *c* peroxidase compound **I**; CcP-II, cytochrome *c* peroxidase compound **II**; CcP-$\mathbf{II}_F$, cytochrome *c* peroxidase compound **II** containing an oxyferryl Fe(IV) heme; CcP-$\mathbf{II}_R$, cytochrome *c* peroxidase compound **II** containing an oxidized Trp-191 radical; c^{2+}, ferrocytochrome *c*; c^{3+}, ferricytochrome *c*; *I*, ionic strength.

0006-2960/95/0434-9991$09.00/0

site at low ionic strength, pH 7.0 (Hazzard et al., 1987, 1988). However, flash photolysis, using specifically modified yeast and horse cytochrome *c*, preferentially reduces the radical site in CcP-**I**, producing CcP-$\mathbf{II_F}$ irrespective of ionic strength (Geren et al., 1991; Hahm et al., 1992, 1993, 1994; Miller et al., 1994; Liu et al., 1994).

The two modes of CcP-**I** reduction, initial reduction of either the Fe(IV) or the Trp-191 radical site, could be related to differences in the interaction between cytochrome *c* and CcP. Pelletier and Kraut (1992) have determined crystal structures of 1:1 complexes of both horse cytochrome *c*/CcP and yeast iso-1 cytochrome *c*/CcP. There are small but significant differences in the interaction between the two proteins in these complexes. These differences could be due to the difference in the structure of the cytochrome *c* or due to the conditions of crystallization. The horse cytochrome *c*/CcP complex was crystallized from low-salt buffer (5 mM potassium phosphate) while the yeast cytochrome *c*/CcP complex was crystallized from a high-salt buffer (150 mM NaCl).

As discussed in the previous paper, there is growing evidence that yeast iso-1 cytochrome *c* interacts with CcP at multiple sites (Matthis & Erman, 1995). In this paper, we report on a stopped-flow investigation of the oxidation of yeast iso-1 ferrocytochrome *c* by CcP-**I** as a function of reactant concentration (0.5−100 μM cytochrome *c*) and ionic strength (0.01−1.0 M). The concentration dependence of ferrocytochrome *c* oxidation is complex and can be explained most simply by a two-binding site mechanism. Electron transfer to the low-affinity binding site dominates at low ionic strength but becomes of minor importance above ~0.10 M ionic strength. The Fe(IV) group in CcP-**I** is reduced prior to the Trp-191 radical at ionic strengths less than 0.3 M. The radical site is reduced first above 0.3 M ionic strength.

Reduction of CcP-$\mathbf{II_R}$ (produced transiently from CcP-**I**) to CcP by yeast iso-1 ferrocytochrome *c* is identical to that reported previously for reduction of CcP-$\mathbf{II_R}$ by horse ferrocytochrome *c* (Summers & Erman, 1988). The observed pseudo-first-order rate constant for CcP-$\mathbf{II_R}$ reduction is independent of the yeast iso-1 ferrocytochrome *c* concentration and depends linearly on the initial concentration of CcP-**I**. This rate constant is also independent of ionic strength between 0.01 and 0.20 M ionic strength. The reduction of CcP-$\mathbf{II_R}$, as observed in these stopped-flow experiments, is too slow to account for the steady-state turnover of CcP during the catalytic oxidation of yeast iso-1 ferrocytochrome *c* (Matthis & Erman, 1995).

MATERIALS AND METHODS

CcP and yeast iso-1 cytochrome *c* were purified and protein solutions prepared as described in the accompanying paper (Matthis & Erman, 1995). Potassium phosphate/potassium nitrate buffers, pH 7.5, were prepared as described previously (Matthis & Erman, 1995). The potassium phosphate concentration in the buffers was kept at ≤10 mM to minimize phosphate binding to cytochrome *c*. Ionic strength was adjusted by the use of KNO_3 as required to make buffers with ionic strengths ranging from 0.01 to 1.0 M.

Kinetic experiments were carried out using a Hi-Tech Scientific PQ/SF-53 stopped-flow spectrofluorometer. The reactant reservoirs, drive syringes, mixer, and observation chamber were thermostated with a circulating water bath at

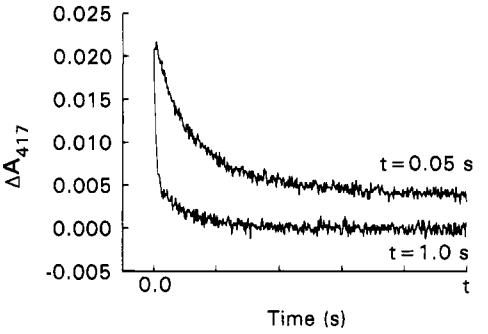

FIGURE 1: Change in absorbance at 417 nm upon mixing 0.19 μM CcP-**I** and 2.0 μM yeast iso-1 ferrocytochrome *c* in a stopped-flow apparatus. The reaction conditions were pH 7.5 in 10 mM potassium phosphate buffer with added KNO_3 to bring the ionic strength to 0.20 M. The upper and lower traces were acquired on 50 ms and 1 s time scales, respectively. The absorbance change was fit to a two-exponential equation with rate constants of 154 and 9.2 s^{-1}. The amplitudes were 0.016 and 0.005 absorbance units for the fast and slow phases, respectively. The total absorbance change is 93% of the theoretical value for the complete reduction of CcP-**I** by ferrocytochrome *c* at this wavelength. The fast phase of the reaction is associated with reduction of the Fe(IV) site in CcP-**I**.

25 ± 1 °C. CcP-**I** was prepared by mixing stoichiometric amounts of CcP and hydrogen peroxide and immediately placed in one syringe of the stopped-flow apparatus. Reduction of CcP-**I**, by mixing with yeast iso-1 ferrocytochrome *c* contained in the second drive syringe, was observed within 1 min of CcP-**I** formation. The reactions were investigated under pseudo-first-order conditions with cytochrome *c* in excess. Final cytochrome *c* concentrations ranged from about 0.5 to 100 μM. Final CcP-**I** concentrations varied from about 0.09 to 4 μM. The reaction was monitored at a variety of wavelengths, including 417, 432, 454, and 550 nm. The wavelength accuracy is ±2 nm.

RESULTS

Initial Site of Reduction in CcP Compound I by Yeast Iso-1 Ferrocytochrome c. When CcP-**I** is rapidly mixed with an excess of c^{2+}, CcP-**I** is reduced to the native, Fe(III) state via a second enzyme intermediate, CcP-**II** (eqs 3 and 4). Reduction of CcP-**I** to CcP-**II** is faster than reduction of CcP-**II** to the native enzyme, and two pseudo-first-order rate processes are observed when the reaction is monitored at 417 nm, the maximum in the difference spectrum between c^{2+} and c^{3+} (Figure 1). The site of reduction in CcP-**I** can be determined from the amplitude of the two kinetic phases. Reduction of the Fe(IV) site in CcP-**I** to Fe(III) makes a very large contribution to the absorbance change at 417 nm while reduction of the Trp-191 radical causes a minor change in the absorbance at 417 nm (Coulson et al., 1971; Ho et al., 1983). The observation that the amplitude of the fast reaction phase in Figure 1 is larger than the amplitude of the slower phase is consistent with preferential reduction of the Fe(IV) site in CcP-**I**. The amplitude of the fast phase is due to the combined, additive absorbance decrease associated with the oxidation of c^{2+} to c^{3+} and reduction of the Fe(IV) site in CcP-**I** to Fe(III) in CcP-$\mathbf{II_R}$. The absorption change of the slower phase of the reaction is due primarily to oxidation of c^{2+} since reduction of the Trp-191 radical causes little change in absorbance at 417 nm. When the reaction is monitored at 432 nm, near an isosbestic point in the c^{2+}/c^{3+} spectra, only the fast phase of the reaction is observed,

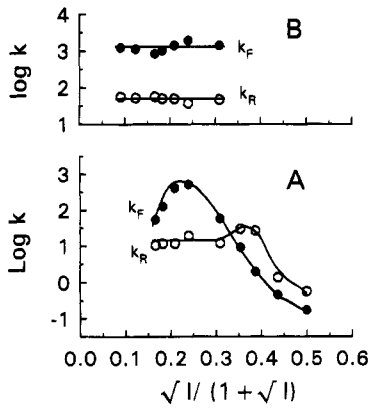

FIGURE 2: Ionic strength dependence of the reduction of the Fe(IV) and radical sites in CcP-**I** at low yeast iso-1 ferrocytochrome *c* concentrations: (●) value of k_F for reduction of the Fe(IV) site; (○) value of k_R for reduction of the Trp-191 radical. (A) 0.20 μM CcP-**I**; 0.98 μM yeast iso-1 ferrocytochrome *c*. (B) 1.0 μM CcP-**I**; 100 μM yeast iso-1 ferrocytochrome *c*. All reactions were carried out in 4.2−10 mM potassium phosphate buffer, pH 7.5, with added KNO_3 to adjust ionic strength.

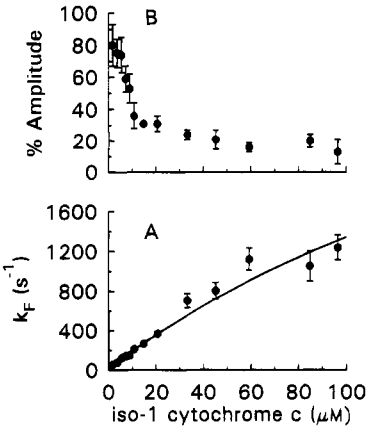

FIGURE 3: (A) Dependence of k_F, the rate of reduction of the Fe(IV) site in CcP-**I**, on the yeast iso-1 ferrocytochrome *c* concentration in 0.01 M ionic strength buffer, pH 7.5. The solid line was calculated from eq 5 of the text. (B) Amplitude of the absorbance change for the fast kinetic phase observed in the stopped-flow instrument in percentage of the theoretical absorbance change for the reaction.

confirming the conclusion that the Fe(IV) site of CcP-**I** is reduced prior to the Trp-191 radical site under the conditions of the experiment shown in Figure 1.

It was previously found that the initial site of reduction in CcP-**I** by horse ferrocytochrome *c* depended upon the ionic strength of the solution (Neuvo et al., 1993). Horse ferrocytochrome *c* reduces the Fe(IV) site of CcP-**I** prior to the radical site below ~0.07 M ionic strength; however, the radical site is preferentially reduced at higher ionic strength. Figure 2 shows the results of a similar investigation with yeast iso-1 ferrocytochrome *c*. The ionic strength dependence of the pseudo-first-order rates constants for the reduction of the Fe(IV) and Trp-191 radical sites using 0.20 ± 0.01 μM CcP-**I** and 0.98 ± 0.01 μM yeast iso-1 ferrocytochrome *c* are shown in Figure 2A. Figure 2B shows a similar experiment except that the CcP-**I** concentration is 1.0 ± 0.1 μM and yeast iso-1 ferrocytochrome *c* concentration is 100 ± 5 μM. The observed pseudo-first-order rate constants for reduction of the Fe(IV) and Trp-191 radical sites are labeled k_F and k_R, respectively. These labels are used irrespective if the sites are reduced in **I** or **II**.

At the higher reactant concentrations (Figure 2B), it is clear that the Fe(IV) site is oxidized prior to the Trp-191 radical site over the entire ionic strength range investigated, 0.01 and 0.2 M *I*. Under these conditions, CcP-**I** is reduced to CcP-$\mathbf{II}_R$.

After completion of our studies on the ionic strength dependence (0.01−0.20 M) of the reduction of CcP-**I** by yeast iso-1 ferrocytochrome *c* and while this paper was in preparation, Liu et al. (1994) reported that the radical site in CcP(MI) compound **I** was reduced prior to the Fe(IV) site by yeast iso-1 ferrocytochrome *c* at 0.40 M ionic strength between pH 6 and 8. Since this was contrary to what we observed for wild-type CcP compound **I** at 0.20 M *I* and below, we extended our studies to 1.0 M *I*, using low concentrations of yeast iso-1 ferrocytochrome *c* (Figure 2A). At 0.3 M *I* and above, reduction of the radical site occurs prior to the Fe(IV) site, consistent with the observations of Miller et al. (1994). Between 0.04[2] and 0.2 M *I*, the Fe(IV) site is reduced faster than the radical site just as observed at higher cytochrome *c* concentrations (Figure 2B). There is a switch in the mechanism of electron transfer between 0.2 and 0.3 M *I*. At the lower ionic strength, CcP-**I** is reduced to CcP-$\mathbf{II}_R$, and at the higher ionic strengths, reduction of CcP-**I** by yeast iso-1 ferrocytochrome *c* produces CcP-$\mathbf{II}_F$.

Concentration Dependence of the Rate of Reduction of the Fe(IV) Site in CcP-I. The pseudo-first-order rate constant for the reduction of CcP-**I** to CcP-$\mathbf{II}_R$, k_F, was measured as a function of concentration between 0.5 and 100 μM c^{2+} and between 0.1 and 1 μM CcP-**I**. The reaction is independent of the initial CcP-**I** concentration but depends upon the c^{2+} concentration. Figure 3A shows values of k_F as a function of c^{2+} concentration at $I = 0.01$ M. The values of k_F range from 10 ± 4 to 1200 ± 100 s^{-1} between 0.49 and 96 μM c^{2+}. The very large rates at high c^{2+} are near the limits of those that can be measured by our stopped-flow instrument, and a considerable portion of reaction is lost in the instrumental dead time for the faster reactions. In Figure 3B, the amplitude of the reaction, as a percent of the expected absorbance change, is plotted versus the c^{2+} concentration. At the lowest concentrations, greater than 80% of the expected amplitude is observed but only ~15% of the reaction is observed at the highest c^{2+} concentrations. However, the continuity of the rate constants and amplitude as a function of the c^{2+} concentration suggests that the rates we measure are a true reflection of the reduction of the Fe(IV) site in CcP-**I**.

At 0.01 M *I*, the value of k_F varies almost linearly with increasing c^{2+}; there is just a hint of saturation of the observed rate constant above 80 μM cytochrome *c*. The data at 0.02 M *I* (not shown) are very similar to those at 0.01 M *I*. This was very surprising initially, in light of the very tight binding between CcP and yeast cytochrome *c* (Corin et al., 1991). However, we now believe that, at low ionic strength, yeast iso-1 ferrocytochrome *c* bound at the high-affinity binding site on CcP-**I** is ineffectual in reducing either the Trp-191 radical or the Fe(IV) site in CcP-**I**. The rates shown in Figure 3A are essentially those for the reduction of the

[2] Below 0.040 M ionic strength, the rate of reduction of CcP-**I** and CcP-**II** using 1 μM yeast iso-1 ferrocytochrome *c* is comparable, and it is difficult to separate the rate of reduction of the radical and Fe(IV) sites. However, above 2 μM ferrocytochrome *c*, it is clear that the Fe(IV) site is reduced prior to the radical site below 0.04 M ionic strength.

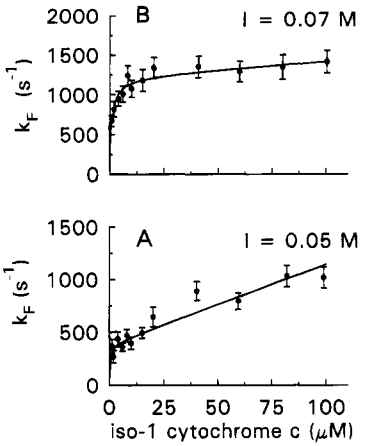

FIGURE 4: Dependence of k_F on the concentration of yeast iso-1 ferrocytochrome *c* concentration: (A) 0.050 M ionic strength; (B) 0.070 M ionic strength. The solid lines were calculated from eq 5 of the text.

Fe(IV) site by yeast iso-1 ferrocytochrome *c* interacting at the low-affinity, secondary cytochrome *c*-binding site on CcP-**I**.

Plots of k_F versus yeast iso-1 ferrocytochrome *c* at intermediate ionic strength are quite different from those at lower ionic strength. The data at 0.05 and 0.07 M *I* are shown in Figure 4A,B, respectively. There is a very rapid increase in k_F at low concentrations of cytochrome *c*, reaching a value of 400 s^{-1} at 4 μM cytochrome *c* at 0.05 M *I* and a value of ~1000 s^{-1} at 10 μM cytochrome *c* at 0.07 M *I*. The observed rate constants then increase in a linear manner up to the highest cytochrome *c* used in this study, ~100 μM. The shapes of the plots shown in Figure 4 are remarkably similar to the steady-state velocity/cytochrome *c* plots at similar ionic strengths (Matthis & Erman, 1995).

At 0.2 M *I*, the plot of k_F reaches saturation over the cytochrome *c* concentration range investigated and can be fit by simple hyperbolic plot (data not shown).

The dependence of k_F on yeast iso-1 ferrocytochrome *c* is quite complex and varies depending upon the ionic strength in the range 0.01−0.20 M. We have found that a three-parameter empirical equation will fit the experimental data, within experimental error, over the yeast iso-1 ferrocytochrome *c* concentration range 0.5−100 μM and between 0.01 and 0.20 M *I* (eq 5). Weighted nonlinear least-squares regression was used to obtain best-fit values for the parameters in eq 5 (Table 1). Values of n_1 and d_1 were obtained between 0.01 and 0.20 M *I*. Above 0.20 M *I*, the reaction was only investigated at low concentrations of yeast iso-1 ferrocytochrome *c* and only n_1, the initial slope of plots of k_F versus cytochrome *c*, was obtained. The value of n_2 is only significant between 0.04 and 0.10 M *I*.

$$k_F = \frac{n_1[c] + n_2[c]^2}{1 + d_1[c]} \quad (5)$$

The values of the empirical parameters are unique, determined only by the experimental data. Interpretation of the empirical parameters n_1, n_2, and d_1 in terms of individual rate and equilibrium constants depends upon the mechanism of the reaction and can be quite complex. In the Discussion section, we will present a two-binding site mechanism for the oxidation of yeast iso-1 ferrocytochrome *c* by CcP-**I** which is consistent with the experimental data. We will use the values of the empirical parameters to extract values for individual rate and equilibrium constants.

Table 1: Empirical Parameters for the Reduction of CcP Compounds **I** and **II** by Yeast Iso-1 Ferrocytochrome *c* at Various Values of Ionic Strength[a]

I (M)	n_1 (μM^{-1} s^{-1})	d_1 (μM^{-1})	n_2 (μM^{-2} s^{-1})	n_3 (μM^{-1} s^{-1})
0.01	22 ± 2	(6.3 ± 1.9) × 10^{-3}		
0.02	15 ± 2	(3.0 ± 2.7) × 10^{-3}		
0.04	130 ± 30	0.51 ± 0.19	3.2 ± 1.6	
0.05	960 ± 780	2.5 ± 2.3	19 ± 20	
0.07	1300 ± 190	1.1 ± 0.2	2.2 ± 1.3	
0.10	940 ± 130	0.55 ± 0.11	0.9 ± 1.5	
0.20	94 ± 5	0.049 ± 0.005		
0.30	9.8 ± 1.2			34 ± 9
0.40	1.9 ± 0.9			36 ± 12
0.60	0.31 ± 0.16			1.3 ± 0.3
1.00	0.12 ± 0.08			0.45 ± 0.19

[a] Parameters n_1, d_1, and n_2 are defined in eq 5 of the text, and parameter n_3 is defined in eq 7.

Concentration Dependence of k_R. The reduction of the Trp-191 radical converts CcP-$\mathbf{II}_R$ to the native enzyme and is characterized by the rate constant k_R. Values of k_R are independent of c^{2+} and increase linearly with increasing initial concentration of CcP-**I**. This is identical to the behavior for reduction of CcP-$\mathbf{II}_R$ by horseheart c^{2+} (Summers & Erman, 1988). In addition, k_R is independent of ionic strength between 0.01 and 0.2 M. Between 0.01 and 0.20 M *I*, k_R can be accurately estimated by eq 6, independent of

$$k_R = (51 \pm 2)\mu M^{-1}\ s^{-1}[\text{CcP-I}]_0 + (0.32 \pm 0.38)s^{-1} \quad (6)$$

the yeast iso-1 ferrocytochrome *c* concentration. Linear least-squares analysis of the dependence of k_R on the initial CcP-**I** concentration gives an intercept of 0.32 ± 0.38 s^{-1} and a slope of 51 ± 2 μM^{-1} s^{-1}. Corresponding values for reduction of CcP-$\mathbf{II}_R$ by horseheart c^{2+} at 0.01 M *I* are 5 ± 3 s^{-1} and 40 ± 25 μM^{-1} s^{-1} for the intercept and slope, respectively (Summers & Erman, 1988).

Above 0.20 M *I*, the reduction of CcP-**I** was only investigated at ≤1 μM yeast iso-1 ferrocytochrome *c*. Under these conditions, the Trp-191 radical site is reduced prior to the Fe(IV) site and k_R represents the conversion of CcP-**I** to CcP-$\mathbf{II}_F$; k_R has a normal concentration dependence in that k_R is independent of the CcP-**I** concentration and increases linearly with increasing ferrocytochrome *c* concentration (eq 7). The parameter represents the limiting slope for the bimolecular interaction of yeast iso-1 ferrocytochrome *c* with CcP-**II**. Values of n_3 are given in Table 1.

$$k_R = n_3[c^{2+}] \quad (7)$$

DISCUSSION

Two-Binding Site Model. The observation that the pseudo-first-order rate constant for reduction of CcP-**I** to CcP-**II** by excess yeast iso-1 ferrocytochrome *c* requires up to three parameters to describe the cytochrome *c* concentration dependence means that a simple one-binding site mechanism is insufficient to explain the data. Exotic one-binding site mechanisms, involving multiple enzyme conformations and coupled rate processes, may be possible but we have not been able to find a satisfactory single-binding site mechanism to rationalize the data presented in this paper. The simplest

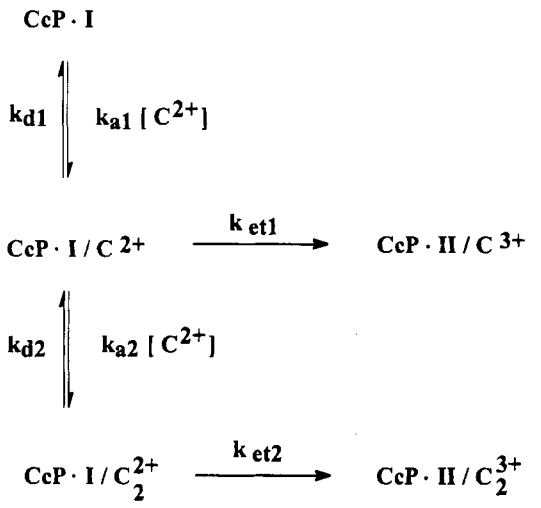

FIGURE 5: Two-binding site mechanism for the reduction of CcP-**I** to CcP-**II**. The association and dissociation rate constants for binding at the high-affinity and low-affinity sites are defined as well as the electron-transfer rates within the 1:1 and the 2:1 cytochrome *c*/CcP complex. The nature of the product depends upon the ionic strength. Below 0.3 M ionic strength, the CcP product is CcP-$\mathbf{II}_R$, and at 0.3 M *I* and above, the product is CcP-$\mathbf{II}_F$. The 2:1 complex makes a significant contribution to reduction of CcP-**I** only below 0.1 M *I*.

mechanism to explain the reduction of CcP-**I** to CcP-$\mathbf{II}_R$ as a function of both concentration and ionic strength is the two-binding site mechanism shown in Figure 5.

There are a number of kinetic constraints that need to be incorporated into the model. Only a single-exponential phase is observed for the reduction of CcP-**I** to CcP-$\mathbf{II}_R$. Both NMR and quenching of the excited triplet state of zinc−CcP indicate that the dissociation of yeast iso-1 cytochrome *c* from the high-affinity site of CcP is slow (Stempf & Hoffman, 1993; Zhou & Hoffman 1993; Yi et al., 1994). The latter observation eliminates a mechanism involving rapid equilibration between free and bound (at the high-affinity site) cytochrome *c* followed by rate-limiting electron transfer within the complex. Since reduction of CcP-**I** to CcP-$\mathbf{II}_R$ is irreversible, dissociation of the product, ferricytochrome *c*, will not influence the rate of CcP-**I** reduction. Use of a steady-state hypothesis for both CcP-**I**/c^{2+} and CcP-**I**/$(c^{2+})_2$ leads to eq 8. The parameters, α_1, α_2, β_1, and β_2

$$k_F = \frac{\alpha_1[c] + \alpha_2[c]^2}{1 + \beta_1[c] + \beta_2[c]^2} \tag{8}$$

are given by eqs 9−12. The parameters, K_{S1} and K_{S2}, are

$$\alpha_1 = k_{et1}/K_{S1} \tag{9}$$

$$\alpha_2 = k_{et2}/K_{S1}K_{S2} \tag{10}$$

$$\beta_1 = \frac{1}{K_{S1}} + \frac{k_{et2}}{(k_{d1} + k_{et1})K_{S2}} \tag{11}$$

$$\beta_2 = 1/K_{S1}K_{S2} \tag{12}$$

defined by eqs 13 and 14.

$$K_{S1} = (k_{d1} + k_{et1})/k_{a1} \tag{13}$$

$$K_{S2} = (k_{d2} + k_{et2})/k_{a2} \tag{14}$$

Equation 8 indicates that four parameters are required to fully explain the concentration dependence of k_F predicted from the mechanism shown in Figure 5. Experimentally, only two or three empirical parameters can be determined at any given ionic strength between 0.01 and 0.20 M and over the cytochrome *c* concentration range used in this study (Table 1). This means that some of the terms in eq 8 become negligible under particular experimental conditions. Below we will describe the conditions under which eq 8 reduces to the two- or three-parameter equations which describe the yeast iso-1 ferrocytochrome *c* concentration dependence of k_F at specific values of ionic strength.

Concentration Dependence of k_F at Low Ionic Strength. Probably the most striking experimental finding is that k_F does not saturate at 100 μM cytochrome *c* at 0.01 M *I* (Figure 3A) while k_F is saturated between 0.1 and 0.2 M *I*. This is contrary to expectation since the binding of cytochrome *c* to CcP is much stronger at low ionic strength than at high ionic strength (Vitello & Erman, 1987; Corin et al., 1991). The two-binding site mechanism provides an explanation for this observation. At 0.01 and 0.02 M *I*, the observed values of k_F are due primarily to electron transfer within the 2:1 cytochrome *c*/CcP-**I** complex while at 0.20 M *I*, k_F is a measure of electron transfer within the 1:1 cytochrome *c*/CcP-**I** complex.

The dependence of k_F on yeast iso-1 ferrocytochrome *c* concentration at 0.01 and 0.02 M *I* only requires two empirical rate parameters, n_1 and d_1 (Table 1). To explain the concentration dependence of k_F at 0.01 and 0.02 M *I*, we make the assumption that k_{et1} is equal to zero. (Essentially, it is assumed that k_{et1} makes no contribution to the observed rate and we estimate that k_{et1} must be less than 20 s^{-1} at these ionic strengths.) If k_{et1} is equal to zero, it can be shown that eq 8 reduces to eq 15 for reasonable values

$$k_F = \frac{(k_{et2}/K_{S2})[c]}{1 + (1/K_{S2})[c]} \tag{15}$$

of the rate constants defined in Figure 5 (See below). Comparison of eq 15 with eq 5 indicates that n_1 is equal to k_{et2}/K_{S2} and that d_1 is equal to $1/K_{S2}$. The values of d_1 in Table 1 translate into values for K_{S2} of 160 ± 50 and 330 ± 300 μM at 0.01 and 0.02 M *I*, respectively. These values are within experimental error of values for K_{D2} determined from equilibrium measurements (Zhou & Hoffman, 1993, 1994; Mauk et al., 1994). The observation that the kinetic parameter, K_{S2}, is within experimental error of K_{D2} provides strong support for the two-binding site mechanism shown in Figure 5 and for our analysis. Since binding at the secondary site is so weak, saturation of the secondary site is not achieved at 100 μM yeast iso-1 ferrocytochrome *c* and the experimental values of K_{S2} ($1/d_1$) have relatively large errors. To minimize propagation of errors in our analysis, we make the assumption that K_{S2} is equal to K_{D2} (*i.e.*, $k_{d2} \gg k_{et2}$) and will use values for K_{D2} calculated from previously published data (See Matthis & Erman, 1995). Calculated values for K_{S2} are shown in the last column of Table 2.

Using the calculated values of K_{S2} from Table 2 and the value of n_1 (eqs 5 and 15) from Table 1, k_{et2} is equal to 3900 ± 200 and 3800 ± 300 at 0.01 and 0.02 M *I*, respectively (Table 2). We can make reasonable estimates of the remaining rate and equilibrium constants from the literature. With k_{et1} negligible, K_{S1} (eq 13) becomes equal to the equilibrium dissociation constant ($K_{D1} = k_{d1}/k_{a1}$), which has a value of 0.05 μM under similar conditions [10 mM

Table 2: Parameters for Reduction of the Fe(IV) Site in CcP Compounds **I** and **II**[a]

I (M)	k_{et1}/K_{S1} (μM^{-1} s^{-1})	k_{et1} (s^{-1})	K_{S1}[b] (μM)	k_{et2}/K_{S2} (μM^{-1} s^{-1})	k_{et2} (s^{-1})	K_{S2}[b] (mM)
0.01	840 ± 340	15 ± 6	[0.018]	22 ± 2	3900 ± 200	[0.20]
0.02	86 ± 140	4 ± 6	[0.047]	15 ± 2	3800 ± 300	[0.27]
0.04	135 ± 52	230 ± 40	1.7 ± 0.7	8.5 ± 1	3300 ± 400	[0.39]
0.05	1200 ± 1500	370 ± 50	0.29 ± 0.35	10 ± 2	4600 ± 700	[0.45]
0.07	1300 ± 300	1200 ± 100	0.93 ± 0.17	4.6 ± 1.0	2700 ± 600	[0.59]
0.10	950 ± 210	1700 ± 100	1.8 ± 0.4	4.1 ± 2.7	3400 ± 2200	[0.83]
0.20	94 ± 13	2000 ± 100	21 ± 2			
0.30	9.8 ± 1.2					
0.40	1.9 ± 0.9					
0.60	0.31 ± 0.16					
1.00	0.12 ± 0.08					

[a] The parameters are defined in Figure 5 and eqs 13 and 14 of the text. [b] Values in brackets were calculated according to the following relationships: $\log K_{S1} = \log K_{D1} = -8.76 + 10.1(I)^{0.5}$ and $\log K_{S2} = \log K_{D2} = -3.98 + 2.85(I)^{0.5}$ (Matthis & Erman, 1995).

potassium phosphate buffer, pH 7.0, I = ~0.02 M (Corin et al., 1991)]. NMR studies (Yi et al., 1994) give a value of ~180 s^{-1} for k_{d1} in 10 mM KNO_3 solutions near neutral pH.

Using the values of the rate and equilibrium constants determined in the prior paragraphs allows us to confirm that eq 8 reduces to eq 15 at 0.01 and 0.02 M I. Substitution of the values of k_{et2}, k_{et1}, k_{d1}, K_{S1} (K_{D1}), and K_{S2} into eq 11 demonstrates that the second term in this equation is small compared to the first term and β_1 equals $1/K_{S1}$ ($1/K_{D1}$) or $\geq 20\ \mu M^{-1}$ at 0.01 and 0.02 M I. If β_1 is $\geq 20\ \mu M^{-1}$ and the cytochrome c concentration is $\geq 0.5\ \mu M$, the initial denominator term of 1 in eq 8 becomes negligible. With α_1 equal to zero, β_1 equal to $1/K_{S1}$, and the initial denominator term of 1 negligible, simple algebra shows that eq 8 reduces to eq 15. Our conclusion is that, at 0.01 and 0.02 M I, the observed rate of reaction is due solely to electron transfer from yeast iso-1 ferrocytochrome c bound in the 2:1 complex to the Fe(IV) site in CcP-**I**.

Concentration Dependence of k_F at Intermediate Ionic Strength. Between 0.04 and 0.10 M I, k_F contains contributions from both 2:1 and 1:1 complexes. Electron transfer from cytochrome c bound at the high-affinity binding site is responsible for the rapid increase in k_F at low cytochrome c concentration in Figure 4. This contribution saturates at relatively low cytochrome c concentrations, but the value of k_F continues to increase due to interaction of cytochrome c at the low-affinity site. The binding of cytochrome c at the low-affinity binding site is quite weak above 0.02 M I, and an apparent second-order rate process causes the linear increase in k_F at high cytochrome c concentrations (Figure 4). The experimental values of k_F can be described by the three-parameter empirical equation (eq 5). Values of n_1, n_2, and d_1 between 0.04 and 0.10 M I are given in Table 1.

Reduction of the four-parameter, theoretical equation for the two-binding-site mechanism to a three-parameter equation is straightforward. At 0.04 M ionic strength and above, K_{S2} increases to such large values that the $\beta_2[c]^2$ term in eq 8 becomes negligible over the cytochrome c concentration range of 1−100 μM. Eliminating the β_2 term in the denominator of eq 8 and assuming that β_1 remains equal to $1/K_{S1}$ above 0.04 M ionic strength reduces eq 8 to eq 16.

$$k_F = \frac{(k_{et1}/K_{S1})[c] + (k_{et2}/K_{S1}/K_{S2})[c]^2}{1 + (1/K_{S1})[c]} \qquad (16)$$

Between 0.04 and 0.01 M I, we can evaluate k_{et1}, K_{S1}, and k_{et2}/K_{S2} (Table 2). Using calculated values for K_{S2} we can also evaluate k_{et2}.

Concentration Dependence of k_F at High Ionic Strength. Above 0.10 M I, binding of cytochrome c to the low-affinity binding site is so weak that it makes negligible contributions to reduction of CcP-**I**. At these higher ionic strengths, the value of k_F is due primarily to oxidation of yeast iso-1 ferrocytochrome at the high-affinity site. Equation 8 reduces to a two-parameter equation at these high ionic strengths because K_{S2} becomes so large that both numerator and denominator terms involving the square of the cytochrome c concentration become negligible below 100 μM cytochrome c. Equation 8 reduces to eq 17. At 0.2 M I, both

$$k_F = \frac{(k_{et1}/K_{S1})[c]}{1 + (1/K_{S1})[c]} \qquad (17)$$

k_{et1} and K_{S1} have been determined (Table 2). Above 0.2 M I, the reaction was only studied at very low yeast iso-1 ferrocytochrome c concentrations that only the limiting slope of the variation of k_F with cytochrome c concentration, k_{et1}/K_{S1}, was determined (Table 2).

Electron Transfer at Low Ionic Strength. The binding of yeast iso-1 cytochrome c to the high-affinity site of CcP is very strong at low ionic strength, with a value for K_{D1} of 0.05 μM in 10 mM potassium phosphate buffer, pH 7 (I = ~20 mM) determined from fluorescence quenching experiments with MgCcP (Corin et al., 1991). The binding of CcP and horse cytochrome c is relatively independent of pH (Vitello & Erman, 1987), and we expect this to be true for yeast iso-1 cytochrome c also. In the previous paper, we have shown that published values of K_{D1} for the 1:1 yeast iso-1 cytochrome c/CcP complex between pH 6 and 7.75 as a function of ionic strength can be correlated by the relationship $\log K_{D1} = -8.76 + 10.1(I)^{0.5}$. This equation predicts that K_{D1} has values of 0.018 and 0.047 μM at 0.01 and 0.02 M I, respectively. In addition, the dissociation rate constant has been estimated to be ~180 s^{-1} between 0.01 and 0.02 M I at neutral pH from NMR relaxation measurements (Yi et al., 1994). This means that CcP-**I** binds rapidly ($t_{1/2}$ < 1 ms) to yeast cytochrome c at the high-affinity site upon mixing in the stopped-flow instrument, with greater than 90% bound even at the lowest cytochrome c concentrations used in this study (0.5 μM). The proposed mechanism suggests that electron transfer from yeast ferrocytochrome c bound at the high-affinity site to either the Fe(IV) or radical sites in CcP-**I** is extremely slow at low ionic strength. Fixing K_{S1} equal to the value of K_{D1} at 0.01 and 0.02 M I gives values of k_{et1} of 15 ± 6 and 4 ± 6 s^{-1} at these two ionic

strengths, respectively. These two values of k_{et1} are consistent with the assumption that K_{S1} and K_{D1} are essentially identical below 0.02 M I since k_{et1} is substantially smaller than the value of k_{d1}, 180 s^{-1}. The observed reduction of CcP-**I** is due primarily to electron transfer from the heme iron cytochrome *c* bound to the low-affinity site to the Fe(IV) group of CcP. Assuming that K_{S2} is identical to K_{D2}, the equilibrium dissociation constant for the 2:1 yeast iso-1 cytochrome *c*/CcP complex gives a value of 3900 s^{-1} for the rate of reduction of the Fe(IV) group in CcP-**I** in the 2:1 complex.

Comparison of Stopped-Flow and Flash Photolysis Results. The value of 15 s^{-1} for k_{et1} is considerably smaller than the value of 250 s^{-1} reported by Hazzard et al. (1988) for the electron-transfer rate between yeast iso-1 ferrocytochrome *c* and CcP-**I** at 0.008 M I in pH 7 buffer from flash photolysis studies. However, the current model offers an explanation for the apparent discrepancy. The flash photolysis studies utilize stoichiometric concentrations of CcP-**I** and ferricytochrome *c*. With very tight binding at the high-affinity binding site at 0.01 M I, essentially all of the yeast ferricytochrome *c* is bound to CcP-**I**. Flash photolysis in the presence of flavins reduces some of the bound cytochrome *c* within 1 μs. At 0.008 M I, electron transfer from reduced cytochrome *c* bound at the high-affinity site is very slow according to our model, less than 15 s^{-1}, but the reduced cytochrome *c* dissociates from the 1:1 complex at a rate of 250 s^{-1} and then is rapidly oxidized by interacting with the low-affinity site of the CcP-**I**. The rate-limiting step in the flash photolysis studies of Hazzard et al. (1988) is the dissociation of cytochrome *c* from the high-affinity binding site on CcP. The value of 250 s^{-1} is similar to the value of 180 s^{-1} for dissociation of yeast iso-1 ferricytochrome *c* from CcP found from NMR experiments under similar conditions (Yi et al., 1994).

Comparison of Unmodified and Ruthenium-Modified Yeast Iso-1 Ferrocytochrome c. Geren et al. (1991) used ruthenium-modified yeast iso-1 cytochrome *c* in flash photolysis studies with CcP-**I** and found results significantly different from those presented here and those of Hazzard et al. (1987, 1988). In low ionic strength buffer (5 mM EDTA and 5 mM phosphate, pH 7, I = ~0.040 M), flash photolytic reduction of the ruthenium resulted in reduction of the cytochrome *c* heme group with a rate in excess of 10^5 s^{-1}. The reduced cytochrome *c* reduced, in turn, the radical site in CcP-**I** with a rate in excess of 50 000 s^{-1}. These results differ in two respects from those we present here. First, at low ionic strength, unmodified yeast iso-1 ferrocytochrome *c* reduces the Fe(IV) group of CcP-**I** prior to the Trp-191 radical, and second, the maximal rate of CcP-**I** reduction is at least 1 order of magnitude slower than reported by Geren et al. (1991). We cannot reconcile these two studies but note that the ruthenium-modified yeast iso-1 cytochrome *c* may interact differently with CcP-**I** than the unmodified yeast cytochrome at low ionic strength. Geren et al. performed experiments at low ionic strength similar to those of Hazzard et al. (1987, 1988) using flash photolysis in the presence of lumiflavin and found that lumiflavin would reduce unmodified yeast iso-1 cytochrome *c* when it was bound to CcP-**I** but not the ruthenium-modified yeast cytochrome *c*.

Electron Transfer at High Ionic Strength. The mechanism of electron transfer from yeast iso-1 ferrocytochrome *c* to the oxidized sites in CcP-**I** changes notably with increasing

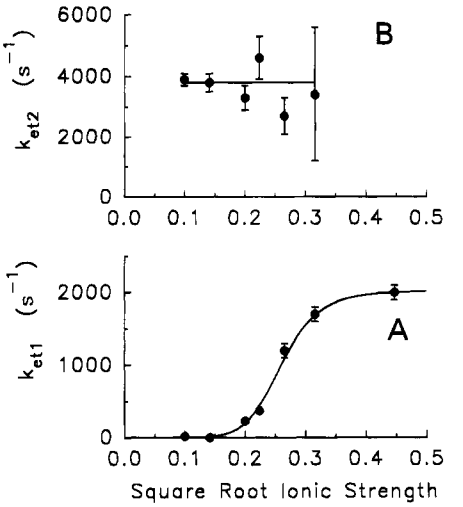

FIGURE 6: Plots of k_{et1} and k_{et2} as functions of the square root of the ionic strength: (A) k_{et1}; (B) k_{et2}; the most accurate values of k_{et2} were obtained at 0.01 and 0.02 M I. The value of k_{et2} appears to be relatively independent of ionic strength with the weighted average equal to 3800 ± 300 s^{-1}.

ionic strength. First, the value of k_{et1} increases substantially with increasing ionic strength, reaching a plateau value near 2000 s^{-1} with the midpoint of the transition occurring near 0.06 M I (Figure 6). Second, even though the value of k_{et2} appears to be essentially independent of ionic strength (Figure 6), oxidation of ferrocytochrome *c* via the 2:1 complex becomes negligible above 0.1 M I for cytochrome *c* concentrations below 100 μM due to the very weak binding at the secondary site. Third, above ~0.2 M I, the radical site is reduced prior to the Fe(IV) site in CcP-**I** in contrast to the initial reduction of the Fe(IV) site at lower ionic strengths (Figure 2).

Hazzard et al. (1988), using flash photolysis, observed saturation kinetics between CcP-**I** and yeast ferrocytochrome *c* at 0.26 M I in pH 7.0 buffers with a maximum electron-transfer rate of ~1500 s^{-1}, similar to the plateau value of 2000 s^{-1} found in our investigation. Hazzard et al. obtained a value of ~200 μM for K_{S1} at 0.26 M I. We have only determined K_{S1} up to 0.2 M I, but extrapolation of our data gives a value of ~60 μM for K_{S1} at 0.26 M I. The values for k_{et1} and K_{S1} obtained in these stopped-flow studies and those obtained using flash photolysis studies are in reasonable agreement considering the differences in pH and buffer composition. Since it is known that many anions bind to cytochrome *c*, it is reasonable to expect that specific ion effects could affect the interaction between cytochrome *c* and CcP. We are not aware of any systematic study of specific ion effects on the binding of these two proteins.

Geren et al. (1991), using flash photolysis to measure the reduction of CcP-**I** by ruthenium-modified yeast cytochrome *c*, found somewhat different results. At pH 7, in a buffer containing 40 mM EDTA and 5 mM sodium phosphate (I = ~0.25 M), they observed reduction of the radical site in CcP-**I** with saturation kinetics. They obtained values of 25 000 s^{-1} and 50 μM for the equivalent of k_{et1} and K_{S1}. The K_{S1} value is similar to our extrapolated value at 0.26 M I, pH 7.5, but k_{et1} is 1 order of magnitude larger. At pH 7.5, our studies show that 0.25 M I is near the transition region between initial reduction of the Fe(IV) site and initial reduction of the radical site (Figure 2).

Electron Transfer via the High-Affinity Binding Site. The ionic strength dependence of k_{et1} (Figure 6A) is remarkable. The value of k_{et1} increases by a factor of 200 over the ionic strength range 0.01−0.2 M *I*, from about 10 to 2000 s^{-1}. The most likely reason for the increase is that the interaction between yeast iso-1 ferocytochrome *c* and CcP-**I** undergoes a subtle rearrangement as the electrostatic contribution to the binding energy is decreased due to increasing ionic strength. The data of Pelletier & Kraut (1992) suggest that the binding interface between cytochrome *c* and CcP can change with increasing salt concentration. An alternative explanation, that the redox potential of the Fe(IV) site (and the driving force for the reaction) is modulated by the ionic environment, is less likely since k_{et2} does not change with ionic strength (Figure 6B), and below 0.2 M ionic strength, both k_{et1} and k_{et2} are measures of the rate of reduction of the Fe(IV) site in CcP-**I**.

Apparent Bimolecular Rate Constants for the Interaction of Yeast Iso-1 Ferrocytochrome c and CcP-I. The ratios of k_{et1}/K_{S1} and k_{et2}/K_{S2} provide *lower limits* for the bimolecular association rate constants for the reaction between yeast iso-1 ferrocytochrome *c* and CcP-**I** at the high- and low-affinity sites, respectively. The value of k_{et1}/K_{S1} goes through an optimum near 0.07 M *I* with a value of 1.3×10^9 M^{-1} s^{-1}. The observation that the value of k_{et1}/K_{S1} goes through an optimum as a function of ionic strength can be understood in terms of the individual rate constants defined in the mechanism shown in Figure 5. The ratio of k_{et1}/K_{S1} is equal to $k_{a1}k_{et1}/(k_{d1} + k_{et1})$ and k_{et1}/K_{S1} underestimates k_{a1} by the factor $k_{et1}/(k_{d1} + k_{et1})$. In theory, the factor of $k_{et1}/(k_{d1} + k_{et1})$ can vary between 0 and 1. We have obtained values for k_{et1} between 0.01 and 0.20 M *I* (Table 2) and could calculate the factor $k_{et1}/(k_{d1} + k_{et1})$ if the value of k_{d1} were known. The only experimentally measured dissociation rate constant comes from NMR relaxation measurements of the yeast iso-1 ferricytochrome *c*/CcP complex (Yi et al., 1994), which give a value of 180 s^{-1} for k_{d1} between 0.01 and 0.02 M *I* at neutral pH. At 0.01 and 0.02 M *I*, the factor $k_{et1}/(k_{d1} + k_{et1})$ averages 0.05 ± 0.04 and k_{et1}/K_{S1} underestimates k_{a1}. Dissociation rate constants are generally independent of ionic stength, and if we assume that k_{d1} is also equal to 180 s^{-1} at 0.20 M *I*, $k_{et1}/(k_{d1} + k_{et1})$ at 0.20 M *I* equals 0.92. The ratio of k_{et1}/K_{S1} provides a good approximation to the true association rate constant at high ionic strength.

With the assumption that k_{d1} is equal to 180 s^{-1} and independent of ionic strength, we can calculate the true value of k_{a1} over the entire ionic strength range investigated. Calculated values of k_{a1} decrease from 1.0×10^{10} M^{-1} s^{-1} at 0.01 M *I* to 1.2×10^5 M^{-1} s^{-1} at 1.0 M *I*, consistent with the interaction of oppositely charged proteins. The value of 1.0×10^{10} M^{-1} s^{-1} at 0.01 M *I* is remarkable. An upper limit for the association rate constant can be estimated from the Smoluchowski equation for diffusion-limited reactions. A value of 6×10^9 M^{-1} s^{-1} is obtained for *neutral* molecules the size of CcP and cytochrome *c*. Electrostatic attraction between these two proteins should enhance the rate at low ionic strength. The electrostatic model of Alberty and Hammes (1958) gives a value of 6×10^{10} M^{-1} s^{-1} for the diffusion-limited rate at zero ionic strength, using the net charges on CcP and cytochrome *c* at neutral pH. These two calculations indicate that the diffusion-limited association rate lies between 6×10^{10} M^{-1} s^{-1} at zero ionic strength and 6×10^9 M^{-1} s^{-1} at infinite ionic strength. The calculated values of k_{a1} are smaller than those for a diffusion-limited reaction but approach the diffusion limit at low ionic strength. This suggests that there are no significant steric or orientational restrictions for complex formation between yeast iso-1 ferrocytochrome *c* and CcP at the high-affinity binding site.

Miller et al. (1994) also found very large association rates for the interaction of yeast iso-1 cytochrome *c* and CcP at the high-affinity site. Using stopped-flow techniques to measure the reaction of CcP-**I** with yeast iso-1 ferrocytochrome *c* between 0.21 and 0.61 M ionic strength (pH 7, 5 mM sodium phosphate buffer with added NaCl to adjust ionic strength), Miller et al. found that the radical site of CcP-**I** was reduced prior to the Fe(IV) site, with k_R decreasing from 8×10^8 M^{-1} s^{-1} at 0.21 M *I* to 2×10^7 M^{-1} s^{-1} at 0.61 M *I* and k_F decreasing from 1.3×10^8 to 4.0×10^6 M^{-1} s^{-1} over the same ionic strength range. These apparent bimolecular rate constants are a factor of ~10 larger than those that we measure over the same ionic strength range but at pH 7.5 in a potassium phosphate/KNO_3 buffer system.

Reduction of CcP-II_R. Reduction of the CcP-II_R produced in the reaction between CcP-**I** and excess yeast iso-1 ferrocytochrome *c* is essentially identical to reduction of CcP-II_R by horse ferrocytochrome *c* (Summers & Erman, 1988). Over the concentration range of the reactants utilized in these studies, the rate of reduction is independent of the ferrocytochrome *c* concentration and depends linearly on the initial concentration of CcP-**I**. The previous study with horse ferrocytochrome *c* was performed only at 0.010 M *I* while the reaction has been investigated between 0.01 and 0.20 M *I* in this study. Reduction of CcP-II_R is essentially independent of ionic strength between 0.01 and 0.20 M *I*. Above 0.20 M *I*, CcP-II_R is not produced in the reaction.

To explain the unusual concentration dependence of CcP-II_R reduction, especially the bimolecular dependence on the initial CcP-**I** concentration, Summers & Erman (1988) proposed a mechanism in which the rate-limiting step is a bimolecular disproportionation of CcP-II_R into CcP and CcP-**I**, followed by rapid reduction of the CcP-**I** by ferrocytochrome *c*. Zhou and Hoffmann (1993) also observed a bimolecular reaction between CcP species in their quenching studies of excited-state zinc cytochrome *c* by native CcP. However, the bimolecular rate constant for that reaction is $\sim 8 \times 10^4$ M^{-1} s^{-1} compared to the value of 5×10^7 M^{-1} s^{-1} we obtain in these studies (eq 6). The independence of the bimolecular rate constant on ionic strength is unusual for an interaction between molecules of the same net charge. It is probable that in our system the bimolecular reaction is between CcP/cytochrome *c* complexes in which the net charge is much smaller than for CcP alone. At pH 7.5, the net charge on CcP is ~−10 (Conroy & Erman, 1978) and the net charge on cytochrome *c* is ~+6 (Marini et al., 1980).

Reduction of CcP-II_R *cannot* be an obligatory step in the steady-state CcP-catalyzed oxidation of ferrocytochrome *c* between 0.01 and 0.20 M *I*. The values of k_R are too slow to accommodate the steady-state turnover. In addition, plots of the steady-state velocity versus yeast iso-1 ferrocytochrome *c* concentration appear to be a scaled version of the plots of k_F versus the cytochrome *c* concentration shown in Figures 3 and 4 (Matthis & Erman, 1995). The scaling factor is a factor of 2−3, suggesting that the steady-state turnover involves two similar rate-limiting steps and that each is equivalent to k_F found in these stopped-flow studies. Summers and Erman (1988) suggested that a form of **I** which

did not involve the Trp-191 radical was initially generated in the reaction between CcP and hydrogen peroxide. This form was called CcP-$\mathbf{I}_A$ and could be directly reduced to CcP-$\mathbf{II}_F$ by ferrocytochrome *c*. If the rates of reduction of CcP-$\mathbf{I}_A$ and CcP-$\mathbf{II}_F$ are similar to the rate of reduction of CcP-**I** found in this study, the steady-state velocity would be as observed. A critical observation in understanding the CcP-catalyzed oxidation of ferrocytochrome *c* will be whether or not CcP-$\mathbf{II}_R$ is formed during the steady-state turnover of the enzyme at low ionic strength. This possibility is under investigation.

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BI950410D