Cytochrome *c* Peroxidase-Catalyzed Oxidation of Yeast Iso-1 Ferrocytochrome *c* by Hydrogen Peroxide. Ionic Strength Dependence of the Steady-State Parameters†

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ABSTRACT: The cytochrome *c* peroxidase-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide can be understood on the basis of a mechanism involving two cytochrome *c*-binding sites on cytochrome *c* peroxidase. Values of the equilibrium dissociation constants for both the high- and low-affinity binding sites determined from the steady-state kinetic measurements agree well with published values obtained by vastly different techniques, providing strong support for the two-binding site mechanism. Maximum enzyme turnover via oxidation of cytochrome *c* bound at the high-affinity site increases from 2 to 860 s^{-1} as the ionic strength is increased from 0.010 to 0.20 M. Oxidation of yeast iso-1 ferrocytochrome *c* is faster in the 2:1 complexes of cytochrome *c* peroxidase compounds **I** and **II** in comparison to the 1:1 complexes. The oxidation rates in the 2:1 complex are macroscopic rate constants equal to the *sum* of the oxidation rates via both the high- and low-affinity sites. The maximum enzyme turnover via the 2:1 complex increases from 1100 to 2700 s^{-1} over the ionic strength range 0.010−0.070 M.

Cytochrome *c* peroxidase (CcP)[1] catalyzes the reduction of hydrogen peroxide to water by utilizing the reducing equivalents from two molecules of ferrocytochrome *c* (eq 1) (Bosshard et al., 1991). A minimal mechanism requires

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

two oxidized enzyme intermediates, cytochrome *c* peroxidase compound **I** (CcP-**I**) and -compound **II** (CcP-**II**), to couple the two-equivalent reduction of H_2O_2 with the one-equivalent oxidation of ferrocytochrome *c* (eqs 2−4). The proton

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

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

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

stoichiometry for this mechanism has been established with the use of a model substrate, ferrocyanide (Conroy & Erman, 1978). One of the product water molecules is released during the reaction of the native enzyme with H_2O_2 (eq 2) (Schonbaum & Lo, 1972). The second water is released when the oxyferryl heme group in the oxidized enzyme intermediates is reduced. The properties of both oxidized intermediates 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). CcP-**II** is produced 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).

There have been several steady-state kinetic studies on the CcP-catalyzed oxidation of ferrocytochrome *c* by hydrogen peroxide, generally using horse or yeast iso-1 ferrocytochrome *c* (Beetlestone, 1960; Yonetani & Ray, 1966; Nicholls & Mochan, 1971; Kang et al., 1977; Kang & Erman, 1982; Kim et al., 1990; Erman et al., 1991, Corin et al., 1991, 1993). At saturating hydrogen peroxide, the steady-state velocities are complex functions of the ferrocytochrome *c* concentration, sometimes showing linear reciprocal plots and sometimes showing biphasic character. Margoliash and co-workers were the first to observe nonlinear reciprocal plots and postulated a two-binding site mechanism for catalysis in which CcP has both a high-affinity and a low-affinity binding site for cytochrome *c* (Kang et al., 1977). Theoretically, both the linear and nonlinear reciprocal plots can be analyzed according to either a one-binding site, multiple pathway mechanism or a two-binding site mechanism (Kang & Erman, 1982).

There is growing evidence that cytochrome *c* interacts with CcP at multiple sites. Stempf and Hoffman (1993) have replaced the heme group of CcP with either zinc or magnesium protoporphyrin IX. The modified CcP is phosphorescent, and quenching of the excited porphyrin triplet state is sensitive to the presence of ferricytochrome *c*. A detailed investigation of the cytochrome *c* concentration dependence of the quenching, using a variety of cytochrome *c*'s, and of the ionic strength dependence of the quenching led to a mechanism involving two cytochrome *c*-binding sites on CcP as proposed earlier by Kang et al. (1977).

Zhou and Hoffman (1993, 1994) observed similar properties using modified horse cytochrome *c* in which the heme iron was replaced with zinc. Zhou and Hoffman investigated the quenching of the excited triplet state of zinc cytochrome *c* by native CcP. They interpreted their data based on a two-

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; CcP-I, cytochrome *c* peroxidase compound **I**; CcP-II, cytochrome *c* peroxidase compound **II**; c^{2+}, ferrocytochrome *c*; c^{3+}, ferricytochrome *c*; *e*, the total enzyme concentration.

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binding site model, with slow electron transfer to cytochrome *c* bound at the high-affinity site and rapid electron transfer to cytochrome *c* bound at the low-affinity site.

NMR experiments show that free and CcP-bound yeast cytochrome *c* are in slow exchange while free and CcP-bound horse cytochrome *c* are in fast exchange on the NMR time scale (Satterlee et al., 1987; Moench et al., 1992; Yi et al., 1993, 1994). Estimates can be made of the rate of dissociation of yeast cytochrome *c* from the high-affinity binding site on CcP. This rate is ~180 s^{-1} in 0.01 M KNO_3 near neutral pH (Yi et al., 1994), too slow to accommodate the steady-state turnover of yeast ferrocytochrome *c*, providing additional evidence that a mechanism involving a single high-affinity binding site is insufficient to account for the CcP-catalyzed oxidation of yeast ferrocytochrome *c* at low ionic strength in the neutral pH region.

In this paper, we report on a detailed steady-state kinetic study of the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide at pH 7.5 as a function of cytochrome *c* concentration (0.5−100 μM cytochrome *c*) and ionic strength (0.01−0.20 M). The concentration dependence of ferrocytochrome *c* oxidation is complex and can be explained most simply based on a two-binding site mechanism. Electron transfer to the low-affinity binding site dominates at low ionic strength but becomes of minor importance at 0.10 M ionic strength and above.

MATERIALS AND METHODS

Proteins. CcP was isolated as previously described (Vitello et al., 1990). PZ values for the multiple preparations used in this study ranged between 1.2 and 1.3. Absorbance ratios at 408/380 nm and 620/647 nm were near 1.5 and 0.76, respectively, at pH 7, indicating that the CcP preparations used were five-coordinate, high-spin Fe(III) forms. CcP concentrations were determined using an absorptivity of 98 mM^{-1} cm^{-1} at 408 nm (Vitello et al., 1990).

Yeast iso-1 cytochrome *c* was isolated from baker's yeast along with CcP. The initial aqueous yeast extract was passed over a DEAE cellulose column, equilibrated in 5 mM potassium phosphate buffer at pH 6.5. CcP was bound to this column while cytochrome *c* passed through in the effluent. The cytochrome *c* in the effluent was bound to a 5 × 10 cm Amberlite CG-50 column, equilibrated with 5 mM potassium phosphate, pH 6. Crude yeast cytochrome *c* was eluted with 0.5 M potassium phosphate buffer, dialyzed against deionized water, and lyophilized. Crude yeast cytochrome *c* was dissolved in 50 mM potassium phosphate buffer, pH 7, oxidized with a small excess of ferricyanide, and chromatographed on a 2.5 × 100 cm column of Sephadex G-75. Separation of isozyme-1 monomer from isozyme-1 dimer and isozyme-2 was accomplished according to the method of Satterlee et al. (1988). Samples of isozyme-1 were either dialyzed against deionized water and lyophilized for storage at −20 °C or dialyzed against the buffer of the experiment. For each steady-state experiment, stock cytochrome *c* solutions were made in the experimental buffer, reduced with a small excess of dithionite, applied to a Sephadex G-75 column to remove any excess dithionite, collected, and kept under nitrogen or argon to minimize autoxidation during the course of the experiment. Percent reduction of the stock solutions was checked before and after each series of experiments, and only data obtained with cytochrome *c* samples greater than 95% reduced were retained. Yeast iso-1 ferrocytochrome *c* concentrations were determined using an absorptivity of 26.9 mM^{-1} cm^{-1} at 550 nm (Van Gelder & Slater, 1962).

Hydrogen peroxide was standardized by the method of Kolthoff and Belcher (1957). Stock solutions of hydrogen peroxide were prepared by diluting standardized 30% Superoxal (Fisher) with deionized, distilled water.

Potassium phosphate/KNO_3 buffers, pH 7.5, were used for the kinetic studies. The buffers contained between 4.2 and 10 mM potassium phosphate, and KNO_3 was added to adjust the ionic strength above 20 mM. A maximum potassium phosphate concentration of 10 mM was used to minimize phosphate binding to cytochrome *c*.

Kinetic Measurements. Oxidation of ferrocytochrome *c* was monitored either at 417 or 550 nm on a Cary Model 219 spectrophotometer, with the cell compartment thermostated at 25.0 ± 0.5 °C. Yeast iso-1 ferrocytochrome *c* concentrations varied between about 0.5 and 100 μM, and CcP concentration varied between 0.1 and 0.7 nM. The reaction was initiated by adding hydrogen peroxide. The final hydrogen peroxide concentration was held constant at 200 μM, sufficient to make the reaction independent of hydrogen peroxide (Loo & Erman, 1975; Kim et al., 1990). Initial velocities were determined from initial slopes of the change in absorbance versus time according to eq 5. $\Delta\epsilon$ is

$$v_0 = -\frac{1}{2}\frac{d[c^{2+}]}{dt} = \frac{1}{2\Delta\epsilon}\frac{\Delta A}{\Delta t} \quad (5)$$

the difference in absorptivity of ferro- and ferricytochrome *c* at the wavelength of observation. Since two molecules of cytochrome *c* are reduced per catalytic cycle (eq 1), the factor of $^1/_2$ is included in eq 5 so that the initial velocity measures enzyme turnover rather than cytochrome *c* turnover. Initial velocities were corrected for the uncatalyzed reaction between ferrocytochrome *c* and hydrogen peroxide. The uncatalyzed rates increased linearly with increasing cytochrome *c* and were generally less than 15% of the enzyme-catalyzed rate. The initial velocities were further corrected for inhibition due to the presence of up to 5% ferricytochrome *c* in the initial reaction mixture (Kang & Erman, 1982).

Data Analysis. The data presented in this paper are part of a much larger data set collected as functions of pH, ionic strength, buffer ions, temperature, hydrogen peroxide concentration, and both the source and concentration of cytochrome *c*. The complete data set was used to assess the precision and reproducibility of the data. Over 700 replicates were analyzed. The precision of the velocities is better than 6% when duplicates, using the same stock solutions, are analyzed on the same day. The day-to-day reproducibility of the initial velocities is 11%.

Preliminary analysis indicated that simple one-binding site, multiple pathway mechanisms did not provide a consistent interpretation of the data and that the simplest explanation of the steady-state oxidation of yeast iso-1 ferrocytochrome *c* involved a two-binding site mechanism in which the affinities of the two sites for yeast cytochrome *c* are substantially different. The two-binding site mechanism that we have used to analyze the data is shown in Figure 1. The mechanism assumes yeast iso-1 ferrocytochrome *c* binds to CcP compounds **I** and **II** in an identical manner. The binding

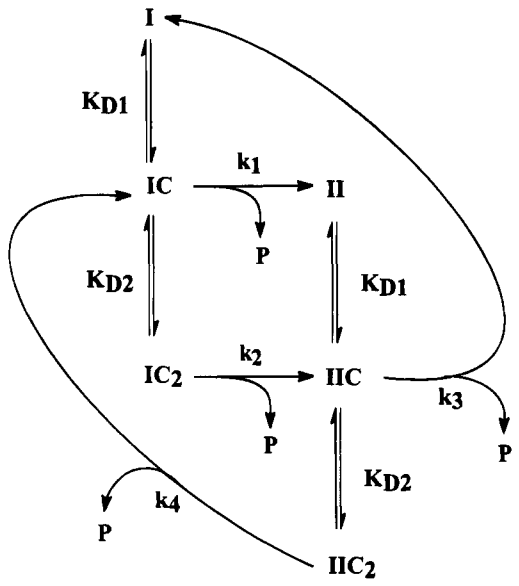

FIGURE 1: Two-binding site mechanism for the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* at saturating hydrogen peroxide. **I** and **II** represent CcP compounds **I** and **II**, respectively. C and P represent ferro- and ferricytochrome *c*, respectively. K_{D1} and K_{D2} are the equilibrium dissociation constants for binding of cytochrome *c* to the high- and low-affinity binding sites, respectively. It is assumed that the binding is independent of the redox state of CcP. The rate constants, k_1, k_2, k_3, and k_4, represent the unimolecular reactions indicated in the figure and could be a measure of conformational reorientation, electron transfer, or product dissociation.

at both the high- and low-affinity binding sites is characterized by equilibrium dissociation constants K_{D1} and K_{D2}, respectively. Conversion of **I** to **II** via the 1:1 and 2:1 complexes is characterized by two rate constants, k_1 and k_2, respectively. Likewise, conversion of **II** to the native enzyme (and subsequent rapid reoxidation of the native enzyme to **I** by hydrogen peroxide under the experimental conditions of these studies) via the 1:1 and 2:1 complexes is characterized by k_3 and k_4, respectively.

The rate expression for the mechanism shown in Figure 1 has been derived previously (Kim et al., 1990). In general the two-binding site mechanism generates an initial velocity expression that includes terms involving cytochrome *c* raised to the third power in both the numerator and denominator of the initial velocity equation. Experimentally, it is found that only squared terms in the cytochrome *c* concentration are required to fit the experimental data. If the two-binding site mechanism is correct, there must be relationships between the parameters that allow at least one power of the cytochrome *c* concentration to be factored and canceled from the numerator and denominator. The least restrictive relationship we have found to reduce the initial velocity expression to one that only contains squared terms in the cytochrome *c* concentration is for the ratios of k_1/k_3 and k_2/k_4 to be equal [see Kim et al. (1990)]. Assuming this relationship holds, the initial velocity expression for the mechanism shown in Figure 1 is given by eq 6. K_{D1} and

$$v_0 = \frac{V_1 K_{D2}[c^{2+}] + V_2[c^{2+}]^2}{K_{D1}K_{D2} + K_{D2}[c^{2+}] + [c^{2+}]^2} \qquad (6)$$

K_{D2} are as defined in Figure 1; V_1/e is equal to $k_1k_3/(k_1 + k_3)$ and V_2/e is equal to $k_2k_4/(k_2 + k_4)$. Under some experimental

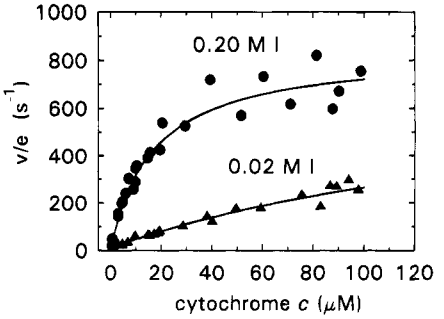

FIGURE 2: Initial velocities for the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide as a function of the cytochrome *c* concentration. Data are shown for experiments conducted at 0.020 M ionic strength (triangles) and 0.200 M ionic strength (circles). The solid lines were calculated according to eq 6 of the text using the steady-state parameters given in Table 1. Experimental conditions: 200 μM hydrogen peroxide, pH 7.5, potassium phosphate/KNO_3 buffers; 25 °C.

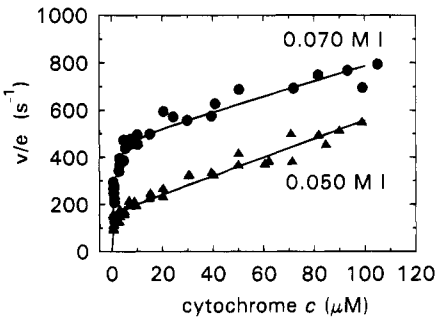

FIGURE 3: Initial velocities for the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide as a function of the cytochrome *c* concentration. Data are shown for experiments conducted at 0.050 M ionic strength (triangles) and 0.070 M ionic strength (circles). The solid lines were calculated according to eq 6 of the text using the steady-state parameters given in Table 1. Experimental conditions: 200 μM hydrogen peroxide, pH 7.5, potassium phosphate/KNO_3 buffers; 25 °C.

conditions, one or more of the parameters become negligible, reducing the four-parameter equation to a two- or three-parameter equation.

Weighted, nonlinear, least-squares regression analysis was used to determine the best-fit values for the parameters in eq 6. Since the coefficient of variation for the initial velocities was independent of the absolute value of the velocity, weights proportional to the inverse of the squared initial velocities were used (Mannervik, 1983).

RESULTS

The initial velocity for the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide has been investigated as a function of the cytochrome *c* concentration between 0.010 and 0.20 M ionic strength. Figure 2 shows the dependence of the initial velocity on the substrate concentration at 0.020 and 0.20 M ionic strength.[2] A striking feature of Figure 2 is that the initial velocities saturate at the higher ionic strength but not at the lower ionic strength. This is *opposite* to expectation for the interaction between oppositely charged proteins (Vitello & Erman, 1987). Figure 3 shows the data at 0.050 and 0.070 M ionic strength. The data at the intermediate ionic strengths are clearly biphasic

[2] The data at 0.01 and 0.10 M ionic strength have been published in a preliminary communication (Erman et al., 1991).

Table 1: Steady-State Kinetic Parameters for the CcP-Catalyzed Oxidation of Yeast Iso-1 Ferrocytochrome *c* by Hydrogen Peroxide as a Function of Ionic Strength[a]

ionic strength (M)	K_{D1} (μM)	V_1/e (s^{-1})	V_2/eK_{D2} (μM^{-1} s^{-1})	K_{D2} (μM)	V_2/e (s^{-1})
0.010	≤0.05	2.1 ± 0.9	5.6 ± 0.5	260 ± 130	1450 ± 730
0.020	≤0.05	8.9 ± 1.2	4.1 ± 0.4	190 ± 70	750 ± 230
0.040	0.48 ± 0.18	80 ± 30	6.8 ± 3.0	[390][b]	[1600 ± 140][b]
0.050	0.29 ± 0.06	170 ± 70	14 ± 4	[460][b]	[2300 ± 140][b]
0.070	0.74 ± 0.12	470 ± 80	4.3 ± 1.3	[600][b]	[2600 ± 360][b]
0.100	2.6 ± 0.3	880 ± 110			
0.200	17 ± 2	840 ± 70			

[a] Data acquired at pH 7.5, 25 °C. K_{D1} and K_{D2} are equilibrium dissociation constants for the 1:1 and 2:1 cytochrome *c*/CcP complexes as defined in Figure 1. V_1/e and V_2/e are equal to $k_1k_3/(k_1 + k_3)$ and $k_2k_4/((k_2 + k_4)$, respectively, with the rate constants defined in Figure 1. [b] The V_2/e values in brackets were obtained from fitting the initial velocity data using fixed values of K_{D2}, calculated from eq 8 in the text. The calculated K_{D2} values are shown in brackets.

with respect to the cytochrome *c* concentration and show no indication of saturation at the highest cytochrome *c* concentrations used in this study.

The cytochrome *c* concentration dependence of the initial velocities was fit to eq 6 in order to extract the "best-fit" values for the steady-state parameters. At 0.10 and 0.20 M ionic strength, the initial velocity is a hyperbolic function of the cytochrome *c*. Equation 6 reduces to a hyperbolic function when K_{D2} is set equal to infinity, i.e., no binding to the low-affinity binding site.

The data at intermediate ionic strengths are biphasic and do not saturate at the highest concentrations of substrate used in this study, Figure 3. Equation 6 can be reduced to a three-parameter equation by dividing the numerator and the denominator by K_{D2}. The squared cytochrome *c* concentration term in the denominator becomes negligible at intermediate ionic strength but the squared cytochrome *c* concentration term in the numerator makes a significant contribution to the observed velocity. The coefficient of the squared cytochrome *c* concentration term in the numerator is equal to V_2/K_{D2}, and best-fit values for this parameter are given in Table 1.

At 0.010 and 0.020 M ionic strength, the initial velocities *almost* fit a simple hyperbolic equation, but there are small deviations at low cytochrome *c* concentrations. Our interpretation is that at the lowest ionic strengths, the "high-affinity" phase of the kinetics is saturated because the value of K_{D1} is so small. The best-fit parameters for eq 6 are given in Table 1.

DISCUSSION

Steady-State Mechanism. We previously investigated the steady-state CcP-catalyzed oxidation of horse ferrocytochrome *c* by hydrogen peroxide under identical experimental conditions (Kim et al., 1990). The oxidation of horse cytochrome *c* was analyzed according to both one- and two-binding site mechanisms. It was concluded that a two-binding site mechanism was inconsistent with the data and that a single-binding site, multiple-pathway mechanism was the best interpretation for the oxidation of horse cytochrome *c*. However, the oxidation of yeast iso-1 ferrocytochrome *c* is substantially different from that of horse cytochrome *c*. We have tried various one-binding site mechanisms to fit the steady-state velocities for yeast iso-1 ferrocytochrome *c*

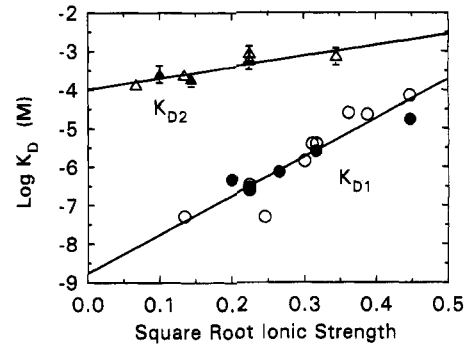

FIGURE 4: Plots of the logarithm of the equilibrium dissociation constants for the 1:1 and 2:1 yeast iso-1 cytochrome *c*/CcP complexes as a function of the square root of ionic strength. The solid circles are K_{D1} values determined in this steady-state kinetic study at pH 7.5 (Table 1). The open circles are published K_{D1} values for the binding of yeast iso-1 ferrocytochrome *c* to CcP between pH 6.0 and 7.75 (McLendon et al., 1993; Stempf & Hoffman, 1993; Mauk et al., 1994). The solid triangles are K_{D2} values determined from the steady-state studies at pH 7.5 (Table 1). The open triangles are published K_{D2} values for the yeast iso-1 cytochrome *c*/CcP complex, pH 6.5−7.75 (Mauk et al., 1994) and the horse cytochrome *c*/CcP complex, pH 7.0 (Zhou & Hoffman, 1993, 1994).

oxidation without success. There is growing evidence that 2:1 yeast cytochrome *c*/CcP complexes can form in solution (Mauk et al., 1994; Zhou & Hoffman, 1993, 1994), and we have been able to achieve a consistent interpretation of the steady-state data using a two-binding-site mechanism for the CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* by hydrogen peroxide.

Binding at the High-Affinity Site. Relatively few equilibrium constants for the binding of yeast iso-1 cytochrome *c* have been published (McLendon et al., 1993; Stempf & Hoffman, 1993; Mauk et al., 1994). The available data have been obtained at a variety of pH values and ionic strengths. Mauk et al. (1994) showed that binding at the high-affinity site is relatively insensitive to pH between 6.0 and 7.75. This small pH dependence is consistent with our observations for the binding of horse cytochrome *c* (Vitello & Erman, 1987). Figure 4 shows a plot of the logarithm of K_{D1} as a function of the square root of ionic strength. The plotted data are published values of K_{D1} which have been obtained between pH 6.0 and 7.75. Also included in the plot are the values of K_{D1} obtained from steady-state kinetic studies between 0.040 and 0.20 M ionic strength (Table 1). There is excellent agreement between the values obtained by steady-state kinetics and other techniques. A linear least-squares correlation of the logarithm of K_{D1} as a function of the square root of ionic strength for all of the data is shown in Figure 4. The linear regression line shown in Figure 4 was calculated according to eq 7.

$$\log K_{D1} = (-8.76 \pm 0.33) + (10.1 \pm 1.1)\sqrt{I} \quad (7)$$

At 0.010 and 0.020 M ionic strength, the binding is so strong that the high-affinity binding site is saturated at the *lowest* cytochrome *c* concentrations used in this study (~0.5 μM). The increase in initial velocity with increasing cytochrome *c* concentration at the two lowest ionic strengths is due to oxidation of ferrocytochrome *c* at the low-affinity binding site. In fitting the kinetic data at 0.010 and 0.020 M ionic strength, we fixed the value of K_{D1} at 0.05 μM and allowed the other parameters to vary in order to obtain the

best-fit parameters for the low-affinity site. It made little difference in the values of the fitted parameters whether K_{D1} was fixed at any value ≤ 0.05 μM.

Binding at the Low-Affinity Site. Very little data are available for binding of cytochrome *c* to the low-affinity binding site. Stempf & Hoffman (1993) concluded that binding of horse and yeast iso-1 cytochrome *c* is similar at the low-affinity site, with comparable values of the equilibrium dissociation constant, K_{D2}. Mauk et al. (1994) have shown the K_{D2} for the binding of yeast iso-1 to CcP is relatively insensitive to pH between 6.5 and 7.75 at 0.050 M ionic strength. In Figure 4 we show a correlation for the logarithm of K_{D2} as a function of the square root of ionic strength for the binding of both yeast iso-1 and horse cytochrome *c*. Included in the plot are the two values of K_{D2} we obtained from the steady-state kinetic studies at 0.010 and 0.020 M ionic strength. The linear least-squares regression line for the logarithm of K_{D2} as a function of the square root of ionic strength for all of the data shown in Figure 4 is given by eq 8. Variation in the value of K_{D2} as

$$\log K_{D2} = (-3.98 \pm 0.15) + (2.85 \pm 0.75)\sqrt{I} \quad (8)$$

a function of ionic strength is much smaller than that of K_{D1}, with the slope of the plot of log K_{D2} versus square root of ionic strength about one-third that of the plot for log K_{D1}.

The internal consistency of the steady-state data and the excellent agreement between the K_{D1} and K_{D2} values obtained from the steady-state results and from other techniques provide strong support for the two-binding site mechanism for CcP-catalyzed oxidation of yeast iso-1 ferrocytochrome *c* shown in Figure 1.

Intracomplex Electron Transfer via High Affinity Site. The steady-state velocities allow evaluation of the rate-limiting conversion of CcP compound **I** to compound **II** and of **II** to the native enzyme via the high-affinity site. Maximum enzyme turnover via the 1:1 complex, as measured by the parameter $k_1k_3(k_1 + k_3)$, is only 2.1 and 8.9 s^{-1} at 0.010 and 0.020 M ionic strength, respectively (Table 1). As will be seen in the accompanying paper (Matthis et al., 1995), the value of k_1 can be measured directly by stopped-flow techniques, and at 0.010 M ionic strength, the value of k_1 is 17 ± 8 s^{-1}. Combining the steady-state and stopped-flow results leads to a value of 2.4 s^{-1} for k_3. Both k_1 and k_3 are significantly smaller than the dissociation rate of yeast iso-1 ferricytochrome *c* from the high-affinity site as determined by NMR exchange dynamics (Yi et al., 1994). This indicates that k_1 and k_3 are a measure of rate-limiting electron transfer from ferrocytochrome *c* to the oxidized sites in CcP compounds **I** and **II**. The very slow rate of electron transfer suggests that conformational gating of the reaction could be occurring and that the conformation of the 1:1 complex is not optimal for efficient electron transfer at low ionic strength (Hazzard et al., 1988). The value of $k_1k_3/(k_1 + k_3)$ is very sensitive to ionic strength (Table 1, Figure 5), increasing with increasing ionic strength until it reaches a plateau value of 860 ± 65 s^{-1} above 0.10 M ionic strength.

Electron Transfer via the 2:1 Complex. At 0.010 and 0.020 M ionic strength, the oxidation of yeast iso-1 ferrocytochrome *c* occurs primarily via the 2:1 complex. The binding is quite weak, but sufficient 2:1 complex exists at 100 μM cytochrome *c* that an extrapolation to the intracomplex electron-transfer rate can be made. The long extrapolation causes relatively large uncertainty in the value of $k_2k_4/(k_2 + k_4)$. Best-fit values of 1450 ± 730 and 750 ± 230 s^{-1} are obtained at 0.010 and 0.020 M ionic strength, respectively. Averaging these two values gives an estimate of 1100 ± 400 s^{-1} for $k_2k_4/(k_2 + k_4)$ at low ionic strength.

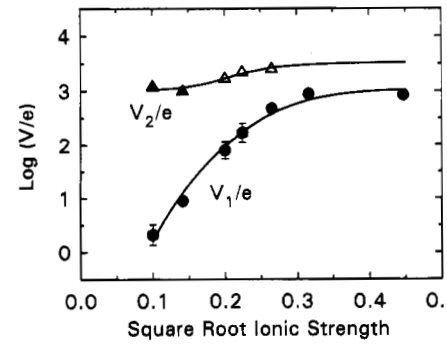

FIGURE 5: Plots of the logarithm of the maximum velocities for yeast iso-1 ferrocytochrome *c* oxidation via the 1:1 and 2:1 cytochrome *c*/CcP complexes as a function of the square root of ionic strength at pH 7.5, 25 °C. The solid circles are the best-fit values for V_1/e, the maximum enzyme turnover rate via the 1:1 cytochrome *c*/CcP complex. V_1/e varies from 2.1 ± 0.9 s^{-1} at 0.010 M ionic strength to 840 ± 70 s^{-1} at 0.200 M ionic strength. The triangles represent the maximum enzyme turnover rate via the 2:1 cytochrome *c*/CcP complex. The solid triangles are determined solely from the steady-state data (Table 1). The open triangles were obtained from the initial velocity data by fixing K_{D2} to the value calculated from eq 8 at the appropriate ionic strength.

Between 0.040 and 0.070 M ionic strength, it is apparent that the low-affinity site is contributing to the observed rate of oxidation of yeast iso-1 ferrocytochrome *c* (Figure 2), but the binding is so weak that extrapolation to the maximum rate is not possible from the kinetic data alone (Table 1). However, if we combine our steady-state kinetic data with K_{D2} values obtained by the correlation shown in Figure 4, estimates of $k_2k_4/(k_2 + k_4)$ can be obtained up to 0.070 M ionic strength, Figure 5. At 0.070 M ionic strength, the value of $k_2k_4/(k_2 + k_4)$ is 2700 ± 360 s^{-1}. The initial velocity data at 0.10 and 0.20 M ionic strength are excellent hyperbolic functions of the cytochrome *c* concentration, and values for $k_2k_4/(k_2 + k_4)$ cannot be determined even if the values of K_{D2} are incorporated into the equation. We have determined that $k_2k_4/(k_2 + k_4)$ cannot be significantly larger than 2700 s^{-1} at 0.10 M ionic strength through simulations of the initial velocities. If $k_2k_4/(k_2 + k_4)$ were as large as 3000 s^{-1}, significant deviations from hyperbolic kinetics would be observed at 0.10 M ionic strength. It is possible that $k_2k_4/(k_2 + k_4)$ could *decrease* substantially above 0.10 M ionic strength and not affect the steady-state velocities, but we consider this unlikely. It is reasonable to assume that intracomplex electron transfer via the low-affinity site increases from a value of 1100 ± 400 s^{-1} at 0.010−0.020 M ionic strength to 2700 ± 400 s^{-1} at 0.070 M ionic strength and above (Figure 5).

Stopped-flow measurements (Matthis et al., 1995) indicate that k_2 has a value of 4200 ± 1100 s^{-1} between 0.010 and 0.020 M ionic strength, which, when combined with the steady-state parameter, gives a value of 1500 ± 500 s^{-1} for k_4 in this ionic strength region.

Conclusions. The contrast in the CcP-catalyzed steady-state oxidation of yeast iso-1 ferrocytochrome *c* and horse ferrocytochrome *c* is remarkable. At pH 7.5, between 0.01 and 0.20 M ionic strength oxidation of yeast iso-1 ferrocy-

tochrome *c* is consistent with a two-binding site mechanism while oxidation of horse ferrocytochrome *c* is not (Kim et al., 1990). The strongest evidence supporting the two-binding site mechanism for yeast iso-1 ferrocytochrome *c* is the consistency between the K_{D1} and K_{D2} values found from the steady-state kinetic studies and published values for K_{D1} and K_{D2} determined by vastly different techniques (Stempf & Hoffman, 1993; Zhou & Hoffman, 1993, 1994; Mauk et al., 1994). On the other hand, fitting the steady-state velocities for oxidation of horse ferrocytochrome *c* to a two-binding site mechanism gives inconsistent values for K_{D1} and K_{D2}. In particular, the value of K_{D2} obtained from the two-binding site kinetic mechanism for horse ferrocytochrome *c* is up to 50 times smaller than published values.

The difference between the steady-state oxidation of yeast iso-1 and horse ferrocytochrome *c* is almost certainly due to the differences in the interaction between these two cytochromes and CcP. The binding of yeast iso-1 cytochrome *c* to the high-affinity site is significantly stronger than binding of horse cytochrome *c*, averaging 40 ± 20 times stronger between 0.01 and 0.10 M ionic strength (Vitello & Erman, 1987; Stempf & Hoffman, 1993; Zhou & Hoffman, 1993, 1994; Mauk et al., 1994). The very high affinity of CcP for yeast cytochrome *c* is also evident in the much slower rate of yeast cytochrome *c* dissociation (~180 s^{-1}) compared to that of horse cytochrome *c* (≥1000 s^{-1}) in 0.01 M KNO_3 solutions at neutral pH (Satterlee et al., 1987; Yi et al., 1994).

At 0.01 M ionic strength, the maximum rate of cytochrome *c* oxidation via the high-affinity site is 250 ± 20 s^{-1} for horse cytochrome *c* and 2.1 ± 0.9 s^{-1} for yeast iso-1 ferrocytochrome *c* at pH 7.5. Oxidation of horse cytochrome *c* via the high-affinity site is sufficiently fast at low ionic strength that contributions from the secondary, low-affinity site are negligible over the cytochrome *c* concentration range investigated. On the other hand, the rate of oxidation of yeast iso-1 ferrocytochrome *c* at the high-affinity site is so slow at low ionic strength that secondary interactions between CcP and yeast cytochrome *c* become of major importance in the rate of yeast iso-1 ferrocytochrome *c* oxidation. As the ionic strength increases, the rate of horse cytochrome *c* oxidation via the high-affinity site remains constant at ~290 ± 30 s^{-1} at pH 7.5 (Kim et al., 1990) while the rate of yeast iso-1 ferrocytochrome *c* oxidation via the high-affinity binding site increases from 2.1 ± 0.9 s^{-1} at 0.01 M ionic strength to reach a plateau value of 860 ± 70 s^{-1} at 0.1 M ionic strength.

The current hypothesis suggests that this increase in rate is due to an increasing mobility between CcP and cytochrome *c* which allows for better alignment of the two proteins for electron transfer as the ionic strength is increased. Another intriguing question is why the rate of yeast iso-1 ferrocytochrome *c* oxidation via the low-affinity binding site is faster than via the high-affinity binding site. Assuming that V_2/e measures the sum of the oxidation rates for yeast cytochrome *c* bound to the high- and low-affinity sites, the corrected rate for oxidation via the low-affinity site only ($V_2/e - V_1/e$) averages 1700 ± 500 s^{-1} over the entire ionic strength range. This is twice the plateau value for V_1/e at high ionic strength and suggests a more efficient electron-transfer pathway via the low-affinity site.

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