Characterization of a Covalently Linked Yeast Cytochrome *c*−Cytochrome *c* Peroxidase Complex: Evidence for a Single, Catalytically Active Cytochrome *c* Binding Site on Cytochrome *c* Peroxidase†

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ABSTRACT: A covalent complex between recombinant yeast iso-1-cytochrome *c* and recombinant yeast cytochrome *c* peroxidase (rCcP), in which the crystallographically defined cytochrome *c* binding site [Pelletier, H., and Kraut, J. (1992) *Science 258*, 1748−1755] is blocked, was synthesized via disulfide bond formation using specifically engineered cysteine residues in both yeast iso-1-cytochrome *c* and yeast cytochrome *c* peroxidase [Papa, H. S., and Poulos, T. L. (1995) *Biochemistry 34*, 6573−6580]. Previous studies on similar covalent complexes, those that block the Pelletier−Kraut crystallographic site, have demonstrated that samples of the covalent complexes have detectable activities that are significantly lower than those of wild-type yCcP, usually in the range of ∼1−7% of that of the wild-type enzyme. Using gradient elution procedures in the purification of the engineered peroxidase, cytochrome *c*, and covalent complex, along with activity measurements during the purification steps, we demonstrate that the residual activity associated with the purified covalent complex is due to unreacted CcP that copurifies with the covalent complex. Within experimental error, the covalent complex that blocks the Pelletier−Kraut site has zero catalytic activity in the steady-state oxidation of exogenous yeast iso-1-ferrocytochrome *c* by hydrogen peroxide, demonstrating that only ferrocytochrome *c* bound at the Pelletier−Kraut site is oxidized during catalytic turnover.

Cytochrome *c* peroxidase (CcP)[1] has played a significant role in the elucidation of the structural basis of heme protein reactivity since CcP was the first heme enzyme whose three-dimensional structure was determined by X-ray crystallography (*1*, *2*). CcP, initially discovered in *Saccharomyces cerevisiae* (*3*), catalyzes the reduction of hydrogen peroxide to water using the reducing equivalents from two molecules of ferrocytochrome *c* (*3*, *4*). During the catalytic process, hydrogen peroxide oxidizes the native Fe(III) form of the enzyme to an intermediate called compound I (*4*, *5*) in which the heme iron is oxidized to an Fe(IV), oxyferryl group (*6*, *7*) and the side chain of Trp-191 is oxidized to a cationic indole radical (*8*−*10*). Two molecules of ferrocytochrome *c* transfer electrons to the Fe(IV) and Trp-191 radical sites and return the enzyme to the native Fe(III) state.

Despite the seeming simplicity of the redox chemistry in the cytochrome *c*−CcP system, unraveling the details of the catalytic mechanism and of the electron transfer reactions has been exceedingly complex. A major problem concerns the binding of cytochrome *c* to CcP. Early studies demonstrated one-to-one (1:1) complex formation with an affinity constant that was strongly dependent upon ionic strength (*11*−*14*). The possibility of a 2:1 complex, with two molecules of cytochrome *c* simultaneously bound to CcP, was first postulated by Margoliash and co-workers in 1977 to explain the steady-state kinetics of the CcP-catalyzed reaction (*15*). Early support for 2:1 complexes came primarily from kinetic studies, studies for which alternative interpretations based on 1:1 complexes could be offered (*16*). As a consequence, the idea of 2:1 complexes remained controversial until 1994 when Mauk and colleagues provided evidence of 2:1 complexes using a nonkinetic technique (*17*). The equilibrium association constant for formation of the 2:1 complex is much smaller than that of the 1:1 complex with the affinity for binding the second cytochrome *c* being 2−4 orders of magnitude weaker than that for binding the first cytochrome *c*, depending upon ionic strength (*14*). There are still unanswered questions about the nature of the 1:1 and 2:1 complexes, including the relative electron transfer activity of cytochrome *c* bound at different locations on the surface of CcP.

Two extreme models for cytochrome *c* binding can be postulated. The first is one in which there is a unique, high-affinity cytochrome *c* binding site on the surface of CcP and this site is defined by the crystal structure of the 1:1 complex as determined by Pelletier and Kraut (*18*). In this model,

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[1] Abbreviations: CcP, cytochrome *c* peroxidase from any source; yCcP, authentic yeast cytochrome *c* peroxidase isolated from *Saccharomyces cerevisiae*; TCEP, tris(2-carboxyethyl)phosphine; rCcP, recombinant cytochrome *c* peroxidase expressed in *Escherichia coli*. The rCcP used in this study has an amino acid sequence identical to that yCcP. Mutations in the amino acid sequences of either CcP or cytochrome *c* are indicated by using the one-letter code for the amino acid residue in the wild-type protein, followed by the residue number and the one-letter code for the amino acid residue in the mutant protein, i.e., C102T represents a mutant in which a threonine residue replaces the cysteine residue at position 102 of the wild-type protein.

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there is a second, much weaker cytochrome *c* binding site on the surface of CcP. In the extreme case, this model assumes that there is no interaction between the bound cytochromes. The second model postulates that there are multiple cytochrome *c* binding sites on the surface of CcP with similar binding affinities (*19*, *20*). Thus, the 1:1 complex is actually a mixture of microscopic species, each with a single cytochrome *c* bound at different locations on the surface of CcP. In this view, the Pelletier−Kraut structure is just one of several possible microscopic forms of the 1:1 complex. In this second model, formation of a 2:1 complex is inhibited by strong electrostatic repulsion between the bound cytochromes.

The ability of cytochrome *c* to bind to multiple locations on the surface of CcP leads to the possibility that rates of electron transfer from bound cytochrome *c* to the Fe(IV) and Trp-191 radical sites in CcP compound I may differ significantly, depending upon the location of the bound cytochrome *c*. Different experimental approaches have led to different conclusions concerning the redox activity of bound cytochrome *c* (*20*−*35*). One mechanism, postulated primarily by Millett, Durham, and co-workers (*21*−*28*), includes a unique high-affinity binding site for cytochrome *c* on the surface of CcP, and only cytochrome *c* bound at this site is catalytically active during enzyme turnover. An alternative hypothesis, primarily championed by Hoffman and co-workers (*20*, *29*−*35*), postulates multiple forms of the 1:1 complex, that cytochrome *c* bound anywhere on the surface of CcP will have electron transfer activity as predicted by the pathway model for electron transfer (*20*), and that the second cytochrome *c* bound in the 2:1 complex has a higher rate of electron transfer to the heme in CcP than the initial cytochrome *c* bound in the 1:1 complex (*31*, *32*).

In an effort to resolve the issue of the redox activity of cytochrome *c* bound at different locations on the surface of CcP, Poulos and colleagues have pioneered the use of covalent complexes of cytochrome *c* and CcP (*36*−*38*). Of particular interest are the synthesis and characterization of two different covalent complexes in which cytochrome *c* is covalently attached to CcP at the Pelletier−Kraut crystallographic site (*36*−*38*). One complex has residue 290 in CcP covalently attached to residue 73 in yeast iso-1-cytochrome *c* through a disulfide linkage, blocking the Pelletier−Kraut site. This complex is much less reactive than wild-type CcP in steady-state turnover experiments. In experiments with horse ferrocytochrome *c* as a substrate, this complex is only 3% as active as wild-type CcP. When using yeast iso-1-ferrocytochrome *c* as a substrate, the covalent complex retains 1−30% of wild-type activity depending upon the experimental conditions (*37*). A second covalent construct links residue 187 in CcP with residue 81 in cytochrome *c* via a disulfide bridge (*38*). An X-ray structure of this covalent complex indicates that the relative positions of the two proteins in the covalent complex are very similar to those in the noncovalent Pelletier−Kraut structure (*18*). This complex retains 3−7% of wild-type activity using yeast ferrocytochrome *c* as a substrate. These studies indicate that blocking the Pelletier−Kraut site substantially decreases the activity of CcP, consistent with the hypothesis that the Pelletier−Kraut site is the primary catalytic site and that cytochrome *c* bound to secondary sites is much less reactive in catalytic turnover.

In this paper, we describe a refinement of the synthesis and purification of the covalent complexes described by Papa and Poulos (*36*) and demonstrate that the residual activity previously reported for the CcP(E290C)−cytochrome *c*(K73C) complex is due to small amounts of free CcP that copurify with the covalent complex. By measuring the activity of fractions containing the covalent complex during a gradient separation procedure, we are able to demonstrate that the covalent complex, in which the Pelletier−Kraut crystallographic site is blocked, is completely inactive.

MATERIALS AND METHODS

Starting Clones and Mutagenesis. J. Satterlee (Washington State University, Pullman, WA) kindly provided the expression system for the recombinant CcP used in this study (*39*). The cloned CcP gene was modified so that the amino acid sequence of the expressed protein is identical to that of baker's yeast CcP (*40*). The CcP gene is inserted into the multiple cloning site of Novagene vector pET24a(+) under control of the T7 promoter. The N-terminal methionine required for initiation of protein synthesis is removed from the recombinant CcP produced by this expression system so that there are no N-terminal modifications in the recombinant CcP compared to baker's yeast CcP (*41*).

G. Pielak (University of North Carolina, Chapel Hill, NC) kindly provided the pBTR(C102T) plasmid containing the gene for yeast iso-1-cytochrome *c* used in this study (*42*). The yeast iso-1-cytochrome *c* gene has been altered, cysteine 102 being replaced with a threonine residue (C102T) to prevent dimerization of the native protein via disulfide bond formation. The plasmid also contains the gene for yeast cytochrome *c* heme lyase to promote covalent attachment of the heme (*43*).

Mutations of both CcP and cytochrome *c* were created using Stratagene Quik-Change mutagenesis and sequenced from 5′ to 3′ and from 3′ to 5′ to ensure that, except for the intended mutation, the protein was identical to the published sequence. Wild-type CcP contains a single cysteine residue at position 128 that could interfere with the desired covalent linkage. The CcP mutant used in this study is a double mutant, C128S/E290K. Likewise, a double mutant of yeast cytochrome *c* was constructed for the synthesis of the covalent complex, K73C/C102T.

Protein Expression and Purification. Recombinant CcP, cytochrome *c*(C102T), and both double mutants were expressed in *Escherichia coli* strain BL21(DE3) and isolated using published procedures (*41*−*45*).

Protein Concentration Determination. Protein concentrations were determined spectrophotometrically. Spectra were determined using either a Varian/Cary model 3E spectrophotometer or a Hewlett-Packard model 8452A diode array spectrophotometer. The extinction coefficients for all heme proteins used in this study were determined using the pyridine hemochromogen method of Berry and Trumpower (*46*). The extinction coefficients and positions of the Soret maxima for the various proteins are as follows: rCcP, 101 ± 3 mM^{-1} cm^{-1} at 408 nm; CcP(E290C), 96 ± 2 mM^{-1} cm^{-1} at 410 nm; reduced cytochrome *c*(C102T), 150 ± 5 mM^{-1} cm^{-1} at 414 nm; and oxidized yeast cytochrome *c*(C102T), 118 ± 4 mM^{-1} cm^{-1} at 408 nm. Cytochrome *c*(K73C) is prone to covalent dimerization with the spectrum of the oxidized form

of the protein being dependent upon the monomer:dimer ratio. Monomeric and dimeric oxidized cytochrome *c*(K73C) have maximum extinction coefficients of 97 mM^{-1} cm^{-1} at 412 nm and 115 mM^{-1} cm^{-1} at 407 nm, respectively. The spectrum of the reduced form of cytochrome *c*(K73C) is independent of the monomer:dimer ratio and has a maximum extinction coefficient of 137 mM^{-1} cm^{-1} at 416 nm. Spectra and spectroscopic properties for all proteins used in this study are given as Supporting Information.

Synthesis of the CcP−Cytochrome c Covalent Complex. Synthesis of the 1:1 covalent complex of rCcP and yeast iso-1-cytochrome *c* via a disulfide link using the cysteine residues engineered into rCcP (position 290) and recombinant cytochrome *c* (position 79) was carried out using a slight modification of the method of Papa and Poulos (*36*, *37*). A detailed synthesis procedure is described in the Supporting Information.

SDS−PAGE Analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using a PhastSystem from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Proteins were incubated at room temperature for 15 min in a 10 mM Tris-HCl buffer (pH 8) containing 2.5% SDS and 1 mM EDTA. To preserve the covalent disulfide cross-links, the denaturing buffer did not contain disulfide reducing agents. Protein samples were loaded onto a PhastGel gradient 10−15 along with standard molecular mass markers. SDS buffer strips were used during the electrophoresis. The gels were stained with Coomassie blue.

Hydrogen Peroxide Concentration. Reagent grade 30% (v/v) hydrogen peroxide was purchased from Aldrich Chemical Co., Inc. The concentration of hydrogen peroxide stock solutions was determined by titration with cerium(IV) sulfate (*47*).

Steady-State Kinetic Studies. Steady-state kinetic studies and activity measurements were performed at pH 7.5 in potassium phosphate buffers at an ionic strength of either 10 or 100 mM. Initial velocities were determined as a function of yeast iso-1-ferrocytochrome *c*(C102T) concentration (generally 1−100 μM) at a constant hydrogen peroxide concentration (200 μM). Initial velocities were determined by measuring the change in absorbance upon oxidation of ferrocytochrome *c* at multiple wavelengths using a Hewlett-Packard model 8452A diode array spectrophotometer. Buffer, cytochrome *c*, and enzyme were thermally equilibrated at 25 °C in the spectrophotometer; initial absorbance readings were taken, and then the reaction was initiated by addition of the hydrogen peroxide. Five different wavelengths, generally chosen from the set of 314, 362, 418, 448, 468, 478, 548, 564, and 574 nm depending upon the substrate concentration, were used to calculate the initial velocity under each set of experimental conditions using eq 1.

$$\frac{v_0}{e_0} = \frac{1}{2(1 - f_{ox})\Delta\epsilon}\frac{\Delta A}{\Delta t} \quad (1)$$

The symbols in eq 1 include the initial velocity (v_0), the total enzyme concentration (e_0), the change in absorbance with time ($\Delta A/\Delta t$), and the difference in the extinction coefficient ($\Delta\epsilon$) between oxidized and reduced yeast iso-1-cytochrome *c*(C102T). Samples of the substrate may contain small amounts of oxidized cytochrome *c* that can inhibit the reaction; f_{ox} is the fraction of oxidized cytochrome *c* in the substrate and is used to make small corrections to the initial velocity. The factor of 2 in the denominator converts cytochrome *c* turnover to enzyme turnover.

RESULTS AND DISCUSSION

Steady-State Activity of the CcP(E290C)−Cytochrome c(K73C) Covalent Complex. The steady-state velocities for both the covalent complex and rCcP as functions of the ferrocytochrome *c* concentration were determined at ionic strengths of 10 and 100 mM in potassium phosphate buffers (pH 7.5). The data are provided as Supporting Information. Under these experimental conditions, the data for both the covalent complex and rCcP can be fit to a simple Michaelis−Menten equation. At an ionic strength of 10 mM, the Michaelis constant (K_M) equals 130 ± 20 and 47 ± 8 μM for rCcP and the covalent complex, respectively. For rCcP, the very large value of K_m is associated with binding of a second molecule of cytochrome *c* to rCcP, with the value of 130 μM similar to reported values of the apparent equilibrium dissociation constant for the 2:1 cytochrome *c*−CcP complex (K_{D2}) at an ionic strength of 10 mM (*14*). The K_M value of 47 μM for the covalent complex sample indicates that secondary cytochrome *c* binding in the covalent complex samples is also weak. It should be noted that the value of K_{D1}, the apparent equilibrium dissociation constant for the 1:1 yeast iso-1-cytochrome *c*−CcP noncovalent complex, is less that 0.1 μM, perhaps as small as 0.01 μM (*14*), at an ionic strength of 10 mM, 3−4 orders of magnitude smaller than the observed K_M values.

At an ionic strength of 10 mM, the V_{max}/e_0 values for rCcP and the covalent complex are 633 ± 64 and 19 ± 2 s^{-1}, respectively. On the basis of the maximum velocities, this sample of the CcP(E290C)−cytochrome *c*(K73C) covalent complex is ∼3% as active as rCcP, similar to values previously reported by Poulos and co-workers (*37*, *38*).

At an ionic strength of 100 mM, the K_M values are 2.0 ± 0.2 and 11 ± 4 μM for rCcP and the covalent complex, respectively. The V_{max}/e_0 values for rCcP and the covalent complex are 625 ± 14 and 18 ± 2 s^{-1}, respectively, again indicating that this sample of the covalent complex is ∼3% as active as rCcP. The K_M value for rCcP is similar to K_{D1} values for the yeast iso-1-cytochrome *c*−CcP complex at an ionic strength of 100 mM (*14*). The K_M value of 11 μM for the covalent complex is intriguing. If this K_M represents the binding of an exogenous cytochrome *c* to a secondary binding site on the covalent complex, this would be the highest affinity yet observed for formation of a 2:1 cytochrome *c*−CcP complex, with the affinity for binding the second cytochrome *c* only 5-fold weaker than the affinity for binding the first. This seems unlikely, and alternative explanations for the activity of the covalent complex were sought.

In overloaded, nonreducing SDS−PAGE gels, it was noticed that the covalent complex samples contained small amounts of impurities that migrated with rates similar to those of the cytochrome *c* monomer, cytochrome *c* dimer, CcP, CcP dimer, and higher-molecular mass aggregates. Figure 1 shows the SDS−PAGE patterns for four fractions of the covalent complex isolated by step gradient elution from a CM52 cation-exchange column. Densitometer tracings give

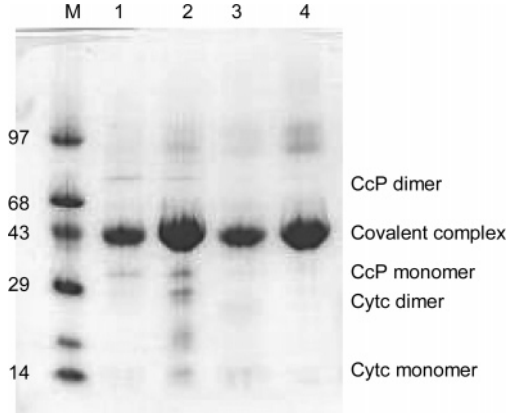

FIGURE 1: SDS−PAGE analysis of four fractions of the CcP(E290C)−cytochrome *c*(K73C) covalent complex purified using a step gradient elution from a CM-Sepharose Fast Flow cation-exchange column: lane M, molecular mass markers with the molecular mass given in kilodaltons; lane 1, leading edge of the eluted peak containing the covalent complex; lane 2, second fraction of the covalent complex peak; lane 3, third fraction of the covalent complex peak; and lane 4, trailing edge of the covalent complex peak.

an estimate of the amounts of CcP monomer and dimer in these samples, and the amount of unreacted CcP ranges between ~3 and 12% of the total protein in the four samples shown in Figure 1. Since this level of contamination of unreacted CcP is similar to the residual activity of the covalent complex, it is reasonable to assume that the observed activity could be due to the small amounts of unreacted CcP that copurify with the covalent complex,

Further Purification of the CcP(E290C)−Cytochrome c(K73C) Covalent Complex. In an effort to improve the purification of covalent dimer, the use of CM-Sepharose Fast Flow resin and gradient elution was evaluated. Figure 2 shows the separation of products from a synthesis reaction using ~17 mg of CcP(E290C) and 29 mg of yeast iso-1-cytochrome *c*(K73C). The separation was performed with a CM-Sepharose Fast Flow column (1.5 cm × 17 cm) equilibrated in 25 mM ammonium acetate. Unreacted CcP(E290C) elutes in the void volume (peak 1, Figure 2). After the sample had been applied, the column was extensively washed using 250 mL (~8 column volumes) of 25 mM ammonium acetate, and then the covalent complex (peak 2) was eluted using a 300 mL (10 column volumes) linear gradient from 25 to 500 mM ammonium acetate. Peak 2, the covalent complex, eluted approximately midway through the gradient. At the end of the linear gradient, peak 3 began eluting and the column was washed with an additional 60 mL (2 column volumes) of 500 mM ammonium acetate. Peak 4 was eluted using a 200 mL (~7 column volumes) linear gradient beginning with 500 mM ammonium acetate and ending with 1.0 M NaCl in 25 mM ammonium acetate. Spectra of each fraction were recorded, and the activities of all fractions through peak 2 and selected other fractions were determined.

After the spectral and activity measurements, appropriate fractions were pooled, dialyzed against deionized water, and lyophilized. The pooled, lyophilized samples were analyzed by nonreducing SDS−PAGE (Figure 3). Overloaded gels were used to look for impurities in the isolated samples. Peak 1 in Figure 2 is predominantly a mixture of monomeric and dimeric CcP but contains detectable quantities of the cytochrome *c* monomer and dimer and covalent complex (Figure 3). Peak 2 is predominantly covalent complex but contains faint traces of the CcP monomer, cytochrome *c* monomer and dimer, and two unidentified, higher-molecular mass (93 and 107 kDa) impurities (Figure 3). Peak 3 is predominantly cytochrome *c* monomer with significant cytochrome *c* dimer and detectable quantities of the covalent complex (Figure 3). Peak 4 is predominantly cytochrome *c* dimer, with significant amounts of cytochrome *c* monomer, and traces of CcP monomer, covalent complex, and an unidentified peak with a molecular mass (19 kDa) between those of the cytochrome *c* monomer and dimer.

One of the more interesting observations concerning the gradient elution of the reaction mixture on the CM-Sepharose Fast Flow column (Figure 2) is the appearance of traces of both the covalent complex and unreacted CcP in all four

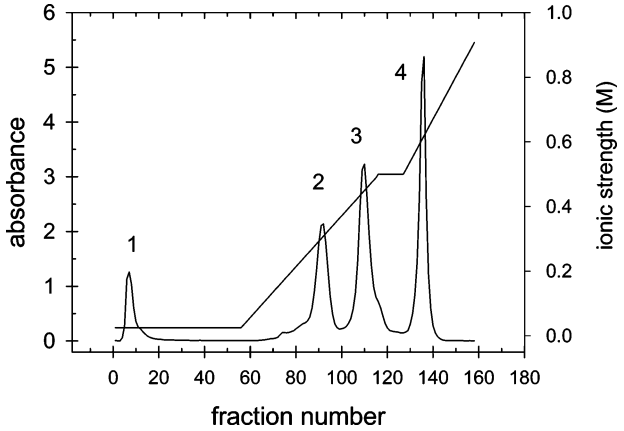

FIGURE 2: Gradient elution of a CcP(E290C)−cytochrome *c*(K73C) reaction mixture on a CM-Sepharose Fast Flow column (1.5 cm × 17 cm). The absorbance of each fraction was determined at 408 nm (left-hand axis), and the ionic strength of the elution buffer is given on the right-hand axis. The column was initially equilibrated in 25 mM ammonium acetate. After application of the reaction mixture, the column was washed with 250 mL of 25 mM ammonium acetate followed by a 300 mL linear gradient from 25 to 500 mM ammonium acetate. At the end of the linear gradient, the column was eluted with an additional 60 mL of 500 mM ammonium acetate. Following the 60 mL hold at 500 mM ammonium acetate, the column was further eluted with a 200 mL linear gradient beginning with 500 mM ammonium acetate and ending with 1.0 M NaCl in 25 mM ammonium acetate. The principal components in each peak are as follows: peak 1, unreacted rCcP(E290C); peak 2, covalent complex; peak 3, unreacted monomeric cytochrome *c*(K73C); and peak 4, unreacted dimeric cytochrome *c*(K73C).

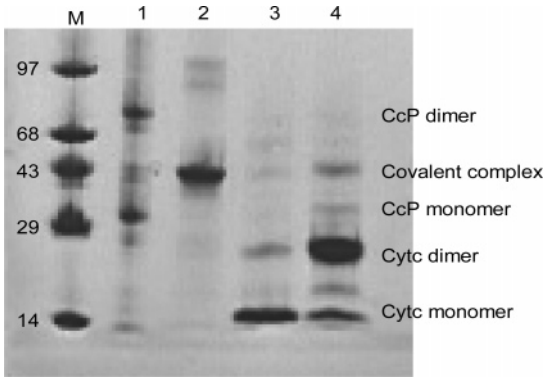

FIGURE 3: SDS−PAGE analysis of peaks 1−4 in the chromatogram shown in Figure 2: lane M, molecular mass markers; lane 1, peak 1; lane 2, peak 2; lane 3, peak 3; and lane 4, peak 4.

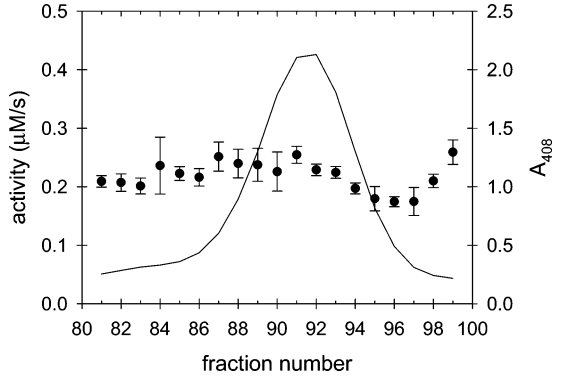

FIGURE 4: Activity of fractions 81−99 from the chromatogram shown in Figure 2. These fractions correspond to the elution of the CcP(E290C)−cytochrome *c*(K73C) covalent complex (peak 2) in the chromatogram shown in Figure 2. The absorbance values at 408 nm (—) for each of the fractions are superimposed over the activities (●). Activities were determined by adding 10 μL of each fraction to 2.0 mL of an assay solution containing 10.2 μM yeast iso-1-ferrocytochrome *c*(C102T) and 200 μM hydrogen peroxide. The assay was performed at pH 7.5 in a 100 mM ionic strength potassium phosphate buffer.

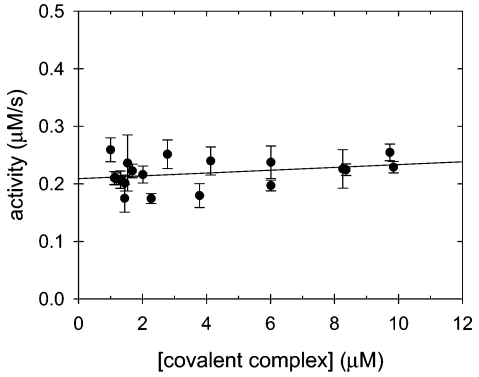

FIGURE 5: Plot of the activities of fractions 81−99 from the chromatogram shown in Figures 2 and 4 as a function of the concentration of the CcP(E290C)−cytochrome *c*(K73C) covalent complex in each fraction. The solid line is a linear least-squares correlation of the data. The best-fit values for the *y*-intercept and slope are 0.20 ± 0.08 μM/s and 0.00 ± 0.02 s^{-1}, respectively.

peaks (Figure 3). A possible explanation is that there are strong noncovalent interactions between all of the protein components in the reaction mixture. The separation will then depend on the competition between binding of the protein components to one another and to the ion-exchange gel.

In a second experiment, a sample of CcP(E290C) that had been separated from a synthesis reaction mixture using step gradient elution from a CM52 column was rechromatographed on the CM-Sepharose Fast Flow column using gradient elution. Most of the sample eluted in the void volume, but a conspicuous brownish-red band remained bound to the top of the column. The column was washed with 100 mL of 25 mM ammonium acetate followed by a 280 mL linear gradient (from 25 to 500 mM ammonium acetate). Fractions were collected, beginning with the sample introduction and ending after 260 mL of the 280 mL gradient was used in the elution. Detectable heme absorbance at 408 nm was observed in all fractions. If the absorbance at 408 nm was attributed to the enzyme, the average CcP(E290C) concentration in the fractions collected during the linear gradient portion of the elution was 0.14 ± 0.10 μM. This experiment suggests that a portion of CcP(E290C) binds tightly to the CM-Sepharose Fast Flow matrix and bleeds off the column at a fairly constant rate throughout the elution, providing an additional source of the unreacted CcP observed in peaks 2−4 in Figure 3.

Activity of Selected Fractions during the Gradient Elution of the Reaction Mixture for Synthesis of the CcP(E290C)−Cytochrome c(K73C) Covalent Complex. Activities of selected fractions from the chromatogram shown in Figure 2 were determined. The activities of fractions including peak 2 are shown in Figure 4, superimposed on the 408 nm absorbance of each fraction. It is readily apparent that the activities are essentially constant across the peak, indicating that the activity is not correlated with the concentration of the covalent complex. This is further illustrated in Figure 5 where the activities in fractions 81−99 are plotted as a function of the concentration of the covalent complex in each fraction. Again, it is obvious that the activity is not directly proportional to the concentration of the covalent complex since, if this were the case, the activity should increase linearly with the increasing concentration of the covalent complex and go to zero in the limit of zero covalent complex. A linear least-squares fit of the data shown in Figure 5 gives a finite activity of 0.20 ± 0.08 μM/s at the intercept (zero covalent complex concentration) and a slope of 0.00 ± 0.02 s^{-1}.

The slope of the plot gives the correlation between the activity and concentration of the covalent complex, and this activity is zero within experimental error. The data presented in Figures 4 and 5 provided very strong evidence that the CcP(E290C)−cytochrome *c*(K73C) covalent complex is inactive. The error in the measurement of the slope can be used to calculate an upper limit for the turnover number of the covalent complex (v_0/e_0). The upper limit for the turnover number is 0.3 s^{-1}, less than 0.06% of that of rCcP under identical experimental conditions.

There are four observations that provide evidence that the residual activity observed in fractions 81−99, the fractions in which the covalent complex elutes, is due to unreacted CcP(E290C). The first observation is that the SDS−PAGE analysis of peak 2 of Figure 3 shows evidence for contaminating CcP(E290C). The second observation is the control experiment described above, in which CcP(E290C) was chromatographed on the CM-Sepahrose Fast Flow column under conditions similar to those of the reaction mixture shown in Figure 2. The control chromatogram shows that small amounts of CcP(E290) bleed from the column throughout the elution and that the average concentration of the enzyme in the region where the covalent complex would elute is 0.14 ± 0.10 μM. The third observation is that the fractions collected during the elution of the covalent complex, peak 2 in Figure 2, have a constant activity not correlated with the concentration of the covalent complex but more appropriately related to a constant value of an impurity (Figures 4 and 5). If the impurity is attributed to unreacted CcP(E290C), the residual activity corresponds to 0.19 ± 0.08 μM CcP(E290C), agreeing quite well with the average concentration of 0.14 ± 0.10 μM CcP(E290C) observed spectroscopically in the control experiment. The fourth observation is that peaks 3 and 4 also have residual enzymatic activity of approximately the same magnitude as observed in peak 2. These experiments provide convincing evidence

that the residual activity observed in the covalent complex samples is due to contaminating unreacted CcP(E290C) and that the covalent complex is inactive.

Other Activities of CcP(E290C) and the Covalent Complex. To demonstrate that the inactivity of the covalent complex is not due to simple inactivation of the bound CcP during the synthesis of the covalent complex, several additional activities were measured at pH 7.5 in 100 mM ionic strength potassium phosphate buffer. The activity of the covalent complex was measured in the presence and absence of tris(2-carboxyethyl)phosphine (TCEP), where TCEP can reduce the disulfide bond in the complex producing monomeric CcP(E290C) and yeast iso-1-cytochrome *c*(K73C). The activity of the covalent complex sample increased 75-fold in the presence of TCEP, demonstrating the greater activity of monomeric CcP(E290K) compared to that of the covalent complex. Assuming the covalent complex is completely inactive, a 75-fold increase in activity corresponds to a 1.3% contamination by unreacted CcP(E290C).

Covalently bound CcP(E290C) reacts with hydrogen peroxide at essentially the same rate as monomeric CcP. The experimentally determined bimolecular rate constants between hydrogen peroxide and yCcP (*48*), rCcP, CcP(E290C), the CcP(E290C)−ferrocytochrome *c*(K73C) covalent complex, and the CcP(E290C)−ferricytochrome *c*(K73C) covalent complex are 44, 33, 54, 44, and 42 μM^{-1} s^{-1}, respectively. Upon reaction of the CcP(E290C)−ferrocytochrome *c*(K73C) covalent complex with hydrogen peroxide, the covalently bound ferrocytochrome *c* is oxidized at the same rate as the hydrogen peroxide, indicating that the rate of electron transfer from the covalently bound ferrocytochrome *c* to the tryptophan radical of compound I is much greater than 400 s^{-1}, consistent with the results of Papa and Poulos (*36*). These data indicate CcP(E290C) was not inactivated during the synthesis of the covalent complex.

Conclusions. It has been convincingly shown that the low levels of catalytic activity associated with the CcP(E290C)−cytochrome *c*(K73C) covalent complex can be attributed to small amounts of contaminating CcP(E290C) that copurify with the covalent complex. This leads to the conclusion that the covalent complex is completely inactive in catalyzing the oxidation of exogenous ferrocytochrome *c* and that only cytochrome *c* bound at the Pelletier−Kraut site (*18*) can be oxidized during the normal catalytic cycle of CcP.

Binding of a second cytochrome *c* to the CcP(E290C)−cytochrome *c*(K73C) covalent complex is still possible, with the second cytochrome *c* binding with affinities similar to those in the native system for formation of the 2:1 complex. Cytochrome *c* binding at a secondary site can influence the steady-state kinetics at low ionic strengths ($\leq$70 mM under our experimental conditions) by facilitating dissociation of the product from the primary site through electrostatic repulsion. This leads to the biphasic nature of the steady-state kinetics previously observed (*15*, *49*).

These results do not obviate the possibility of direct electron transfer from cytochrome *c* bound at a secondary site to the heme iron of CcP when using zinc-substituted cytochrome *c* and photoinitiation of the electron transfer process (*20*, *29*−*35*). The photoinitiated electron transfer reactions have much higher driving forces than the thermally activated CcP-catalyzed oxidation of ferrocytochrome *c* by hydrogen peroxide.

SUPPORTING INFORMATION AVAILABLE

A detailed description of the synthesis of the covalent complex, the spectroscopic properties of the proteins, including spectra and extinction coefficients, and the kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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