Effects of Surface Amino Acid Replacements in Cytochrome *c* Peroxidase on Complex Formation with Cytochrome *c*†

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ABSTRACT: Site-directed mutagenesis was employed to examine the role played by specific surface residues in the activity of cytochrome *c* peroxidase. The double charge, aspartic acid to lysine, point mutations were constructed at positions 37, 79, and 217 on the surface of cytochrome *c* peroxidase, sites purported to be within or proximal to the recognition site for cytochrome *c* in an electron-transfer productive complex formed by the two proteins. The resulting mutant peroxidases were examined for catalytic activity by steady-state measurements and binding affinity by two methods, fluorescence binding titration and cytochrome *c* affinity chromatography. The cloned peroxidases exhibit similar UV–visible spectra to the wild-type yeast protein, indicating that there are no major structural differences between the cloned peroxidases and the wild-type enzyme. The aspartic acid to lysine mutations at positions 79 and 217 exhibited similar turnover numbers and binding affinities to that seen for the "wild type-like" cloned peroxidase. The same change at position 37 caused more than a 10-fold decrease in both turnover of and binding affinity for cytochrome *c*. This empirical finding localizes a primary recognition region critical to the dynamic complex. Models from the literature proposing structures for the complex between peroxidase and cytochrome *c* are discussed in light of these findings.

Cytochrome *c* peroxidase (CcP;[1] EC 1.11.1.5) is a soluble heme enzyme that catalyzes the oxidation of ferrocytochrome *c* [cytc(II)] by hydrogen peroxide:

$$2\text{cytc(II)} + H_2O_2 + 2H^+ \rightarrow 2\text{cytc(III)} + 2H_2O \quad (1)$$

This reaction has been extensively studied as a model for understanding protein–protein recognition and the mechanism controlling biological redox reactions. As a result of these intense research activities, there exists a rich body of biophysical data to facilitate detailed mechanistic studies. Yonetani and Ray (1966) conducted the first in-depth kinetic study on highly purified CcP and determined optimal solution conditions for the steady-state kinetic assay of CcP catalytic activity with both horse and yeast reduced cytc. Subsequent kinetic and thermodynamic studies support an increasingly complex picture of the reaction mechanism. In a recent report, Summers and Erman (1988) proposed a reaction scheme involving 10 species of enzyme and enzyme–substrate (product) complexes. Two sequential one-electron transfer steps are central to the redox mechanism. Each occurs within a complex composed of reduced cytc and one of the two oxidized forms of the enzyme, CcP(IV,$R^{\bullet+}$) or CcP(IV), in equilibrium with CcP($R^{\bullet+}$). CcP(IV,$R^{\bullet+}$) represents a 2-equiv oxidation of wild-type ferric CcP by hydrogen peroxide resulting in an oxyferryl iron, IV (Hewson & Hager, 1979), and an amino acid radical cation, $R^{\bullet+}$ (Yonetani et al., 1966). The equilibrium mixture of CcP($R^{\bullet+}$) and CcP(IV) results from a one-electron reduction of CcP(IV,$R^{\bullet+}$). Complex formation was first suggested to explain observed saturation kinetics (Chance, 1950; Nicholls, 1964; Yonetani & Ray, 1966; Nicholls & Mochan, 1971). Since then a thermodynamically well-characterized complex has been substantiated from the results of sedimentation velocity and gel filtration (Mochan, 1970, 1971; Nicholls & Mochan, 1970; Margoliash et al., 1976; Kang et al., 1977), NMR (Gupta & Yonetani, 1973; Satterlee et al., 1987), absorption spectroscopy (Erman & Vitello, 1980), and fluorescence quenching (Leonard & Yonetani, 1973; Kornblatt & English, 1986; Vitello & Erman, 1987) experiments. Contention about the stoichiometry of binding has been addressed most recently by Vitello and Erman (1987) in favor of a 1:1 complex between the proteins.

The three-dimensional structures of both cytc (Swanson et al., 1977; Takano & Dickerson, 1981; Louie et al., 1988) and CcP (Poulos & Kraut, 1980; Edwards et al., 1987), for various oxidation states, are known at atomic resolution. Recent efforts to obtain cocrystals of the two proteins, however, have led to ambiguous results. In the absence of crystallographic data for the complex, computer graphics techniques have been used to construct a model for the CcP–cytc electron-transfer complex (Poulos & Kraut, 1980) on the basis of optimization of the hydrogen-bonding interactions between complementary charged groups. In this model the heme crevices of the two proteins are aligned by matching a region rich in lysines surrounding the cytc heme crevice with a preponderance of negatively charged residues surrounding the heme edge where

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[1] Abbreviations: yCcP, native cytochrome *c* peroxidase from yeast; ECcP, cloned peroxidase expressed in *Escherichia coli*; MgCcP, magnesium porphyrin yCcP; D37K, D79K, and D217K, cloned cytochrome *c* peroxidase in which aspartic acid was replaced by lysine at positions 37, 79, and 217, respectively; cytc, cytochrome *c*.

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the heme propionates are attached in CcP. The interface so constructed includes cytc lysine residues inferred from chemical modification studies to be involved in the reaction of this protein with CcP (Kang et al., 1978). The complementary carboxyl groups of CcP in the purported binding interface are consistent with a number of those protected in differential chemical modification studies conducted with CcP free in solution vs bound to cytc (Waldmeyer et al., 1982; Bechtold et al., 1985). The original model, based on the unrefined 2.5-Å CcP (Poulos & Kraut, 1980) and 2.0-Å tuna cytc structures (Swanson et al., 1977), paired Asp 37, 79, and 217 of CcP to Lys 13, 27, and 72 of tuna cytc, respectively. Upon refinement of both structures to higher resolution, 1.7 Å for CcP (Finzel et al., 1984) and 1.8 Å for cytc (Takano & Dickerson, 1980), the model was revised. Many of the original intermolecular contacts were preserved and a number of additional contacts observed. However, the interaction between Asp 79 of CcP and Lys 27 of Cytc was absent since Asp 79 in CcP was found to be positioned incorrectly in the original model. Nonetheless, this negatively charged residue still remains on the periphery of the postulated interface.

In order to probe the nature of the ionic interactions involved in the purported CcP–cytc binding interface, amino acid replacements of lysine for aspartic acid were affected in CcP at positions 37, 79, and 217. The resultant mutant enzymes were then assessed kinetically for their catalytic competence to oxidize reduced cytc and thermodynamically for their ability to form a complex with this protein. Binding of the various peroxidases to cytc was evaluated independently by two different approaches. One exploits the fluorescent magnesium derivative of CcP while the other utilizes cytc affinity chromatography. Parallel effects on catalytic competence and binding affinity were observed for the altered enzymes.

Materials and Methods

Materials

Horse heart cytochrome *c* (Sigma, type VI) was used without further purification. Hydrogen peroxide (30%) without preservatives was supplied by Eastman Kodak Co. Reagent-grade potassium or sodium phosphate, as indicated, was used to prepare all buffers. CcP was isolated as described in Cheung et al. (1986). Yeast iso-1 cytochrome *c* was extracted and purified according to the methods described by Sherman et al. (1968). Mature cytochrome *c* peroxidase was expressed in *Escherichia coli* TB1 and subsequently isolated according to the methods outlined by Holzschu et al. (1988). Due to plasmid instability, *E. coli* colonies were screened by ELISA for cloned peroxidase (ECcP) production prior to fermentor inoculations. Point mutations of aspartic acid to lysine were made by site-directed mutagenesis methods previously described (*ibid.*) at positions 37, 79, and 217 to obtain the altered peroxidases D37K, D79K, and D217K, respectively.

Methods

Isolation and Purification of the Cloned Peroxidases. Cultures (10 mL) of a clone freshly assayed for protein expression by ELISA were grown in LB media with 2% (w/v) glucose (the plasmid is under control of the lac operon) and 100 mg/L ampicillin by agitating at 37 °C. After ca. 10 h cells were near the end of log-phase growth and the culture was used to inoculate 300 mL of the same media mixture with 2–3 drops of P2000 (Sigma) and no glucose in a 2.8-L Fernbach flask. After 6–8 h the 300-mL culture was used to inoculate a 10-L fermentor and the cells were grown at 36 °C with aeration at 10 L/min and mechanical agitation at a setting of 400–550 rpm. The fermentation media consisted of the following: 30 g/L Bactotryptone (Difco), 15 g/L Bacto yeast extract (Difco), 10 g/L NaCl, 0.5 g/L L-proline (Sigma), 0.1 g/L ampicillin (sodium salt, Sigma), and 1 mL of P2000. Dissolved oxygen was monitored; a significant increase in dissolved oxygen (>20%) indicated the end of log-phase growth and signaled the appropriate time for harvesting. The pH was strictly controlled at 7.0 ± 0.05 by microprocessor-controlled addition of 8.5 M ammonium hydroxide and 3.675 M phosphoric acid. Typically 200–400 g of wet cells were harvested by centrifugation at 4 °C in a Sorvall RC-5C using a GC3 rotor spinning at 6000 rpm for 10 min; all subsequent isolation procedures were conducted at 4 °C.

The cell pellet was resuspended in a lysis buffer (1:1 v/v) consisting of the following: 50 mM pH 6 acetate, 0.5 mM dithiothreitol, 1 mM EDTA (disodium salt), 0.2 mM phenylmethanesulfonyl fluoride, and 0.5 mg/mL poly(L-lysine) (MW 1000–4000, Sigma). Polylysine is added to prevent cleavage of the protein by an acid-activated membrane-associated protease produced in response to production of ECcP (Ciccarelli, unpublished data). Lysis was done in a stainless steel 350-mL bead beater (Bio-Spec Products) with 150 mL of 0.15-μm acid-washed glass beads (Sigma). Just prior to use the beads were washed in lysis buffer. Cell suspension was added to the bead beater so as to exclude air and was cycled five times with 2-min alternating cycles. The resulting slurry was allowed to settle for 3–5 min on ice, and the lysate was decanted into a 600-mL beaker. The glass beads were washed three times with lysis buffer and the washes added to the lysate. Lysis is indicated by the suspension turning a darker brown in color. This mixture was then sonicated on a Heat Systems Ultrasonics processor outfitted with a microtip for three cycles of 2 min on and 2 min off employing 1-s pulses at 50% duty cycle. The suspension became darker and exhibited a floating, presumably membranous, phase. Protamine sulfate (Sigma grade x) was sifted into the lysate as a solid with swirling to 2 mg/mL to precipitate DNA. Cell debris was pelleted by centrifugation at 13000 rpm for 25 min in a GSA rotor.

The resulting green translucent supernatant containing apo-ECcP was pooled and reconstituted by the addition of heme. A stock solution of hemin was prepared from 300 μL of 1 M NaOH, 300 μL of a 10% solution of Tween 80 (Sigma), and 30 mg of bovine hemin (Porphyrin Products). After vigorously vortexing, 2.4 mL of water was added and the solution vortexed again. Then 2.5 mL of Tween 80 (10% solution) and 2.5 mL of the hemin stock solution was added per 250 mL of lysate. Reconstitution was evidenced by a change in color from the green characteristic of heme to a dark brown. After centrifugation for 20 min at 20000 rpm in an SS34 rotor the solution was ready for anion-exchange chromatography.

Although the remaining purification steps apply to all four peroxidases, column chromatography elution profiles differ for each of the enzymes. The different profiles are summarized in Table I. The conditions for ECcP will be used as an example in the text that follows. The protein was pumped onto a column (2.5 × 15 cm) of Q-Sepharose (Pharmacia) preequilibrated with 50 mM potassium phosphate, pH 6. A cloudy red-brown material passed through and was discarded. The column was then washed with equilibration buffer until the optical density monitored at 280 nm fell to a constant base-line reading. For ECcP, a 500-mL linear gradient in KCl (0–0.5 M) at pH 6 in 50 mM potassium phosphate was run. Elution was monitored at 280 or 405 nm and ECcP eluted between 0.2 and 0.3 M KCl. The elution profile was also checked by running column fractions on SDS–PAGE. Gel electrophoresis

Table I: Column Chromatography Elution Profiles for Purification of Cytochrome c Peroxidase

peroxidase	column material[a]	gradient, low salt/high salt[b]	center of elution profile:[c] [salt] (M)
ECcP	Q-Sepharose	60 mM KPO_4/60 mM KPO_4, 0.6 M NaCl	0.28
	Bio-Gel HT	0.1 M/0.35 M KPO_4	0.18
	Q-Sepharose	0.05M/0.75 M NaOAc	0.42
D37K	Q-Sepharose	50 mM KPO_4/50 mM KPO_4, 0.5 M NaCl	0.18
	Bio-Gel HT	0.1 M/0.35 M KPO_4	0.24
	Q-Sepharose	0.1 M/0.35 M NaOAc	0.17
D217K	Q-Sepharose	50 mM KPO_4/50 mM KPO_4, 0.5 M NaCl	0.27
	Bio-Gel HT	0.1 M/0.35 M KPO_4	0.17
	Q-Sepharose	0.05 M/0.38 M NaOAc	0.24
D79K	Q-Sepharose	50 mM KPO_4/50 mM KPO_4, 0.5 M NaCl	0.32
	Bio-Gel HT	0.1 M/0.35 M KPO_4	0.22
	Q-Sepharose	0.25 M/0.75 M NaOAc	0.30

[a] The pH of the chromatography steps was the same for all four peroxidases and was as described for ECcP in the text: pH 6 for Q-Sepharose treatment 1, pH 6 for Bio-Gel HT, and pH 5 for Q-Sepharose treatment 2. [b] Potassium phosphate and sodium acetate are abbreviated as KPO_4 and NaOAc, respectively. [c] The molar concentration of salt constituting the linear gradient corresponding to a maximum in the peroxidase elution profile as determined by the absorbance at 280 or 405 nm.

confirmed the presence of a 34000-dalton protein and provided a means of qualitatively monitoring the purification achieved by this and subsequent chromatographic steps. Monitoring of the Soret absorption is not sufficient because there is at least one other heme-binding protein in significant quantities.

Fractions containing ECcP were pooled and centrifuged for 10 min at 20000 rpm in an SS34 rotor to remove any precipitate. The solution was concentrated in an Amicon concentrator, diluted at least 5-fold with water, and then reconcentrated before loading onto a Bio-Gel HT (hydroxyapatite) column (2.5 × 6 cm) which was preequilibrated with 50 mM potassium phosphate at pH 6. The brown band that formed in the top third of the column was washed with equilibration buffer until the absorbance at 280 nm decreased to a constant value near zero (ca. 1.5 column volumes). A linear potassium phosphate gradient was run from 50 to 350 mM, with ECcP eluting at ca. 240 mM. Again, fractions were examined by SDS–PAGE and the 34000 MW band corresponding to ECcP showed a marked increase in purity. Fractions containing ECcP were pooled, concentrated to 5–10 mL, and diluted 5-fold with water. This solution was pumped onto a second Q-Sepharose column (2.5 × 5 cm) preequilibrated with 50 mM sodium acetate, pH 5. In a linear acetate gradient (50–750 mM) ECcP eluted at ca. 450 mM and appeared >95% pure as judged by SDS–PAGE. Typically, the ECcP fractions were pooled and then concentrated to low volume and diluted 5-fold into 50 mM potassium phosphate, pH 7. The concentration and dilution steps were repeated. After centrifugation to remove any precipitate, the peroxidase solution was divided into small aliquots, quickly frozen in liquid nitrogen, and stored at −80 °C.

The purity criteria for all four enzymes was a single band on SDS–PAGE and $R_z = A_{\text{Soret max}}/A_{280} = 1.15–1.35$.

Analytical Determinations. The extinction coefficients at the Soret band maxima for the cloned peroxidases, ECcP, D37K, D79K, and D217K, were determined to be ϵ (λ_{max}) = 99 (409.5), 109 (411), 98 (410), and 104 (409) mM^{-1} cm^{-1}, respectively. These were obtained by using the modified Lowry procedure (Markwell et al., 1981) to determine the absolute protein concentration of a solution of known Soret absorbance for each of the enzymes and assuming 1 mol of heme/mol of protein. Concentrations of enzyme stock solutions were measured spectroscopically using the appropriate absorptivity for each. Ferrocytochrome c was prepared by gel filtration on Sephadex G-25 of ascorbic acid reduced horse heart cytochrome c (Sigma, type VI). The concentration of fully reduced cytochrome c was determined from the maximum absorption at 550 nm using ϵ_{550} = 27.6 mM^{-1} cm^{-1} in the presence of a slight excess of dithionite (Margoliash & Frohwirt, 1959). Hydrogen peroxide was standardized against potassium permanganate (Fowles, 1957) and this solution used to determine the extinction coefficient at 230 nm in pH 7.0 phosphate buffer to be ϵ_{230} = 72.4 M^{-1} cm^{-1}. The concentration of stock solutions in subsequent measurements were determined spectroscopically using this absorptivity.

SDS–PAGE and isoelectric focusing procedures were performed on minigels in the Phastsystem (Pharmacia).

All UV–visible spectral measurements including those for steady-state enzyme kinetics assays were performed on a Perkin-Elmer Lambda array Model 3840 spectrophotometer interfaced to a series 7500 personal computer, except for the spectrum of ECcP which was measured on a Perkin-Elmer Lambda Model 4C dual-beam instrument.

Preparation of the Fluorescent Derivative, Magnesium Cytochrome c Peroxidase. In order to prepare magnesium CcP (MgCcP), yeast apo-CcP was first generated by extraction of the heme from yCcP with 2-butanone (Yonetani, 1967). Protoporphyrin IX was purchased from Porphyrin Products (Logan, UT). Mg protoporphyrin was prepared as described by Cowan and Gray (1989). Addition of Mg protoporphyrin to the apoprotein, according to the method previously described for reconstitution of apoprotein with heme (Yonetani, 1967), yielded MgCcP.

Steady-State Kinetic Measurements. For the kinetic measurements the cell compartment was thermostated at 25 °C. Assay mixtures were made by pipetting appropriate amounts of stock buffer, enzyme, EDTA, and water to a final volume of 1 mL. The reaction was initiated by addition of H_2O_2 to a final concentration of 180 μM. The time course of the reaction was monitored by using the difference in extinction coefficients for the reduced and oxidized cytochrome c, $\Delta\epsilon = \epsilon^{red} - \epsilon^{ox}$ at 550 or 475 nm of 19500 (Kang & Erman, 1982) or −4800 M^{-1} cm^{-1}, respectively. The latter was obtained by a difference spectrum of the reduced and oxidized proteins generated as follows. Cytochrome c, fully oxidized by excess ferricyanide, was gel filtered on Sephadex G-25 to remove unreacted ferricyanide and ferrocyanide and the spectrum recorded and digitally stored. An excess of ascorbic acid was then added to reduce the protein. The quantities of ascorbic acid needed did not contribute to the absorbance at 475 nm. The reduced spectrum was recorded and digitally stored and the difference between the oxidized and reduced spectra computed. Enzymatic activities were determined by measuring the initial rate of the reaction as a function of ferrocytochrome c. The concentration dependence of the initial reaction velocity as a function of reduced cytc was fit to the Michaelis–Menten equation using the nonlinear least-squares fitting program, Enzfitter (Elsevier-Biosoft, Cambridge, U.K.).

Affinity Column Chromatography. A cytc affinity column was prepared by employing slight modifications of the method of Azzi et al. (1982). Briefly, activated thio–Sepharose (Pharmacia) was rinsed well with doubly distilled water (ca. 10 swollen bead volumes) and then stirred under a nitrogen blanket for 30 h in 10 mM potassium phosphate, pH 7.2, with

reduced iso-1 cytc from *Saccharomyces cerevisiae*. Cytc was in large excess compared to the number of activated thiols on the beads, specified by Pharmacia to be 4.6 mmol/g of dried beads. This protein contains a free thiol at position 107 that can react with the activated thiol bead to form a disulfide linkage. After incubation the slurry was poured into a 2.5 × 2.5 cm column and extensively rinsed with 1 M potassium phosphate, pH 7.2, buffer saturated with KCl until no more cytc was eluted as determined by monitoring the wash-through spectrally. The cytc remaining was assumed to be covalently bound, as a reducing agent such as mercaptoethanol was required to elute it. The ratio of moles of bound cytc (total incubated minus that which washed free) to moles of activated thiols indicated that all of the free thiols were occupied. The column was then rinsed with 1 M $CuSO_4$ to oxidize the cytc. The protein was completely oxidized as judged by UV–visible spectra of a sample of the suspended beads. For this measurement interfering light scattering of an equal suspension of unlabeled beads was subtracted. The cytc-derivatized Sepharose was then used for binding studies of the peroxidases.

Glass columns (20 × 3.5 mm) were calibrated for volume and packed with 1 mL of cytc beads. Each column was tested to assess the amount of peroxidase that could be eluted reproducibly and completely at a given pH. Ten milligrams of pure enzyme was introduced to the column in 1 mL of 5 mM potassium phosphate buffer, pH 6 or 7. Under these conditions all of the peroxidase was bound. The enzyme was then eluted with 1 M phosphate buffer and the total quantity of eluted protein was determined spectrally. No irreversible binding of protein was observed. Binding affinity profiles for a given peroxidase were generated as follows: For each point of the profile, a known concentration of peroxidase in 5 mM potassium phosphate was loaded onto the affinity column which was preequilibrated with the same buffer. Then the column was washed with doubly distilled water. Five milliliters of the appropriate pH and phosphate buffer concentration was passed through the column and the resulting 5-mL fraction collected. The peroxidase concentration of this fraction was determined spectrally and normalized to the total peroxidase initially bound. Any protein still bound was then eluted with a high-salt wash. A subsequent wash at 5 mM phosphate prepared the cytc affinity material for use in obtaining the next point in the profile. The process was repeated until the entire elution profile was generated.

Fluorescence Titrations. Fluorescence titrations were performed on a Perkin-Elmer MPF-44A fluorimeter. The fluorescent derivatives were found to be light sensitive; therefore, exposure to irradiation was minimized by protecting the samples from light during storage and using the smallest excitation slit widths permitting good signal-to-noise ratios. Aliquots of titrant were typically added using a Hamilton microliter syringe. In all cases the fluorescence intensities were corrected for dilution. Inner-filter effects were negligible at the wavelengths used.

Fluorescence quenching resulting from the formation of a 1:1 complex between an emitting donor, D, and an acceptor molecule, A, capable of quenching that emission, can be described by

$$D + A \underset{}{\overset{K}{\rightleftharpoons}} AD \qquad K = [AD]/[D][A] \tag{1a}$$

$$I/I_0 = \{1 + \alpha K[A]\}/\{1 + K[A]\} \tag{1b}$$

Equation 1b relates the ratio of the observed fluorescence, I, in the presence of A to the fluorescence observed in the absence of quencher, I_0. The experiment proceeds by monitoring I as a function of increasing concentrations of A. K is the association constant describing the complexation between fluorophore and quencher, and α is the ratio of the relative fluorescence quantum yields of complex to that of pure donor. A derivation of the general case for any binding stoichiometry is presented in the appendix. Equation 1b contains the concentration of uncomplexed acceptor [A]. In order to calculate this quantity, I, as a function of $[A]_T$, the total concentration of acceptor present, is fit to the parametric equation

$$I = (I_0 - I_\infty)[K'/(K' + [A]_T)] + I_\infty \tag{2}$$

in order to obtain I_∞, the fluorescence intensity at infinite quencher concentration. In this equation K' is a parametric constant that is related to the equilibrium constant for complex formation between the fluorophore and the quencher. The observed fluorescence intensity and the total intensity change caused by complex formation, $I_0 - I_\infty$, was used to calculate the concentration of unbound quencher, [A]. A nonlinear least-squares fit of I/I_0 as a function of [A] according to eq 1b yielded α and K.

Fluorescence Recovery Simulation. The following expression relates normalized observed fluorescence as a function of donor concentration free from, [D], or bound to, $[D]_b$, an acceptor.

$$I/I_0 = (1/[D]_T)\{[D] + \alpha[D]_b\} \tag{3a}$$

In competitive binding equilibria a nonfluorescent molecule, C, competes with the fluorescent donor, D, exclusively, for the binding of a quenching acceptor, A, as summarized by eq 1a and

$$C + A \underset{}{\overset{K_c}{\rightleftharpoons}} AC \qquad K_c = [AC]/[C][A] \tag{3b}$$

where K_c is the equilibrium constant for binding of competitor. In a competition experiment C is added to a mixture of A and D. As a result of the competitive equilibria, as the concentration of C increases, more unbound D is released into solution and a recovery of the fluorescence is observed. Simulation of the behavior of I/I_0 as a function of [C] requires the calculation of [D] and $[D]_b$. For competitive binding of D and C to A with 1:1 stoichiometry for both, it can be shown that the concentration of unbound donor is the solution of the cubic equation

$$\{K^2 - KK_c\}[D]^3 + \{[C]_T K^2 + KK_c[D]_T + K - K_c - \Phi K^2 + \Phi KK_c\}[D]^2 + \{2K_c[D]_T - K[D]_T - \Phi KK_c[D]_T\}[D] - K_c[D]_T^2 = 0 \tag{3c}$$

where $\Phi = [D]_T + [C]_T - [A]_T$. Equation 3c simplifies to a quadratic only when the donor and competitor bind to A with equal affinity, i.e., when $K = K_c$. Fluorescence recovery was simulated by calculating I/I_0 under the experimental conditions used for the desired set of K and K_c values.

Results

Cloned Peroxidase Enzymes. The cloned peroxidases were typically expressed at levels of 15–25 mg of protein per 10-L fermentation. The mature expressed ECcP differs in sequence from that published for bakers' yeast (Takio et al., 1980) at two positions. ECcP has isoleucine for threonine at position 53 and aspartic acid for glycine at position 152. By a number of criteria this cloned protein behaves similarly, if not identically, to the yeast enzyme. They coelectrophorese on SDS–PAGE and exhibit almost identical UV–vis spectra with maxima at 408 nm and 409.5 for yCcP and ECcP, respectively [Holzschu et al. (1988) and Figure 1]. The cloned enzyme displays similar catalytic activity in oxidizing reduced cytc (discussed further below) and gives a single band with the same $pI = 5.34$ on isoelectric focusing gels as the predominate band

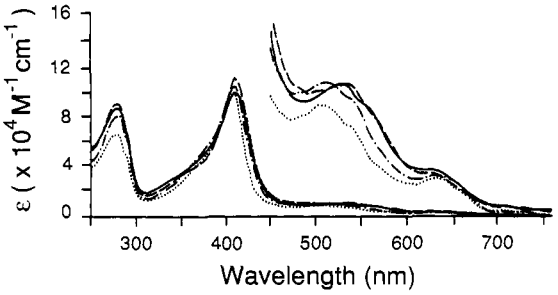

FIGURE 1: UV–visible absorption spectra for four cloned cytochrome c peroxidases. The spectra are ECcP (—), D37K (– –), D79K (···), and D217K (–·–). The spectral window from 450 to 760 nm has been magnified 10-fold. The solution conditions are 50 mM phosphate, pH 7. Proteins used were previously stored at −70 °C.

Table II: Isoelectric Points and Soret Absorption Maxima for Peroxidase

peroxidase	pI[a]	Soret λ_{max} (nm)
ECcP	5.4	409.5
D37K	5.6	411
D79K	5.75	410
D217K	5.8	409

[a] Samples of 100 ng/μL were dissolved in 50 mM phosphate buffer, pH 7.0, and were electrofused on a gel with a pH gradient of 4–6.5.

observed for yCcP. Hence, ECcP was used as the basis protein for investigating the effects of surface charge changes on complex formation with cytc. Three peroxidase mutants, D37K, D79K, and D217K, were generated by site-directed mutagenesis in which a single aspartic acid was replaced by lysine. For the enzyme in solution at neutral pH each of these replacements is expected to affect a double charge change at or near the surface believed to form an interface with cytc. Hence, the choice of Asp → Lys substitution at these sites was engineered anticipating the maximum disruptive effect on complex formation and, according to current kinetic models for the redox reaction, on enzyme catalysis.

The altered peroxidases coelectrophoresed in SDS–PAGE with the *E. coli* produced clone, and each yielded a single band on isoelectric focusing gels. All three proteins exhibited slightly different isoelectric points, which were higher than that for ECcP (Table II). All four enzymes, as isolated, exhibit UV–visible spectra with the same overall shape (Figure 1, full spectra), slightly different red-shifted Soret bands (Table II), and somewhat different ratios of the Soret band absorbance to the protein absorbance at 280 nm. The standard purity index used, A_{408}/A_{280}, for pure yCcP ranges between 1.20 and 1.35 for reported values and for values observed from preparations in our laboratory. Red-shifted Soret maxima have been noted previously and attributed either to an incompletely folded protein, which folds completely upon binding to cytc (Hake et al., 1990), or to the presence of contaminant, damaged forms of the enzymes (Ferrer et al., 1991). In the latter report FPLC ion exchange was used to remove a contaminant responsible for the red-shifted Soret maximum of a native-like recombinant peroxidase. The identical procedure as described by Ferrer et al. was used to further purify ECcP. This did reveal small amounts of a protein contaminant. Given the small amount of contaminant and the substantial loss of enzyme incurred upon using this chromatographic step, in our hands, the FPLC step was replaced by a cytc affinity chromatography step that gave equivalent spectra and purity with lower protein losses. However, it should be noted that spectra taken of the freshly isolated recombinant proteins in 50 mM phosphate buffer, pH 6, before affinity column purification typically showed purity indices between 1.25 and 1.35.

Expansion of the region between 450 and 760 nm reveals charge-transfer transitions for all four recombinant peroxidases between 450 and 750 nm (Figure 1, magnified region) as observed previously for yCcP (Yonetani et al., 1966; Yonetani & Anni, 1987). Notable differences are apparent in comparing the recombinant protein spectra to each other and to the published spectrum for yCcP (Yonetani & Anni, 1987). The positions of these electronic absorption bands have been correlated with the coordination number and spin state of the iron. These spectral differences suggest that surface mutations effect variations at the active site. Other notable features of the spectra compared in Figure 1 include differences in the Soret region. In particular, the size and position of the shoulder found between 340 and 380 nm vary for the four proteins. At pH 7 the A_{Soret}/A_{280} ratios of the freshly purified recombinants fall in a range similar to that for yCcP, i.e., 1.20–1.35. As previously noted, these ratios can vary depending on the conditions of storage and handling. The spectra shown in Figure 1 are for proteins that had been stored frozen at −80 °C in 50 mM phosphate buffer, pH 7. For the spectra of ECcP, D37K, and D217K the purity ratios are 1.14, 1.21, and 1.28, respectively. That for D79K is somewhat larger at 1.5. The spectral differences evident in Figure 1 suggest that the recombinant proteins, as used, contained a mixture of five- and six-coordinate, high- and low-spin ferric states (Iizuka et al., 1971; Hori & Yonetani, 1985; Yonetani & Anni, 1987).

Enzyme Kinetics. All four peroxidases were active in oxidizing horse heart cytc(II) in the presence of hydrogen peroxide. Steady-state kinetic measurements were performed as a means of assessing the relative specific activity of the individual mutants. The results are presented in Figure 2 in the form of Eadie–Hofstee plots. Preliminary results were previously reported by McLendon et al. (1989). The initial velocities as a function of cytc(II) concentration were first fitted to monophasic kinetics using the Michaelis–Menten equation to obtain values of K_M and the turnover number, $V_{max}/[e]_0$. However, it has been previously shown that the steady-state kinetics of native yeast CcP are more complex. Under most conditions biphasic Eadie–Hofstee reciprocal plots have been observed (Margoliash et al., 1976; Kang et al., 1977, 1978; Kang & Erman, 1982). This is also the case for yCcP presented in Figure 2A using 50 mM phosphate buffer at pH 6. A computer fit to monophasic kinetics is poor (solid line) and is made more apparent when compared to the manually constructed lines (dashed) for a biphasic response that better describes the data. A similar treatment also reveals biphasic kinetics for ECcP (Figure 2B). The same is presented for D217K (Figure 2C); the need to consider biphasic kinetics for this enzyme is less apparent. Although more complex kinetic behavior cannot be excluded for D79K and D37K, only a fit to monophasic kinetics is offered. Table III lists steady-state kinetic parameters for all five peroxidase enzymes. For yCcP and ECcP estimates for K_M and the turnover number are given for each of two phases, faster and slower. Each phase of the biphasic profiles was treated independently. Estimates for the turnover numbers were obtained by extrapolation of each phase to the v-axis intercept of the Eadie–Hofstee plot (Figure 2A,B), and the associated K_M values were obtained from the slopes of each phase. In Table III the three mutant peroxidases, D37K, D79K, and D217K, are characterized by a fit to monophasic kinetics. Given the complex nature of K_M values for this multistep reaction system, no discussion will be attempted here.

A comparison of the turnover numbers shows that the relative activities of the peroxidases in decreasing order are

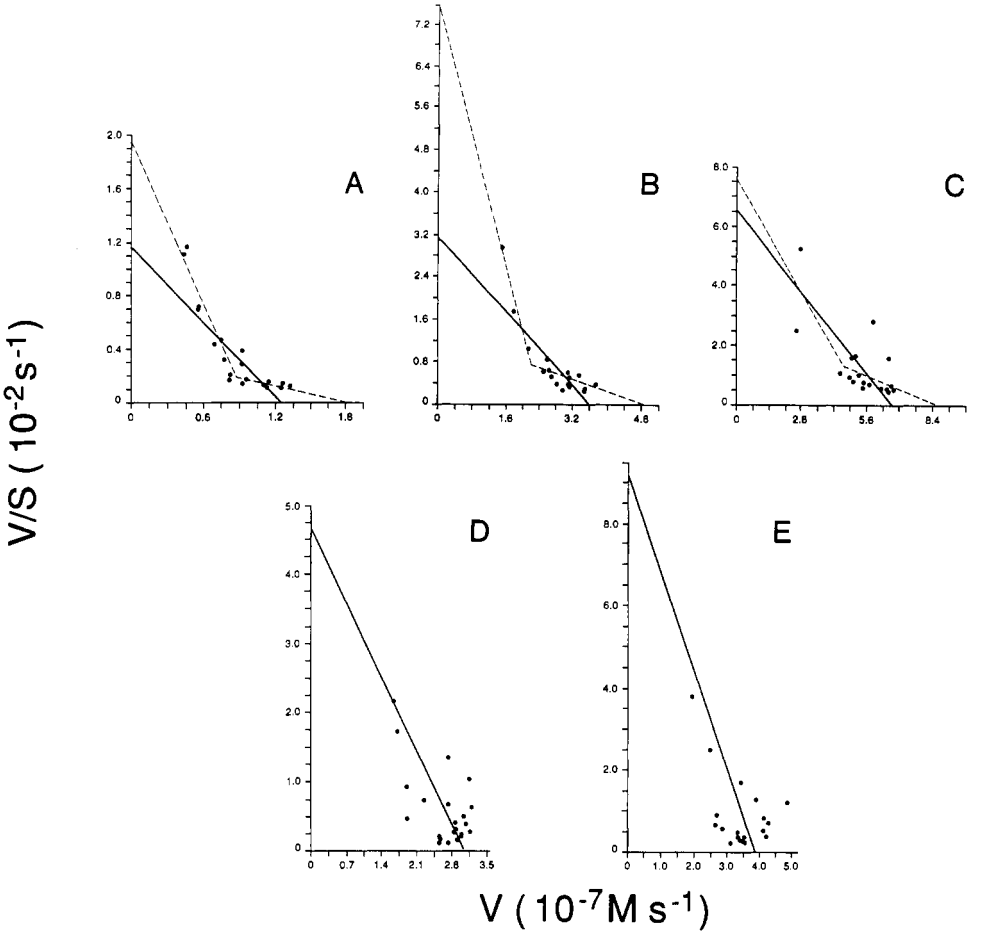

FIGURE 2: Steady-state kinetic data for native and recombinant yeast cytochrome *c* peroxidases. Eadie–Hofstee plots for (A) yCcP, (B) ECcP, (C) D217K, (D) D79K, and (E) D37K at 0.5, 1.0, 1.0, 1.0, and 20 nM enzyme concentrations, respectively. The solution conditions were 50 mM phosphate, pH 6. The solid lines are computer fits to the monophasic Michaelis–Menten equation, and the dashed lines in panels A–C are hand-drawn "fits" to two kinetic phases. Kinetic parameters are listed in Table III.

Table III: Steady-State Kinetic Parameters for Cytochrome *c* Peroxidase and Horse Heart Cytochrome *c*[a]

	K_M (μM)			$V_{max}/[e]_0$ ($\times 10^2$ s^{-1})		
complex	faster	slower	mono-phasic	faster	slower	mono-phasic
cytc·yCcP[b]	50	5		1.8	1.0	
cytc·ECcP	38	3.8		4.9	2.5	
cytc·D37K[b]			4 ± 2			0.2 ± 0.01
cytc·D79K			6.5 ± 1.7			3.0 ± 0.1
cytc·D217K			9.5 ● 3			6.5 ± 0.3

[a] Assay conditions were 1 nM peroxidase enzyme, unless otherwise indicated, 180 μM H_2O_2, and 50 mM pH 6.0 phosphate buffer. Enzyme kinetics are parametrized by using either two pairs of K_M and turnover number estimates corresponding to the faster and slower phases of a manual biphasic kinetics "fit" or a single pair of these parameters obtained from a least-squares computer fit to the monophasic Michaelis–Menten equation. Turnover number estimates for biphasic kinetics were obtained by extrapolation to the initial rate axis, *v*, for each phase of an Eadie–Hofstee plot of the steady-state kinetic data shown in Figure 2. Corresponding K_M values are derived from the slope of the line describing that phase. The kinetic data characterized by a single K_M and turnover number were fit using the computer software package Enzfitter. The reported standard errors were derived using simple weighting, i.e., assuming equal error for each data point. [b] The concentration of yCcP was 0.5, and that of D37K was 20 nM.

as follows: D217K > ECcP ≈ D79K > yCcP ≫ D37K. For this comparison an average of the turnover numbers for the two phases was taken for yCcP and ECcP. D37K exhibits approximately a 20-fold decrease and D217K nearly a 2-fold increase in activity over that of the unaltered cloned peroxidase. The catalytic activities with yeast cytc as substrate (data not shown) yield similar relative activities for the mutants. However, with yeast cytc as substrate the rates are, in general, higher, indicating that peroxidase can catalyze oxidation of the physiological substrate more efficiently. A more sophisticated analysis of the biphasic nature of the data, however, was not attempted here but can be found for yCcP by Kang and Erman (1982).

In light of the multistep reaction scheme alluded to above and proposed by Summers and Erman (1988), K_M and V_{max} are both complicated functions of several rate constants describing both complex formation and electron-transfer steps. Therefore, steady-state kinetic measurements are useful for screening functional effects resulting from an amino acid replacement but not for disclosing the nature of this effect. In order to determine whether complex formation is influenced by a site alteration, independent binding experiments were performed.

Complex Formation. Fluorescent derivatives of both cytc and CcP have been previously used to study the binding interaction of these two proteins to each other (Kornblatt & English, 1986; Koloczek et al., 1987; Vitello & Erman, 1987). In these studies it is assumed that the binding interactions involving the derivatized proteins are the same as those for the native species. However, as this has not been directly demonstrated, it still remains a point of concern. Complexation is a sensitive function of solution conditions (i.e., pH and salt) (*ibid.*). In previous investigations differences in solution conditions used and the manner in which analytical techniques have been implemented have created considerable discrepancy

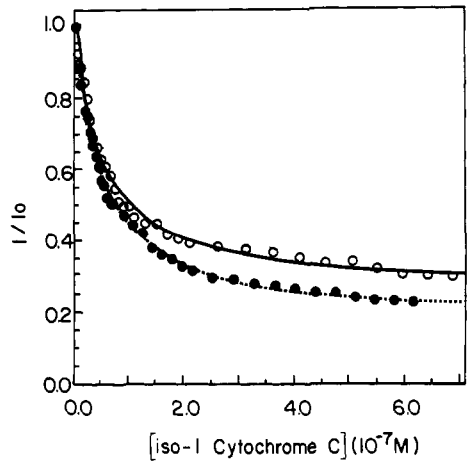

FIGURE 3: Fluorimetric titration of MgCcP with iso-1 yeast cytochrome c. The fluorescence of MgCcP at 9.7×10^{-8} M concentration was excited at 556 nm and observed at 597 nm. The curves are theoretical fits to eq 1b. In 10 mM pH 7 phosphate (●), the fitted parameters K_{Mg} and α (defined in the text) are $(2.0 \pm 0.1) \times 10^7$ M^{-1} and 0.17 ± 0.02, respectively, and in 50 mM pH 6 phosphate (○), they are $(2.0 \pm 0.08) \times 10^7$ M^{-1} and 0.26 ± 0.008, respectively.

concerning the stoichiometry and reported values for the binding affinity between CcP and cytc. In order to provide a self-consistent thermodynamic picture of the relative binding, two different but complementary studies were employed here.

Case 1. Competitive binding measurements between the fluorescent derivative, MgCcP, and underivatized peroxidases for yeast iso-1 cytochrome c were performed to establish whether the various altered peroxidases could compete with the unaltered enzyme for the binding of cytc. Apo-yCcP was reconstituted with MgCcP, producing a fluorescent derivative with an emission maximum at 596 nm. Addition of yeast iso-1 cytc to a solution of this metal-substituted CcP in 50 mM pH 6 or 10 mM pH 7 phosphate buffer resulted in quenching of the fluorescence. The quenching profile approached a constant value at higher [cytc] (Figure 3) and, when fit to eq 1b with a 1:1 stoichiometry of MgCcP:cytc, yielded a binding constant of $(2.0 \pm 0.1) \times 10^7$ M^{-1} in 10 mM phosphate, pH 7, buffer. The fit to a 1:1 complex was good by visual criteria; fits to other stoichiometries were examined but not deemed necessary. The same binding constant was observed at the higher ionic strength of 50 mM phosphate. This weak dependence of peroxidase affinity for iso-1 cytc on ionic strength has been previously observed (Leonard & Yonetani, 1974) and contrasts with the stronger ionic strength dependence exhibited for binding to horse heart cytc (Vitello & Erman, 1987). This binding constant is considerably larger than the values of 4.5×10^5 M^{-1} and 2.9×10^5 M^{-1} at 20 mM ionic strength and pH 6 and 7.5, respectively, reported previously for association of CcP with horse heart cytc (Vitello & Erman, 1987). The nearly 100-fold greater association constant found for yeast iso-1 cytc compared to horse heart cytc reflects a much greater specificity of the physiological redox partner for CcP.

With the simple binding interaction between MgCcP and cytc established, the resulting complex was used to examine the ability of each cloned peroxidase to compete with MgCcP for the binding of iso-1 cytc. Typically, the fluorescence of a 0.3 μM solution of MgCcP was recorded and successive additions of iso-1 cytc were made until a 1:1 molar ratio of the two proteins was achieved. At these concentrations the two proteins are largely complexed. Addition of submicromolar concentrations of ECcP, D79K, or D217K effected an increase in fluorescence from the partially "quenched" level whereas even a 20-fold excess of D37K over MgCcP changed

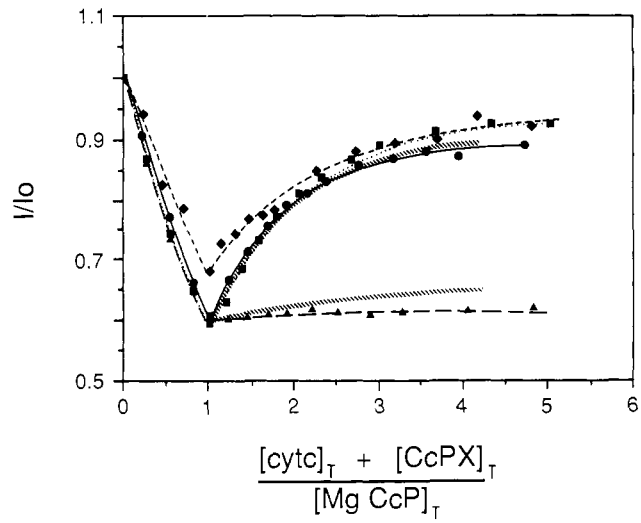

FIGURE 4: Competitive binding between four cloned peroxidases and MgCcP for iso-1 cytc monitored fluorimetrically. Excitation and emission wavelengths used were the same as in Figure 3. Note that the abscissa is the ratio of the sum of total solution concentrations of iso-1 cytc, $[\text{iso-1 cytc}]_T$, and a cloned peroxidase, $[CcPX]_T$, to the total concentration of MgCcP, $[MgCcP]_T$. For each titration, additions of cytc alone were made up to a one molar equivalent with MgCcP (1 on the abscissa); thereafter, aliquots of one of the four peroxidases, ECcP (–●–), D37K (– –▲– –), D79K (– – ■– –), and D217K (– ◆ –), were added. The solution was buffered with 10 mM pH 5.7 phosphate. Typically, $[MgCcP]_T$ was 0.3 μM. The four curves running through the data have no theoretical significance. The hatched curves, top and bottom, are simulations of the fluorescence recovery with $K_x = K_{Mg}$ and $K_x = 0.1(K_{Mg})$, respectively.

the fluorescence intensity only slightly (Figure 4). Hence, ECcP, D79K, and D217K effectively competed with MgCcP for binding to the yeast cytochrome. D37K, however, showed only weak competition over the range investigated. There is some variation observed in α for titration of MgCcP with cytc for different samples of MgCcP used. This effectively raises or lowers the titration curve and is responsible for the difference in competition curves seen for D217K compared to the other three peroxidases. The cause of this is not known.

The following equation summarizes the competitive binding equilibrium assuming a simple stoichiometry:

$$\text{MgCcP—iso-1 cytc} + \text{CcPX} \overset{K_{ce}}{\rightleftharpoons} \text{MgCcP} + \text{CcPX—iso-1 cytc} \quad (4)$$

CcPX represents one of the cloned peroxidases. Complexes are designated by a long dash (—) between two components. K_{ce} is the equilibrium constant for competitive binding exchange and is equal to K_x/K_{Mg}, where K_x and K_{Mg} are defined by the respective equilibria

$$\text{CcPX} + \text{iso-1 cytc} \overset{K_x}{\rightleftharpoons} \text{CcPX—iso-1 cytc} \quad (5)$$

$$\text{MgCcP} + \text{iso-1 cytc} \overset{K_{Mg}}{\rightleftharpoons} \text{MgCcP—iso-1 cytc} \quad (6)$$

Since the relative affinities of these proteins for iso-1 cytc are evident with respect to the same competitive binder, i.e., MgCcP, then they can be directly compared to each other. Peroxidase mutants D79K and D217K yield affinities close to that for ECcP while a double charge change engineered at position 37 decreases binding dramatically. The fluorescence recovery caused by competitor binding to a 1:1 mixture of MgCcP and iso-1 cytc was simulated according to the recipe outlined under Methods. For $K_x = K_{Mg}$ the simulated recovery was very close to the experimental data found for ECcP, D217K, and D79K (Figure 4), affirming that the binding affinity of all three proteins was similar to that of MgCcP for

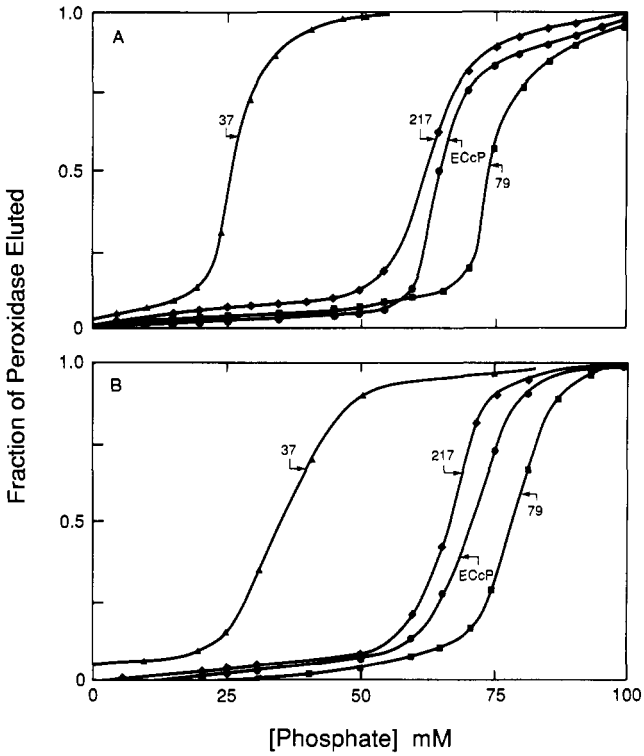

FIGURE 5: Elution profiles of peroxidase from an iso-1 cytc [Fe(III)] affinity column. The elution was conducted with 5-mL aliquots of increasing phosphate concentration at pH 6 (A) and 7 (B) for ECcP (●), D37K (▲), D79K (■), and D217K (◆). Note that the data are not adjusted for the differences in ionic strength at pH 6 vs 7 for phosphate buffer caused by a shift in the distribution of ionic species, especially HPO_4^{2-} and $H_2PO_4^-$. Details of the procedure are outlined under Methods.

cytc. A second simulation is shown for much weaker competitor binding, $K_x = 0.1(K_{Mg})$. The extent of fluorescence recovery caused by D37K is less than this simulation, showing that the mutation at position 37 lowered the binding constant at least 10-fold compared to that of MgCcP. Hence this places an upper limit on the binding constant for D37K of $<2 \times 10^6$ M^{-1}.

Case 2. A cytc affinity column was employed to probe the relative binding affinities of the mutant peroxidases. Iso-1 cytc was covalently linked at the free thiol of cysteine 107 via a disulfide linkage to activated thiol–Sepharose beads. The surface-bound cytc behaved the same as the native protein in solution; it displayed the same UV–visible spectrum, had the same redox potential, and caused the same spectral shifts in CcP upon binding each of the mutant peroxidases (Hake et al., 1990). Measured quantities of each peroxidase were bound to the cytc affinity column and eluted in steps using 5-mL aliquots of increasing phosphate salt. At both pH 6 and pH 7 the same trends were observed (Figure 5); D37K displayed an elution profile at much lower ionic strength than the other three peroxidases. The elution profiles of the other three enzymes appeared in the order of increasing ionic strength, D217K, ECcP, and D79K. All four profiles appeared steeper at pH 6 compared to pH 7 although it should be noted that a comparable change in phosphate concentration at the two pHs encompasses different changes in ionic strength (see Figure 5 legend).

The peroxidase binding profiles generated using a cytc affinity column complement the data obtained from fluorescence titration experiments by providing a view of enzyme affinity over a large range of electrolyte concentration. A measure of the difference in binding constants (but not the absolute values) can be obtained from the column data by measuring the relative eluted fractions as a percentage of total protein. Qualitatively, this can be visualized in Figure 5 by drawing a vertical line through the data. For example, at 50 mM phosphate, pH 6, the differences in binding affinity are negligible for D79K and ECcP while both bind much more strongly than does D37K. It is only at higher electrolyte concentration that the differences between D79K and ECcP are revealed.

Discussion

The construction of surface mutations on peroxidase permits a detailed investigation of the role played by specific charged amino acid residues in the recognition of this molecule for a physiological partner, cytc. These recombinant mutant proteins are readily constructed and expressed in quantities permitting physical studies. The selection of amino acid changes was, in large part, guided by the computer model for the complex of CcP with tuna cytc presented by Poulos and Kraut (1980), henceforth referred to as the PK model. This model and data from chemical modification/cross-linking experiments (Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985) strongly implicated the importance of the acidic residues between positions 33 and 37 in the sequence. Of these, the aspartic acid at position 37 was chosen for mutation to lysine. The PK model pairs CcP Asp 37 with Lys 13 on tuna cytc. Lum et al. (1987) have suggested a computer model (referred to, henceforth, as LB) between the yeast iso-1 cytc and CcP in which Asp 37 is hydrogen bonded to Arg 18, an analogue to Lys 13 of tuna cytc. In this approach an energy minimization procedure was performed refining the basic strategy taken by Poulos and Kraut. In addition, Brownian dynamics computer simulations (BDCS) of the diffusion-controlled association of the two proteins prior to electron transfer show a high probability of successful docking with cytc in the region of the acidic residues (Northrup et al., 1988). Successful docking is defined as an association between the two proteins with a heme–heme edge distance within 20 Å and heme planes parallel to within 60°.

Our results are consistent with Asp 37 playing an important role in recognition as predicted by all three computer studies. A mutation to lysine at this site significantly impaired the catalytic activity and the binding affinity for cytc. Both of these functions decreased by more than an order of magnitude compared to the wild-type-like ECcP (Table III and Figure 4, respectively). Given this functional correlation between activity and affinity, it is concluded that lowered affinity is responsible, at least in part, for the observed decrease in catalytic activity. This is in agreement with previous binding and kinetic studies demonstrating that complexation is a necessary, if not sufficient, prerequisite for a successful redox reaction between the two proteins.

It is important to note that the binding studies presented here examine the interaction between Fe(III) cytc and Fe(III) peroxidase, products of the reaction between the redox-active species, Fe(II) cytc and compound I. Hence, the lower binding exhibited by D37K compared with the other recombinant peroxidases directly demonstrates the important role played by Asp 37 of resting peroxidase in the molecular recognition of oxidized cytc. Extending this to the interaction between Fe(II) cytc and compound I requires that the four recombinant compound I peroxidases exhibit a similar ordering of affinities for reduced cytc; i.e., D37K(IV,$R^{\bullet+}$) exhibits much weaker binding to Fe(II) cytc than do the compound I species of ECcP, D79K, and D217K. Binding isotherms of the redox-active pairs cannot, however, be directly obtained as they do

not reach equilibrium but react. Nevertheless, two points of evidence exist that point to a similar ordering for the recombinant binding affinities of the redox-active species as exist for the Fe(III) cytc/Fe(III) CcP species. First, competition binding data on the various compound I complexes to oxidized cytc are indistinguishable from the results obtained with the Fe(III) cytc/resting peroxidase system (unpublished data). Second, the same relative trend is observed for binding of Fe(II) cytc to the four resting recombinant peroxidases (Hake et al., unpublished results). Hence, either the decreased catalytic rate for D37K is caused by diminished affinity or the electron-transfer process has been independently affected. Independent measurements of the electron-transfer process indicate that D37K is capable of electron exchange at rates comparable to those of ECcP and D217 (D79K is anomalous; unpublished data). This work is still in progress. Therefore, the available evidence is consistent with Asp 37 of compound I playing an important role in recognizing the Fe(II) cytc binding interface prior to electron transfer.

A mutation of Asp 217 to Lys has less of an effect. The turnover number is somewhat larger than and the binding affinity for oxidized cytc comparable to that seen for ECcP. This is contrary to the expected result if an important electrostatic interaction at this position exists with a surface residue on cytc in the complex. Such an interaction is predicted by the PK model that electrostatically pairs Asp 217 on CcP with Lys 72 on tuna cytc. The analogous interaction is not explicitly stated in the LB model. Lum et al. note that Lys 77, the analogue to Lys 72 on iso-1 cytc, is trimethylated, but their calculations indicate that this does not prevent formation of any of the other interprotein contacts cited by the PK model. In BDCS an ensemble of productive encounter complexes form a domain about Asp 217 on CcP. Three such regions were formed, two of which are centered about Asp 37 and Asp 217. The latter was clearly less populated. As shown here by "disruptive mutation", this region does not appear to play a critical role in complex formation.

As stated in the introduction, the original PK model (Poulos & Kraut, 1980) paired Asp 79 of CcP with Lys 27 of cytc. Upon refinement of both CcP and cytc structures a reexamination of the model revealed that this interaction was absent. Nonetheless, studies of this mutant were done for two reasons. (1) Asp 79 provides a negative electrostatic residue on a side of the peroxidase molecule close to the region purported to form the binding surface for cytc. Even though no specific contact was found in the proposed model, the charge so positioned might still be significant. (2) Chemical modification studies on horse heart cytc showed that Lys 27 was included in the kinetically significant domain that interacts with peroxidase and Asp 79 was the only point of contact on CcP which had been thus far implicated. However, our results show that a double charge change at position 79 did not dramatically affect catalytic activity of the protein, as measured by steady-state kinetics, or affinity for cytc.

Given that the complex is undoubtedly dynamic, the activity profile of a given surface amino acid must be examined in the wider context of all species participating in the redox reaction. Along these lines we have found that D217K and D79K show different relative affinities for cytc depending on the oxidation state of the latter. For Fe(II) cytc the order of binding affinities is D217K > ECcP > D79K whereas for Fe(III) cytc the order is reversed, D79K > ECcP > D217K (Hake et al., unpublished results). In general, reduced cytc is bound much more strongly than the oxidized cytc for all the peroxidases. It is expected that for a good enzyme catalyst the substrate [i.e., Fe(II) cytc] would be bound more tightly than the product [i.e., Fe(III)]. The catalytic activity of these three peroxidases, as assessed by the steady-state measurements in this paper, parallels the relative binding affinities for substrate.

A more general observation gleaned from all three mutations is that all surface aspartic acid positions are not created equal. This is best demonstrated by comparing the small but distinct differences in the isoelectric points for the four cloned peroxidases. The same putative double charge change of Asp → Lys introduced at three distinct sites affected different overall charge changes for CcP. From this it can be inferred that the effective charge for the same surface residue depends on its immediate environment. If surface electrostatics play an important role in steering the protein partners into a productive complex, then the effective Coulombic force fields might be determined, to a first approximation, by positioning charged residues at appropriate surface locations. Fine-tuning the effective electrostatic field is then achieved with the residues surrounding those charges.

Another observation is that reactivity of CcP with cytc is rather tolerant of large changes. Even though a change of Asp to Lys at position 37 on CcP resulted in decreased binding and catalytic activity with cytc as substrate, these activities were not eliminated. Such tolerance is consistent with a plasticity of binding, as noted already for cytc (Zhang, 1990; Margoliash & Bosshard, 1983). Tolerance to surface charge mutations is also consistent with the emerging picture of dynamic recognition for the two redox partners, cytc and CcP. The BDCS of Northrup et al. (1988) predict numerous configurations in which an electron-transfer competent complex can be achieved. The initial associated is the result of diffusion and orientationally nonspecific Coulombic attraction. During the relatively long residence time of the complex the proteins explore each others' surface via quasi one-dimensional rotational diffusion with a multitude of rotational degrees of freedom. During this rotational search the appropriate relative orientations are achieved for electron transfer. If the reactive surface of a protein is a limited part of the whole system, then such reduced dimensional searching increases the efficiency and speed of a successful recognition event compared to that attainable for collisions governed by three-dimensional diffusion. This method of biological target location has been previously proposed (Berg et al., 1981) to explain the kinetics of lac repressor binding to its DNA operator region (Winter et al., 1981).

Of concern in any study in which a protein is mutagenetically altered is whether such changes affect only local structural perturbations, thereby permitting a relatively isolated region of the molecule to be examined, or if the modifications are translated into major conformational distortions. UV–visible spectra for the four recombinant proteins indicate that these surface mutations do not drastically alter the overall peroxidase structure. Differences in the UV–visible spectra do, however, evidence more subtle structural differences that are "sensed" by the electroactive heme chromophore. For example, as already noted, charge-transfer spectral bands are correlated to specific iron ligand field and spin states (Iizuka et al., 1971; Hori & Yonetani, 1985).

In addition, all three mutations result in functional enzymes with considerable, albeit different activities. Enzyme function is typically quite sensitive to significant structural alterations. Both wild-type yCcP and the recombinant native-like ECcP exhibit similar biphasic steady-state kinetics. This is evidence supporting the structural similarity of these two enzymes. Such complex kinetics appears to be less pronounced (D217K)

or, perhaps, not present at all (D79K and D37K) for the mutants. However, whether such different steady-state kinetic profiles underscore structural differences is not clear. The functional form (i.e., linear or nonlinear) of Eadie–Hofstee plots of the initial velocity as a function of ferrocytochrome *c* concentration are a complicated function of the buffer concentration and/or ionic strength of the solution (Kang et al., 1977, 1978). Hence, changing a charged surface amino acid might effectively change the functional dependence of the kinetic profile, independent of structural effects that extend beyond the mutation site. As already mentioned, subtle structural differences present between the mutants and wild-type-like peroxidase await more detailed analysis.

Finally, such surface mutations, by affecting the mode of docking of the proteins, may also affect electron flow between cytc and peroxidase within a complex. As mentioned above, this issue will be addressed in more detail in a subsequent study.

Appendix

The quenching profile resulting from the binding between a fluorescent donor, D, and an acceptor molecule that statically quenches that fluorescence, A, is derived for the general case. Consider the binding equilibrium

$$nD + mA \rightleftarrows A_mD_n \quad \text{(A1)}$$

where n donors and m acceptors form a complex, A_mD_n. The total fluorescence of the solution can be written as

$$F = f_D[D] + f_A[A] + f_{AD}[A_mD_n] \quad \text{(A2)}$$

where f_i (i = D, A, AD) is the fluorescence signal per unit concentration of species i under the instrumental conditions of excitation and detection of emission. If the quencher has no detectable fluorescence at the monitoring wavelength, then $f_A = 0$. The total fluorescence intensity of a solution of fluorophore or a mixture of fluorophore and quencher, respectively, are given by

$$I_0 = f_D[D]_T \quad \text{(A3a)}$$

$$I = f_D[D] + f_{AD}[A_mD_n] \quad \text{(A3b)}$$

$[D]_T$ is the total concentration of fluorophore. Using the equilibrium constant expression for eq A1, $K = [A_mD_n]/([D]^n[A]^m)$, and letting $\alpha = f_{AD}/f_D$, the ratio of the solution fluorescence in the presence of quencher to that in the absence of quencher is given by

$$I/I_0 = \{1 + \alpha K[D]^{n-1}[A]^m\}/\{1 + nK[D]^{n-1}[A]^m\} \quad \text{(A4)}$$

By substituting the mass balance equations expressing the total concentrations of fluorophore, $[D]_T = [D] + n[A_mD_n]$, and acceptor, $[A]_T = [A] + m[A_mD_n]$, eq A4 can be recast into

$$I/I_0 = \{1 + \alpha K([D]_T - \gamma([A]_T - [A]))^{n-1}[A]^m\}/\{1 + nK([D]_T - \gamma([A]_T - [A]))^{n-1}[A]^m\} \quad \text{(A5)}$$

where $\gamma = n/m$, which reduces to eq 1b for a 1:1 complex.

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Kinetics of Extension of O^6-Methylguanine Paired with Cytosine or Thymine in Defined Oligonucleotide Sequences†

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ABSTRACT: The frequency of extending m^6G·C or m^6G·T pairs, when the 3′ and 5′ flanking neighbors of m^6G are either cytosines or thymines, was investigated using primed 25-base-long oligonucleotides and the Klenow fragment of *Escherichia coli* DNA polymerase I (Kf). The efficiency, V_{max}/K_m, of extension to the following normal base pair was up to 40-fold greater than for the formation of the m^6G·T or m^6G·C pair. The frequencies of inserting either dCMP or dTMP opposite these m^6G bases did not appear to be different in the two sequences, C-m^6G-C and T-m^6G-T, but extension was favored in the C-m^6G-C sequence. The m^6G·T pair extended to a C·G pair most efficiently, indicating that it was not a strong block to continued replication past the template lesion. Thus, m^6G·T flanked by cytosines replicates more readily than when flanked by thymines, increasing G → A transitions. These data lend further support to the importance of sequence context in mutagenesis.

The ability of modified bases in an oligonucleotide template to direct incorporation of normal dNTPs has been studied in several laboratories (Watanabe & Goodman, 1982; Eritja et al., 1986; Randall et al., 1987; Singer et al., 1989; Dosanjh et al., Shibutani et al., 1991). The emphasis has been placed on the specificity of the insertion step, using a variety of DNA polymerases. For the carcinogenic alkyl derivatives, O^6-methylguanine (m^6G) and O^4-methylthymine (m^4T), pairing with T and G, respectively, is favored in certain sequences. For example, when a 5′ C precedes a template m^6G, the preference for forming m^6G·T over m^6G·C is 6–7-fold greater than when there is a 5′ T (Singer et al., 1989).

Extension from a mismatch is an important factor in in vivo replication. Petruska et al. (1988), Perrino and Loeb (1989), Mendelman et al. (1990), and Shibutani et al. (1991) produced mismatched base termini by annealing an appropriate primer to template and then extending with different DNA polymerases. Extensions of purine–pyrimidine mismatches (e.g., G·T, A·C) were generally favored over the other mispairs.

In the present work, the kinetics of insertion and extension have been measured in the same experiment. These data allowed conclusions to be drawn concerning the relative ability of polymerases to perform the two steps consecutively. In these experiments, changes were made in the 3′ and 5′ bases flanking m^6G to allow measurements of insertion and extension individually and also simultaneously. The effects of sequence and nature of mismatch on mismatch extension efficiencies were measured by a primer extension kinetic assay. The sequences studied were C-G-C, C-m^6G-C, T-G-T, and T-m^6G-T. These sequences and the technique used allow comparison of any dNTP insertion of bases opposite G or m^6G and extension kinetics from newly formed termini to the following normal base pairs.

Experimental Procedures

Materials

The cloned Klenow fragment of *Escherichia coli* DNA polymerase I (Kf) and dNTP substrates (FPLC purified) were

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