Effects of Surface Amino Acid Replacements in Cytochrome *c* Peroxidase on Intracomplex Electron Transfer from Cytochrome *c*†

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ABSTRACT: Transient absorption techniques were used to measure the intracomplex electron transfer rates between four recombinant yeast cytochrome *c* peroxidases and iso-1 cytochrome *c* (cytc). The binding affinities and catalytic activities with cytc were previously examined [Corin et al. (1991) *Biochemistry 30*, 11585]. The four include a wild-type peroxidase (ECcP) and three others, each of which has one surface aspartic acid converted to lysine at position 37, 79, or 217. These sites have been suggested to be within or proximal to the recognition site for cytc. These mutants conduct electron transfer with cytc but differ with respect to the ionic strength profiles of their limiting rate constants. At pH and μ = 114 mM, ECcP and D217K show similar limiting rate constants for electron transfer with cytc, k_{lim}, of ca. 2000 s^{-1}. In the same peroxidase concentration range, the D37K mutant exhibits a k_{obs} of ca. 100 s^{-1}. Instability of the compound I form of D79K prevented a complete study of the intracomplex kinetics of this mutant by this technique. At pH 6 and low ionic strength (8 mM), D37K exhibits a dramatic increase in k_{obs} to ca. 800 s^{-1} while the other two recombinants show a marked decrease to values <150 s^{-1}. D37K displays much lower affinity for cytc than do the other peroxidases at higher ionic strengths [Hake et al. (1992) *J. Am. Chem. Soc. 114*, 5442], thus preventing adequate complexation necessary for efficient electron transfer. Variations in binding affinity do not explain the more subtle ionic strength kinetic profile observed for D217K. The "optimal" ionic strength for this mutant is shifted to higher ionic strength; e.g., at 212 mM D217K exhibits an electron transfer rate constant of 6000 s^{-1}, almost 3-fold that of ECcP under the same conditions. In this case, coupling to the interfacial motions and conformational geometries within the complex must be invoked. Steady-state kinetic measurements conducted with iso-1 cytc yield turnover numbers that are higher than those observed with horse heart cytc but exhibit a similar ordering with D217K > ECcP ≥ yCcP ≫ D37K. The maximal turnover obtained from these measurements compare favorably with the limiting rate constants observed from single-turnover kinetics. In general, such experiments help to examine separately the functional roles that specific amino acids play in molecular recognition and electron transfer through the protein matrix.

A major focus of research in biological electron transfer is directed toward a detailed understanding of the mechanism whereby two proteins exchange electrons. In these systems, the redox reaction occurs within a transient complex formed between the two proteins, each housing one or more redox centers often buried deep within the polypeptide matrix. Typically, electrons must traverse distances of 10–30 Å between the redox centers of the complex (McLendon, 1988). The oxidation–reduction reaction between cytochrome *c* (cytc)[1] and cytochrome *c* peroxidase from yeast (yCcP) provides a well-studied paradigm for protein electron transfer where the redox centers in both proteins are the organometallic cofactor heme. The protein matrix packaging provides both solubility to the iron center and the appropriate level of specificity to the redox reaction within the cell. It is this essential polypeptide packaging that typically constrains electron exchange to occur over a relatively long distance of separation. The closest approach between the redox active centers is determined by the van der Waals contact distance permitted by the protein matrix. The nature of the contact site, or interface, is of great interest for two reasons: it is the recognition site coding for specificity between the two proteins, and it is presumed to contain the entrance(s) to the path(s) along which electrons flow into and out of the polypeptide.

Of primary interest is the electrostatic nature of the regions of the two proteins that form the interface between cytc and yCcP in the complex. In a docking model first proposed by Poulos and Kraut (1980), lysines surrounding the exposed heme edge on cytc are paired with acidic residues clustered about a region on the surface of yCcP. Chemical modification studies conducted on both cytc (Kang et al., 1978) and yCcP (Waldmeyer et al., 1982; Bechtold et al., 1985) have demonstrated the functional importance of these charged surface residues. In a previous report (Corin et al., 1991),

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[1] Abbreviations: yCcP, wild type cytochrome *c* peroxidase from yeast; ECcP, cloned cytochrome *c* peroxidase expressed in *E. coli*; D37K, D79K, and D217K, point mutants of the cloned peroxidases in which aspartic acid was replaced by lysine at positions 37, 79, and 217, respectively; CcPX·I, the compound I species of peroxidase X oxidized by two equivalents compared to the resting (Fe[III]) enzyme; CcPX·II, the compound II species of peroxidase X oxidized by one equivalent compared to the resting enzyme; cyt, cytochrome *c*; cytc(II) and cytc(III), the reduced and oxidized species of cytc, respectively; EDTA, ethylenediaminetetraacetic acid.

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site-directed mutagenesis was employed to examine the functional role played by selected aspartic acids on the surface of peroxidase in recognizing cytc. Aspartic acid residues 37, 79, and 217 were converted to lysine yielding the corresponding recombinant proteins D37K, D79K, and D217K. All three mutant peroxidases displayed catalytic activity with ferrous horse heart cytc as substrate and showed no signs of major structural differences from the native-like recombinant peroxidase, ECcP, or the native yeast peroxidase, yCcP. The resting, or Fe(III), species of ECcP, D79K, and D217K exhibited similar but not identical binding affinities for cytc(III). Mutation at position 37 resulted in more than a 10-fold decrease in affinity for cytc and, hence, is clearly functionally important in defining the cytc binding site. Decreased binding affinity is responsible, at least in part, for the lower catalytic rates observed for this recombinant enzyme in steady-state kinetic measurements. Still undetermined, however, is how such surface mutations affect electron transport into and out of the peroxidase independent of their role in recognition.

In order to address this question, we have conducted laser flash photolysis experiments to investigate the effect of the three aspartic acid to lysine surface mutations on electron transfer from cytc to CcP within the complex between them. In a preceding communication (Corin et al., 1991), steady-state kinetics measurements were conducted for the enzymes using reduced horse heart cytc as substrate. In the present study, the steady-state measurements were repeated using the physiological redox substrate iso-1 cytc. The rates of substrate turnover determined from these measurements are compared with the rates of intracomplex electron transfer determined from the flash photolysis experiments using the same substrate.

MATERIALS AND METHODS

Yeast iso-1 cytc was extracted and purified according to the methods described by Sherman et al. (1968). The recombinant enzymes, CcP, D37K, D79K, and D217K, were obtained as previously described (Corin et al., 1991), and all protein concentrations were calculated spectroscopically using the extinction coefficients stated therein. 5-Deazariboflavin (5-DRF) used for photoreduction of cytc was synthesized by Dr. M. C. Walker using the procedure of Smit et al. (1986).

Laser flash photolysis kinetic studies were performed as previously described (Hazzard et al., 1987, 1988b). A typical sample was prepared for photolysis by addition of 0.5 mL of a 100 μM argon-sparged solution of 5-deazariboflavin the appropriate aliquots of concentrated stock solutions of reduced iso-1 cytc and one of the recombinant peroxidases. Iso-1 cytc was oxidized with an excess of potassium ferricyanide. Both ferrocyanide and unreacted ferricyanide were removed by passing the protein solution through a Q-Sepharose column equilibrated in phosphate buffer, pH 6. The high p*I* cytc passed through, and iron hexacyanide compounds were bound. The fully oxidized state of peroxidase, also called compound I, CcP·I, was then generated by titration to equivalence of the resting enzyme with a buffered 5 mM solution of H_2O_2. This species is known to decay slowly in the absence of a reducing substrate (Erman & Yonetani, 1975) for the wild-type protein. This slow decay did not interfere with the flash photolysis measurements, except in the case of D79K, for which a more rapid irreversible decay ensues, making kinetic measurements with the ferryl species unreliable or impossible. Note that the steady-state measurements previously reported for this mutant (Corin et al., 1991) are not plagued by the same problem. The reasons stem from the manner in which the experiment is performed. For flash photolysis experiments, compound I of a given peroxidase is prepared and must remain stable for seconds to minutes in the cuvette before the laser flash is executed and data acquisition is begun. For steady-state measurements, peroxide is typically the last component added to the cuvette. Given that the reduced cytc substrate present is in great excess, the compound I formed rapidly turns over substrate being reduced successively to compound II and then to the resting, Fe(III), species before the catalytic cycle starts over again. Under these conditions, compound I of D79K is reduced much faster than it can spontaneously decay. The result is that reliable steady-state kinetics data can be obtained. The linkage between amino acid position 79 and the heme active site will be considered in more detail elsewhere (in preparation); therefore, this recombinant will not be discussed further here.

At the free flavin concentration used, pulsed laser excitation at 405 nm generated 5-DRF semiquinone (5-DRFH$^{\bullet}$) at a concentration in the monitoring beam that is ≤ 1 μM (Hazzard et al., 1991). Since the oxidized protein concentrations for both proteins were always ≥ 5 μM, all experiments were conducted under pseudo-first-order conditions. The kinetics were examined at various ionic strengths at both pH 6 and pH 7 using ethylenediaminetetraacetic acid, EDTA, and sodium phosphate buffer. The reduction of cytc(III) and CcPX·I and the reoxidation of cytc(II) were followed at 550 nm. X refers to any one of the peroxidases.

As was previously shown, free CcP·I is not reduced directly by 5-DRFH$^{\bullet}$ on a time scale relevant to the flash photolysis experiment. However, prior reduction of cytc(III) by the semiquinone does lead to subsequent rapid reduction of the oxidized peroxidase by cytc(II) (Hazzard et al., 1987). Using the highly reactive photogenerated semiquinone 5-DRFH$^{\bullet}$ as the reductant ensures that the initial reduction of cytc is not rate limiting to the ultimate reduction of CcPX·I.

UV–vis spectral measurements were performed on an IBM Model 9430 dual-beam spectrophotometer. Steady-state kinetic measurements were conducted as outlined by Corin et al. (1991) except that iso-1 cytc was reduced with an excess of sodium ascorbate. The unreacted ascorbate was removed by passing the protein solution through a Q-Sepharose column equilibrated in phosphate buffer, pH 6. Calculation of the turnover numbers, $V'_{max}/[e_0]$, is based on the maximal velocity of cytc turnover, $V'_{max} = d[\text{cytc(II)}]/dt$.[2] The fluorescence titration was conducted and analyzed as previously described (Corin et al., 1991).

RESULTS AND DISCUSSION

Intracomplex Electron Transfer. Flash photolysis of 5-DRF solutions containing EDTA, cytc(III), and equimolar amounts of H_2O_2 and one of the recombinant peroxidases exhibited a transient absorption response similar to that seen previously with the native yeast enzyme (Hazzard et al., 1987). As shown in Figure 1 for D217K, an initial rapid rise in the absorption at 550 nm is followed by a slower decay to below

[2] The velocity of the reaction, so defined, is twice the value used to define enzyme turnover. Enzyme turnover is formally calculated by dividing the rate of change of cytc by 2 to normalize for the stoichiometric coefficient of this reactant in the balanced chemical equation, i.e., 2cytc(II) + H_2O_2 + 2H^+ → 2cytc(III) + 2H_2O. We report cytc turnover, rather than enzyme turnover, in order to maintain consistency with the steady-state data previously reported from our laboratories (Corin et al., 1991). Please note that in that work the turnover numbers reported are actually cytc turnover numbers, $V'_{max}/[e_0]$, where $V'_{max} = 2V_{max}$, not $V_{max}/[e_0]$, as indicated.

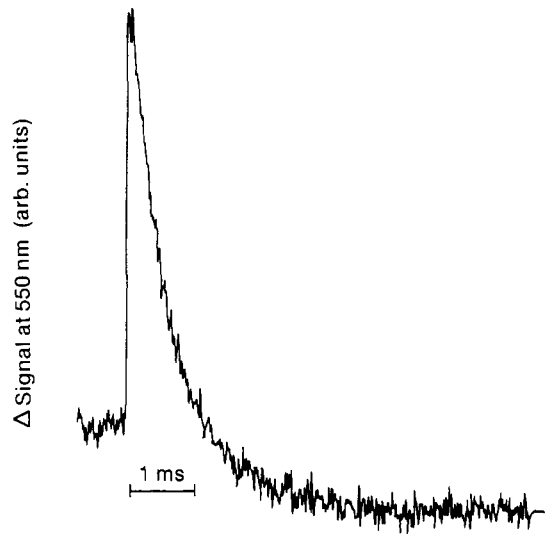

FIGURE 1: Laser flash-induced transient absorption response for the reduction and reoxidation of iso-1 cytc in the presence of 5-deazariboflavin and D217K compound I. The concentrations of iso-1 cytc and D217K were both 10 μM, and the concentration of 5-deazariboflavin was 100 μM. The laser excitation wavelength was 405 nm and the monitoring wavelength was 550 nm. The solution was buffered by 100 mM, pH 6, phosphate buffer and contained 0.5 mM EDTA. The observed rate constant of cytc reoxidation, k_{obs}, = 1800 s^{-1}. The experimental error in k_{obs} is estimated to be about 10%.

the preflash baseline. By analogy to the earlier studies conducted with yCcP, the rapid rise in absorbance corresponds to the reduction of cytc(III) to cytc(II). The slower decay is the time-dependent reoxidation of cytc(II) and concomitant reduction of the compound I of D217K. The net decrease in absorbance below the preflash baseline is consistent with a negative redox difference spectrum resulting from the reduction of the compound I species as seen for yCcP (Hazzard et al., 1987). Hence, D217K must have a redox difference spectrum similar to yCcP in this region. Similar transient absorption responses were observed for all four recombinant peroxidases.

The absorbance decays were well fit by single-exponential functions, characterized by time constants, $\tau = 1/k_{obs}$, as expected for pseudo-first-order kinetics. At pH 6 and μ = 114 mM, the observed pseudo-first-order cytc(II) reoxidation rate constant was dependent on the concentration of CcPX·I. As previously found for yCcP (Hazzard et al., 1988b), the nonlinear concentration dependence of k_{obs}, for D217K and ECcP (Figure 2), is consistent with the formation of a transient complex between cytc(II) and CcPX·I followed by an intracomplex electron transfer step. At high CcPX·I concentrations, the process(es) controlling intracomplex electron transfer (k_{lim}) becomes(become) rate limiting as predicted by the minimal mechanism of the form

$$\text{cytc(II)} + \text{CcPX·I} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{complex} \xrightarrow{k_{lim}} \text{cytc(III)} + \text{CcPX·II} \quad (1)$$

The initial rapid reduction of cytc(III) to cytc(II) ($t_{1/2} \approx 50$ μs under these conditions) by the photogenerated semiquinone is not explicitly shown. As the system is at equilibrium just prior to the laser flash, this initial reduction step involves two species of cytc(III), that which is free in solution (shown explicitly in eq 1) and that which is bound to CcPX·I (not shown). In as much as both reduction reactions are rapid (Figure 1), they do not contribute to the kinetic process being considered here. The rapid generation of two different species

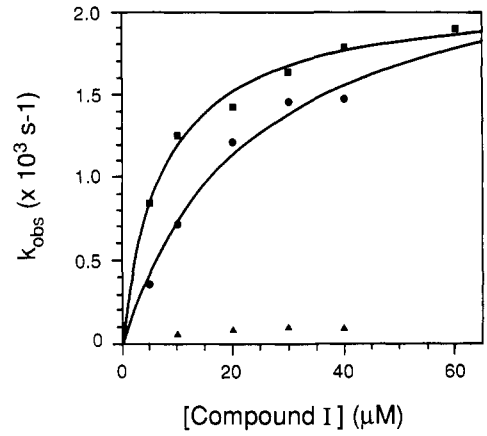

FIGURE 2: Observed rate constants for reduced iso-1 cytochrome *c* oxidation by compound I peroxidase as a function of compound I concentration. Symbols: (●) ECcP, (■) D217K, (▲) D37K. The solid curves represent fits of the data using eq 2 according to procedures outlined in the text. The fitted parameters, k_{lim}, appear in Table I. Solution conditions were as stated for Figure 1, and the concentration of iso-1 cytc was 10 μM.

of cytc(II) is discussed further below. CcPX·II, or compound II, is the species of peroxidase which is oxidized by one equivalent compared to the Fe(III) resting enzyme; it is capable of oxidizing a second equivalent of reduced cytc. However, this latter reaction does not occur during the present experiment because of the relatively small amount of cytc(II) generated by the flash. In the experiments reported here, and previously, we observe only the reaction between cytc(II) and the oxyferryl, Fe(IV)O, species. For example, identical kinetics are found in the absence of any amino acid radical species. In recent work (Hahm et al., 1992) similar rates for the reaction within the CcP/cytc(II) complex have been observed. In the same work, a significantly faster process ($k_{obs} \sim 10^5$ s^{-1}) was reported and assigned to reaction with the amino acid radical site. The observation of such different rates for the reactions with these isolated sites provides a simple rationale of the need for interfacial dynamics interconverting between the "radical site" and the "ferryl heme site". In our work, reported here, this faster process is not resolved, and so it will not be further considered. The mechanism of eq 1 predicts saturation when the rate constant for the process(es) controlling intracomplex electron transfer, k_{lim}, $< k_1$ [cytc(II)] + k_{-1}. Thus, the value of k_{lim} can be obtained from the limiting asymptote of the data in Figure 2. As will be discussed below, k_{obs} for D37K cannot be treated similarly in the concentration range studied.

All reactions were conducted under pseudo-first-order conditions [see Hazzard et al. (1991) for a more complete description] permitting eq 1 to be treated as two consecutive first-order reactions with the second step irreversible. Therefore, an explicit mathematical solution of the rate equations was used to simulate and then to fit the data as previously outlined (Ahmad et al., 1982). Unlike the rate limiting value, k_{lim}, specific values for k_{-1} are not well determined by a particular property of the analytical solution to the mechanism of eq 1. However, simulations clearly demonstrated (data not shown) that the overall apparent binding constant describing formation of an electron transfer competent complex between cytc and peroxidase, K_{app} ($=k_1/k_{-1}$), which is determined by the curvature of the plot of k_{obs} ($=1/\tau$) vs the total initial concentration of CcPX·I, is accurately evaluated by this procedure. Values for both k_{lim} and K_{app} were obtained for the data plotted in Figure 2 and the resulting fitted values are listed in Tables I and II, respectively. As previously noted, the laser flash photolysis generates two species of cytc(II),

Table I: Rate-Limiting Rate Constants, k_{lim}, and Observed Pseudo-First-Order Rate Constants, k_{obs}, for the Reaction between Compound I Peroxidases and Reduced Cytochrome c[a]

	pH = 6			pH = 7	
peroxidase	k_{obs} ($\times 10^3$ s^{-1}) μ = 8 mM	k_{lim} ($\times 10^3$ s^{-1}) μ = 114 mM	k_{lim} ($\times 10^3$ s^{-1}) μ = 212 mM	k_{obs} ($\times 10^3$ s^{-1}) μ = 180 mM	k_{lim} ($\times 10^3$ s^{-1}) μ = 270 mM
ECcP	0.13	1.95	2.02	0.19	1.38
D217K	0.11	2.05	6.00	0.54	0.39
D37K	0.79	0.1[b]	ND[d]	0.005[c]	ND

[a] Listed are the rate-limiting first-order rate constants for the electron transfer step of eq 1, k_{lim}, or the observed pseudo-first-order rate constants, k_{obs}. For a number of conditions k_{obs} is reported when k_{lim} could not be determined (i.e., k_{obs} showed no apparent dependence on the peroxidase concentration over the accessible concentration range). For k_{obs}, the values reported are the averages of k_{obs} at compound I concentrations of 10 and 20 μM unless otherwise indicated. The error in all parameters is conservatively estimated at <20%. As mentioned in the text, values of k_{lim} are obtained from fits to eq 1 and are well-determined by the limiting asymptote of the data in Figure 2. The k_{obs} values were manually extracted from semilogarithmic plots of the transient decay data. The solution at pH 6, μ = 8 mM was buffered with 5 mM phosphate. The remaining solutions at pH 6 and 7 were buffered using 100 mM phosphate. EDTA accounted for the remainder of the ionic strength. [b] The average of k_{obs} values determined at 30 and 40 μM peroxidase. [c] Determined at 10 μM peroxidase only. [d] Note that ND means that the experiments for these conditions were not done.

Table II: Estimates for the Apparent Association Constants, K_{app}, for the Complexation of Reduced Cytochrome c to Recombinant Peroxidases[a]

	K_{app} ($\times 10^5$ M^{-1}), pH = 6		K_{app} ($\times 10^4$ M^{-1}), pH = 7
peroxidase	μ = 114 mM	μ = 212 mM	μ = 270 mM
ECcP	1.3	1.0	4.3
D217K	2.0	0.7	7.1

[a] These association constants are the ratio of the forward and reverse rate constants for complexation fitted to a basic two-step mechanism represented by eq 1. The values for these fitted parameters are highly coupled, rendering a meaningful error analysis difficult. Therefore, the association constants are presented as estimates.

cytc(II) bound to CcPX·I and cytc(II) free in solution. Equation 1 describes the bimolecular electron transfer process from the latter. A more complete mechanistic description also includes the unimolecular process of electron transfer in the preformed complex. This unimolecular process does not yield a concentration-dependent pseudo-first-order k_{obs}, contrary to what is observed (Figure 1). This must mean that the bimolecular process is dominant for the range of CcPX·I concentrations investigated where k_{obs} is concentration-dependent ($\leq$20 μM) and, according to eq 1, that K_{app} reflects the binding affinity of cytc(II) to CcPX·I.[3] Dominance of one molecular process is also consistent with monophasic transient decay curves (e.g., Figure 1) describing cytc(II) oxidation. Both paths, unimolecular and bimolecular, yield the same limiting rate constant.

As is evident by inspection of Figure 2 and the fitted values of k_{lim} (Table I), ECcP and D217K behave almost identically at pH 6 and μ = 114 mM and exhibit similar intracomplex electron exchange rate constants of ca. 2000 s^{-1}. A much smaller rate constant, k_{obs}, is observed for reduction of D37K CcP·I, 100 s^{-1} (Table I and Figure 2). This rate constant does not clearly reach a limiting value in the concentration range examined, and, therefore, no fit was attempted. If an equivalent mechanism were assumed, then a very low K_{app} would be needed to fit the data. As will be discussed in more detail below, this is consistent with previous equilibrium measurements which show that this mutation dramatically decreases binding to cytc (Corin et al., 1991). The apparent binding constants for ECcP and D217K extracted from the kinetic data are within a factor of two for both enzymes under a number of solution conditions (Table II). For example, at μ = 114 mM K_{app} is ca. 1.3 $\times$ 10^5 M^{-1} and 2.0 $\times$ 10^5 M^{-1} for ECcP and D217K, respectively. Values similar to these can be obtained by simply treating the curves in Figure 2 as (kinetic) binding isotherms and manually calculating binding constants or fitting as mentioned above. For comparison, direct fluorescence quenching equilibrium measurements of MgCcP/Fe(III)cytc binding yield association constants of (4 $\pm$ 1) $\times$ 10^4 M^{-1} at 110 mM, pH 6, phosphate and (2.5 $\pm$ 0.5) $\times$ 10^5 M^{-1} at 80 mM phosphate (Zhang et al., 1993), a range that clearly brackets the K_{app} values derived from single-turnover kinetics.

All observations are then consistent with the mechanism of the form shown in eq 1 in that a complexation step precedes electron transfer and the rate reaches a limiting value upon complete complexation of cytc to peroxidase. As mentioned above, this condition is not attained in the flash photolysis experiments for D37K. The binding affinity for this mutant was found to be at least 10-fold less than that found for ECcP or D217K by florescence competition binding (i.e., <2 $\times$ 10^6 M^{-1}) (Corin et al., 1991). This experimental method does not provide a good estimate of the binding constant for a weak competitor; therefore, only an upper limit of the binding affinity can be extracted. Cytochrome c affinity column binding experiments also show lower binding affinity of D37K to cytc(III) compared with that of ECcP and D217K (Corin et al., 1991; Hake et al., 1992). This same technique also reveals that all three recombinant enzymes display stronger binding to reduced cytc than to oxidized cytc. The relative affinities for the three peroxidases are different toward reduced cytc vs oxidized cytc, i.e., D217K > ECcP $\gg$ D37K and ECcP > D217K > D37K, respectively. In these affinity column experiments, binding was examined from μ = 0 to 1.0 M for reduced cytc and from μ = 0 to 0.1 M for oxidized cytc. One advantage in using the cytc affinity column for inspecting peroxidase binding affinity is that a broad profile of the ionic strength dependence of the enzyme's binding affinity for cytc can be conveniently probed. However, the affinity chromatography technique does not easily permit determination of the absolute binding constant. If, as proposed above, the concentration dependence of the kinetics in Figure 2 reflects the binding of peroxidase to cytc(II), then the somewhat steeper curve for D217K, compared with that for ECcP, indicates slightly higher affinity between the former and oxidized cytc. This is consistent with the ordering of relative affinities just stated.

Recognition vs Electron Transfer. The above discussion suggests that the ratio of the kinetic rate constants describing complexation, k_1/k_{-1}, rather than the limiting rate constant for intracomplex electron transfer, k_{lim}, can account for the

[3] In fact, assessing the binding affinity between cytc(II) and CcPX·I by laser flash photolysis kinetics may be one of the few, if not only, means of doing so. Equilibrium methods do not work as the two do not come to equilibrium but quickly react.

lower observed rates for D37K in the concentration range investigated. This is consistent with the fact that D37K binds much less tightly to cytc than does ECcP or D217K under a number of solution conditions. To test this further, a kinetic study was performed at lower ionic strength.

For an ionically driven complexation step, protein–protein association is predicted to be inversely related to ionic strength. Therefore, it should be possible to compensate for weak complex formation between D37K and cytc by decreasing the ionic strength. This has been previously demonstrated for the CcP mutants discussed herein (Hake et al., 1992). As shown in Table I, k_{obs} for D37K increased dramatically from ca. 100 s^{-1} at μ = 114 mM to ca. 790 s^{-1} at μ = 8 mM. This demonstrates that D37K CcP·I can, indeed, form a competent electron transfer complex with cytc under solution conditions permitting complex formation.

Hence, a competent complex between D37K and cytc can be achieved at low ionic strength. On the basis of this, we conclude that Asp 37 has a role in the electrostatic recognition of the positively charged surface of the substrate, cytc, and that this surface site does not play a critical role in electron conduction into or out of the peroxidase binding interface.

In contrast to D37K, ECcP and D217K both display a dramatic decrease in k_{obs} at μ = 8 mM compared to μ = 114 mM. Such behavior has been observed previously for yCcP reacting with either iso-1 or iso-2 cytc (Hazzard et al., 1988a). One explanation is that the complex formed at low ionic strength is not structurally or dynamically "optimal" for electron transfer. In addition, surface mutations change the ionic strength dependence for electron transfer between the various peroxidases and cytc. For example, at μ = 212 mM, k_{lim} for D217K has increased to ca. 6000 s^{-1}, from 2000 s^{-1} at μ = 114 mM, while comparable values of k_{lim} for ECcP are essentially unchanged under the same two ionic strength conditions (Table I).

There are at least two events which must occur prior to the electron transfer step. The first is complex formation, as explicitly described by eq 1, and the second is achieving the proper geometry(ies) within that complex. At ionic strengths <500 mM, ECcP and D217K both exhibit high affinities for cytc(II) which are relatively ionic strength independent (Hake et al., 1992). Hence, binding is a necessary but insufficient prerequisite to electron transfer. Within the complex, motions of the two molecules are critical for achieving the correct geometry to permit electron transfer. Since the ionic strength profile for electron exchange differs for different surface mutants, these motions/geometries must have an ionic strength dependence. According to theory (Marcus & Sutin, 1985), the *magnitude* of the rate is determined by (1) the geometric arrangement(s) of the two partners in the "cocked" complex as reflected in the electronic (coupling) matrix elements, (2) the specific thermodynamic driving force in that arrangement-(s), and (3) the reorganization energy associated with the movement of charge from cytc to CcP. This picture is consistent with the observation that the binding (sub)domains on peroxidase for cytc(II) and cytc(III) differ (Hake et al., 1992). Thus, some "ionic strength dependent" interfacial motion is required to move between these (sub)sites. Evidence for such populations composed of different geometrial orientations is provided by a comparison of the decay profiles of the complex between the fluorescent CcP analog, MgCcP, and cytc at 300 and 77 K (Zhang et al., 1990). At 300 K the decay profile is simplified but is complex at 77 K. "This pattern suggests that molecular recognition in the protein complex produces individual conformational states which are trapped at low temperature and reequilibrate at room temperature on the subnanosecond time scale, via lower dimensional diffusion." Ultimately, a complete model of the electron transfer reaction between these two proteins must ntain a description of the coupling of such ionic strength dependent motions and/or orientational states of the two molecules in the complex with the electron transfer step.

Table III: Steady-State Kinetic Parameters for Cytochrome *c* Peroxidase and Yeast Iso-1 Cytochrome *c*[a]

complex	K_M (μM)	$V'_{max}/[e]_0$ ($s^{-1} \times 10^2$)
cytc/yCcP	13 ± 0.6	8.0 ± 0.1
cytc/ECcP	10 ± 0.2	8.8 ± 0.05
cytc/D37K[b]	2 ± 0.3	0.4 ± <0.01
cytc/D217K	8.8 ± 0.5	10.9 ± 0.15

[a] Assay conditions were 1 nM peroxidase enzyme, unless otherwise indicated, 180 mM H_2O_2, and 50 mM pH 6.0, phosphate buffer at 25 °C. The data were fit to a simple Michaelis–Menten equation. [b] The concentration of CcPK37 was 10 nM. Standard errors are calculated by the fitting program Enzfitter (Elsevier-Biosoft, Cambridge, U.K.), using simple weighting, i.e., assuming errors are the same for each data point.

Values of k_{lim} are significantly lower at pH 7 and ionic strengths of 180 and 270 mM for both ECcP and D217K than those found at a comparable ionic strength range at pH 6 (Table I). This is consistent with a pH optimum between 5 and 6 for initial rates of peroxidase oxidation of iso-1 cytc observed for yCcP (Yonetani & Ray, 1966). At μ = 270 mM, the rates for both recombinants were a hyperbolic function of compound I concentration, whereas neither enzyme displayed a strong concentration dependence at μ = 114 mM, pH 7 (data not shown).

Steady-State Kinetics with Iso-1 Cytc. In a previous study, we reported steady-state kinetic data for reaction of these peroxidase mutants with horse heart cytc (Corin et al., 1991). These steady-state kinetic measurements were among the first physical data collected in our laboratory to identify the functional effects resulting from mutation at position 37 in the peroxidase. The flash photolysis experiments, however, were conducted with the physiological redox partner iso-1 cytc. In order to relate the single-turnover kinetic data to steady-state kinetic measurements, the latter were repeated using reduced yeast iso-1 cytc as substrate. A fit to the simple Michaelis–Menten equation of the initial reaction velocities as a function of iso-1 cytc concentration yielded the K_M and turnover numbers, $V'_{max}/[e]_0$, listed in Table III. V'_{max} is the maximal cytc reaction velocity, as defined in the experimental section, at saturating substrate concentration, and $[e]_0$ is the peroxidase concentration. Plots of the resulting Eadie–Hofstee transformations of the fits to these data are shown in Figure 3. The Eadie–Hofstee plots for all four peroxidases in 50 mM phosphate, pH 6.0, are monophasic over the range of cytc concentrations from 5 to 100 μM. Kang et al. (1977), reporting maximal turnover in the same manner as is done here, observed two kinetic phases for the oxidation of reduced yeast cytc by yCcP in 100 mM phosphate, pH 6.0. In addition to the difference in phosphate concentrations of the two reports, measurements by Kang et al. including the lower range of cytc concentrations, i.e., 1–5 μM. As the initial phase is observed only with cytc concentrations $<\sim 5$ μM this may explain the absence of the initial phase in Figure 3. The monophasic turnover number of 800 s^{-1} reported here is close to the turnover number associated with the initial phase, V_{max1}, of the steady-state kinetics reported by Kang et al. (1977), 750 s^{-1}. However, as demonstrated by these authors there is a dependence of V_{max1} on the buffer concentration; V_{max1} at 100 mM phosphate is 1.5–2 times that found at 50 mM

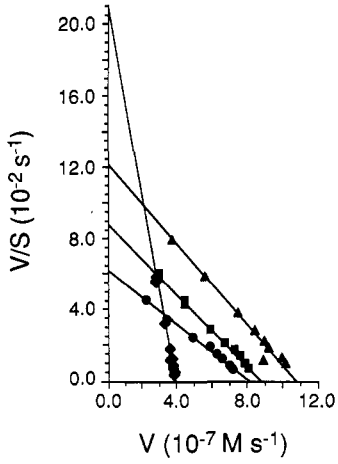

FIGURE 3: Steady-state kinetic data for the recombinant yeast cytochrome c peroxidases ECcP, D217K, D37K: Eadie–Hofstee plots for (●) yCcP, (■) ECcP, (▲) D217K, and (◆) D37K. The enzyme concentrations were 1 nM for the first three and 10 nM for D37K. The iso-1 cytc concentrations were varied from 5 to 100 μM. The solution conditions were 50 mM phosphate, pH 6. The solid lines are the resulting Eadie–Hofstee transformations of computer fits to the monophasic Michaelis–Menten equation.

phosphate. If it is assumed that this dependency extends to our data, then our turnover number of 800 s^{-1} becomes 1200–1600 s^{-1} at the higher phosphate concentration. Effectively, therefore, we observe a turnover that is 1.5–2 times as large as that of Kang and co-workers. The K_M value obtained here, 13 μM (Table III), is more than 4 times their value of the Michaelis–Menten constant describing the initial phase, K_{M1} = 2.7 μM. They report K_{M2}, the Michaelis–Menten constant describing the second phase of the steady-state kinetics, as >100 μM, but do not indicate a value for the corresponding V_{max2} under these solution conditions. The steady-state kinetics of ECcP is characterized by parameters similar to those for yCcP; i.e., K_M = 10 μM, $V'_{max}/[e]_0$ = 880 s^{-1} (Table III). The above comparison of our data with that of Kang and co-workers is so presented for the sake of continuity with the published literature, but as the mono- or biphasic nature of the steady state kinetic data is not central to the present report, we will not pursue it further.

The catalytic efficiencies of the peroxidases varied with D217K > ECcP ≥ yCcP ≫ D37K. The differences in rates between ECcP and yCcP are small, i.e., <20%. Therefore, the overall trend is similar to that observed when horse heart cytc was used as substrate under the same solution conditions. The absolute turnover numbers with iso-1 cytc as substrate for all three peroxidases are larger than those found using horse heart cytc. For example, the fast and slow phase of the yCcP oxidation of reduced horse heart cytc exhibit turnover numbers of 180 and 100 s^{-1}, respectively, compared to the monophasic turnover number of 800 s^{-1} using iso-1 cytc as substrate (Table III). This agrees, qualitatively, with the previous observation that the yeast cytc is more active at higher ionic strength than is horse heart cytc. Using 0.2 M Tris-HCl, pH 7.0, the yeast cytc was found to be 10–20-fold more active than horse heart cytc. The reverse is true at lower ionic strength; i.e., in 0.01 M Tris-HCl, pH 7.0, the mammalian cytc is twice as active as the fungal protein (Kang et al., 1977).

We note that for all three recombinants, ECcP, D37K, and D217K, the steady-state rates of maximum catalytic turnover, $V'_{max}/[e]_0$ (Table III), are less than or equal to half the rates of intracomplex electron transfer, k_{lim} (Table I), as measured by flash photolysis at the same pH but twice the buffer concentration. Such differences in rate may be attributed to the difference in the buffer concentrations used in the two experiments. As noted above, Kang et al. (1977) observed a 1.5–2-fold increase in V_{max} for yCcP and iso-1 cytc in going from 50 mM to 100 mM phosphate. Assuming this observation can be extended to our results, then the turnover numbers reported here for 50 mM phosphate can be adjusted to buffer conditions of 100 mM phosphate. The turnover numbers become 1320–1760 s^{-1} and 1635–2180 s^{-1} for ECcP and D217K, respectively. These values, so corrected, do not differ greatly from the values of k_{lim} reported in Table II.

Such large turnover rates do not agree with the simple unimolecular dissociation rate constant extracted from pre-existing data. Under the solutions conditions used for the steady-state kinetics reported here, 50 mM, pH 6, phosphate, the association constant for both cytc(III) and cytc(II) binding to the Fe(III)CcP analogue, MgCcP, is 2 × 10^7 M^{-1} as determined by fluorescence titration (Corin et al., 1992; Zhang et al., 1993). The literature provides two estimates for the bimolecular association rate constant, k_1. The first, obtained from steady-state kinetic measurements, is 5.6 × 10^8 M^{-1} s^{-1} (μ = 0.2 M, sodium acetate buffer, pH 6; Yonetani & Ray, 1966) and the second, from stopped flow experiments, is > 4.7 × 10^8 M^{-1} s^{-1} (μ = 0.01 M, phosphate buffer, pH 7.5; Summers & Erman, 1988). These data predict a simple dissociation rate of ~25 s^{-1} which is far too slow to permit the substrate turnover rates observed from steady-state kinetic data. Previous work suggests that more than one binding site exists (Kornblatt & English, 1986; Das et al., 1988, Northrup et al., 1988; Hake et al., 1992). This second site must be much weaker than ~10^7 M^{-1} as fluorescence titration data describing a site of this affinity fit a single site binding model well. NMR data show that the exchange, or dissociation, rate of complex formation for the CcP/iso-1 cytc complex is concentration dependent (Moench et al., 1992). One mechanism that would reconcile these data involves formation of a ternary complex, i.e., two cytc molecules bound to one CcP, with one site occupied primarily by substrate, cytc(II), and the other by product, cytc(III). The binding of the second (reduced/substrate) molecule of cytc might then enhance the rate of release of the first (oxidized/product). Such a model invoking substrate-facilitated product release would serve to explain two key observations: (1) the apparent concentration dependence of the exchange rate and (2) a higher overall dissociation of cytc than is possible by simple unimolecular dissociation. The overall dissociation rate would then be the sum of two terms, the slow unimolecular exchange, k_{-1}, plus a much larger concentration dependent second term, k'_{-1}. Such substrate-facilitated product release has precedent in other enzyme-catalyzed reactions such as dihydrofolate reductase (S. Benkovic, personal communication). As there are, undoubtedly, other mechanisms that might account for the data, this model is only suggested as one possibility.

CONCLUSIONS

From these and previously reported experiments, we can draw the following specific conclusions about the functional role played by two acidic surface residues in peroxidase in cytc recognition and electron exchange.

I. Aspartate 37 plays a role in forming the complex with cytc but need not be directly involved in electron flow into and out of the molecule. This constitutes a case in which these two steps can be functionally distinguished.

II. Aspartic acid 217 also participates in establishing the configuration of the cytc/CcP complex, but the effects are subtle. The mutant peroxidase D217K exhibits different

relative affinity for the reduced and oxidized cytc substrate (Hake et al., 1992) compared to that of the native-like recombinant, ECcP. Overall, a smaller change is observed in binding affinity for cytc when Asp → Lys mutations are made at position 217 compared to the same change at position 37 (Corin et al., 1991).

III. The basic general mechanism of complex formation followed by electron transfer does not account for the different ionic strength profile of the D217K mutant compared to that of the native-like peroxidase. The rate-limiting step for electron transfer for D217K shows a stronger, positive ionic strength dependence than that for the native-like enzyme. Both these enzymes, ECcP and D217K, have similar binding affinities for reduced cytc in the range μ = 100–250 mM that are relatively independent of ionic strength. The observed difference in ionic strength dependencies of k_{lim} can be explained by invoking an ionic strength dependence of the interfacial motions of these enzymes in complex with cytc. The mutation of Asp 217 → Lys changes this dependence, thereby implicating a role for this surface residue in guiding the dynamic interactions and conformational geometry within the complex. These interfacial interactions, in turn, affect the efficiency of electron flow.

In a more general sense, kinetic and thermodynamic studies on site specific mutants of CcP are leading to a more detailed picture of the dynamic roles played by individual amino acids. This, in turn, permits the construction of a more complete picture of the electron transfer process between such biological macromolecules. The simple model describing complex formation, guided, at least in part, by electrostatics, followed by electron exchange and separation of the product species, is still the foundation of the overall reaction scheme. However, a comprehensive mechanism accounting for all the thermodynamic and kinetic data available requires more complexity. One possible mechanism is suggested whereby a ternary complex between two molecules of cytc and one of peroxidase is formed and substrate-assisted product release occurs. Studies such as the one presented here provide insight into the molecular structure–function relations and subtle dynamics of the process.

While this work was in review, the X-ray crystal structure for the 1:1 complex between cytc(III) and ferric CcP was solved by Pelletier and Kraut to 2.3-Å resolution (1992). This structure is quite different from the Poulos and Kraut computer model for the complex previously published (1980) and provides a solid structural foundation upon which to base future discussions of the reaction mechanism. The most striking result is that the cytc binding domain on CcP is centered about two alanine residues rather than the negatively charged residues emphasized in the "electrostatic" model. Nonetheless, included in a list of CcP surface residues that make contact (i.e., are within 4 Å) with cytc is Asp 34. This is one of five such negatively charged residues in the short stretch of sequence spanning residues 33–37. We refer to this as the "negative patch". Recently, we have mutated another member of this group, Asp 33, and found that it too shows similarly large decreases in catalytic turnover and binding affinity to cytc (in preparation). Though not directly implicated in the binding of cytc in crystals of the complex, these two residues are not far from the binding domain. Functionally, they clearly are important for proper molecular recognition. Perhaps further study of the structure of the CcP/cytc complex as well as additional structural investigations using mutant proteins will provide insight into the dynamic behavior of these biological redox proteins.

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