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Apolar Distal Pocket Mutants of Yeast Cytochrome *c* Peroxidase: Hydrogen Peroxide Reactivity and Cyanide Binding of the TriAla, TriVal, and TriLeu Variants

Anil K. Bidwai, Cassandra Meyen, Heather Kilheeney, Damian Wroblewski, Lidia B. Vitello, and James E. Erman*

Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, IL 60115

Abstract

Three yeast cytochrome *c* peroxidase (CcP) variants with apolar distal heme pockets have been constructed. The CcP variants have Arg48, Trp51, and His52 mutated to either all alanines, CcP(triAla), all valines, CcP(triVal), or all leucines, CcP(triLeu). The triple mutants have detectable enzymatic activity at pH 6 but the activity is less than 0.02% that of wild-type CcP. The activity loss is primarily due to the decreased rate of reaction between the triple mutants and H₂O₂ compared to wild-type CcP. Spectroscopic properties and cyanide binding characteristics of the triple mutants have been investigated over the pH stability region of CcP, pH 4 to 8. The absorption spectra indicate that the CcP triple mutants have hemes that are predominantly five-coordinate, high-spin at pH 5 and six-coordinate, low-spin at pH 8. Cyanide binding to the triple mutants is biphasic indicating that the triple mutants have two slowly-exchanging conformational states with different cyanide affinities. The binding affinity for cyanide is reduced at least two orders of magnitude in the triple mutants compared to wild-type CcP and the rate of cyanide binding is reduced by four to five orders of magnitude. Correlation of the reaction rates of CcP and 12 distal pocket mutants with H₂O₂ and HCN suggests that both reactions require ionization of the reactants within the distal heme pocket allowing the anion to bind the heme iron. Distal pocket features that promote substrate ionization (basic residues involved in base-catalyzed substrate ionization or polar residues that can stabilize substrate anions) increase the overall rate of reaction with H₂O₂ and HCN while features that inhibit substrate ionization slow the reactions.

Keywords

cytochrome *c* peroxidase; distal pocket mutants; spectroscopic properties; hydrogen peroxide reaction; cyanide binding

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*Corresponding Author: Phone: (815) 753-6867. Fax: (815) 753-4802. jerman@niu.edu. Department of Chemistry and Biochemistry Northern Illinois University DeKalb, IL 60115 U.S.A. .

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Appendix A. Supplementary Data Figures showing the spectra of the triple mutants at various pH values, CD spectra, steady-state activity, stopped-flow data for the triAla/H₂O₂ reaction, pH dependence of the spectra of the CcP(triLeu)/cyano complexes, and the pH dependencies of the kinetic parameters for cyanide binding to the triVal and triLeu mutants along with tables containing values of *K*_{DA} and *K*_{DB} for cyanide binding to the triple mutants and of the kinetic parameters, *k*_a, *k*_d, *k*_{slow}, P1, P2, and P1/P3 are provided in the supplementary data, which is available free of charge via the Internet at doi:.

1. Introduction

Yeast cytochrome *c* peroxidase (CcP)¹ catalyzes the reduction of hydrogen peroxide to water by ferrocycytochrome *c*, Eq. (1) [1]. In Eq. (1), C²⁺ and C³⁺ represent the Fe(II) and Fe(III) forms

of cytochrome *c*, respectively. The redox sequence involves initial oxidation of the native ferric enzyme by hydrogen peroxide to form an enzyme intermediate called compound I and one of the two product water molecules, Eq. (2). CcP compound I (CcP-I) retains the second oxygen atom

of the hydrogen peroxide as part of a ferryl heme group. To complete the catalytic cycle, CcP compound I is reduced back to the native Fe(III) state of CcP by two molecules of ferrocycytochrome *c*. The very rapid initial reaction between enzyme and hydrogen peroxide, Eq. (2), can be considered the distinguishing feature of the peroxidases. Structurally, the peroxidases have very polar heme pockets with invariant distal arginine and histidine residues [2-6]. A distal histidine residue is essential for catalytic activity, accelerating the reaction between hydrogen peroxide and enzyme by at least a factor of 10⁵ [7,8].

Other classes of heme proteins tend to have less polar heme pockets than the peroxidases and react less readily with hydrogen peroxide to form compound I-like intermediates [9,10]. In some cases, the porphyrin ring is oxidized more readily than the heme iron leading to destruction of the heme group in the presence of hydrogen peroxide [10]. Heme degradation limits the study of hydrogen peroxide activation by the less reactive heme proteins. In our continuing efforts to explore the structural basis of hydrogen peroxide activation utilizing CcP mutants, we have found an interesting correlation between the rate of CcP-I formation and the rate of cyanide binding [11]. This implies that the rate-limiting step in CcP compound I formation is binding of hydrogen peroxide to the heme iron rather than the redox chemistry involved in compound I formation. Structural features that are important for accelerating cyanide binding are also important for accelerating the rate of hydrogen peroxide binding to the heme iron.

Studies on the binding of small ligands to heme proteins have a long history and have provided useful information concerning the structural basis for heme protein activity [12]. Ferrous and ferric heme proteins bind distinctly different groups of small ligands. Among the ferrous heme ligands, O₂, CO, and NO are most important. Common ferric heme ligands include cyanide, azide, fluoride, and imidazole. Ligand binding to the ferric state of heme proteins is most relevant to peroxidase chemistry and, since ferric heme ligands tend to be weak acids or bases, can provide an abundance of information relevant to discrimination between neutral and charged forms of the ligand. Rates of ferric heme ligand binding can be

¹**Abbreviations and Textual Footnotes** CcP, generic abbreviation for cytochrome *c* peroxidase whatever the source; yCcP, authentic yeast cytochrome *c* peroxidase isolated from *S. cerevisiae*; rCcP, recombinant cytochrome *c* peroxidase expressed in *E. coli*, the rCcP used in this study has an amino acid sequence identical to that of yCcP; CcP(MI), recombinant CcP expressed in *E. coli* with four amino acid variations compared to yCcP, a Met-Ile N-terminal extension and mutations T53I and D152G; mutations in the amino acid sequences of CcP are indicated by using the one letter code for the amino acid residue in the wild-type protein, followed by the residue number and the one letter code for the amino acid residue in the mutant protein, *i.e.*, R48A represents a mutant in which an alanine residue replaces the arginine residue at position 48 of the wild-type protein; CcP(triAla), triple mutant of rCcP with R48A/W51A/H52A; CcP(triVal), triple mutant of rCcP with R48V/W51V/H52V; CcP(triLeu), triple mutant of rCcP with R48L/W51L/H52L. CcP-I, CcP compound I; CD, circular dichroism.

sensitive reporters of the acid/base chemistry, electrostatic environment, and accessibility of the heme site.

The monooxygenases are a class of heme proteins with significantly less polar heme pockets than the peroxidases [13]. The monooxygenases utilize molecular oxygen to oxygenate a wide variety of generally apolar substrates. The catalytic cycle involves binding of molecular oxygen to the Fe(II) heme, reduction of the oxygen complex to generate a ferryl heme intermediate, and transfer of the ferryl oxygen to the apolar substrate. Oxygen transfer requires proximity between the ferryl heme and the substrates. Apolar heme pockets facilitate substrate binding near the heme iron. The monooxygenases can also use H₂O₂ to generate the ferryl heme in a less effective reaction called the peroxide shunt pathway [14].

We have constructed three CcP variants with apolar heme pockets for a planned series of studies including the effect of the mutations on hydrogen peroxide activation, binding of small ligands such as cyanide and imidazole, and potential peroxxygenase activity of CcP. The apolar distal heme pockets were constructed by simultaneously replacing Arg48, Trp51, and His52 with either all alanines, CcP(triAla), all valines, CcP(triVal), or all leucines, CcP(triLeu). It is anticipated that the hydrogen peroxide reactivity will be severely diminished but that the peroxxygenase activity may be enhanced. In this study, we report on the initial characterization of the three CcP triple mutants including their spectroscopic properties, their catalytic activity toward yeast iso-1 ferrocycytochrome α (C102T), their reaction with hydrogen peroxide, and both the kinetic and equilibrium properties of cyanide binding. As anticipated, the three triple mutants of CcP have very low reactivity toward hydrogen peroxide in the formation of the initial oxidized intermediate, CcP compound I. Moreover, oxidative destruction of the heme by hydrogen peroxide precludes extensive studies of compound I formation. Cyanide binding can act as a surrogate for the hydrogen peroxide reaction and we report a detailed study of cyanide binding to these three mutants over the pH stability range of CcP, pH 4 to 8. Overall, the rates of cyanide binding are about four to five orders of magnitude slower than for wild-type CcP [15]. In spite of the very low rates of reaction with H₂O₂, preliminary studies indicate that the peroxxygenase activities of these mutants can be enhanced up to fifteen-fold compared to wild-type CcP.

2. Materials and Methods

2.1. Cloning, Expression, and Purification of CcP Mutants

James Satterlee, Washington State University, kindly provided the expression system for the recombinant CcP (rCcP) used in this study [16]. The CcP gene was modified to include an N-terminal methionine for bacterial expression and the exact amino acid sequence of mature baker's yeast CcP [17]. The CcP gene is inserted into the multiple cloning site of the Novagene vector pET24a(+) under control of the T7 promoter. The CcP mutants were created using the Stratagene QuikChange mutagenesis kit and sequenced in both directions to assure that, except for the intended mutations, the gene was identical to the published sequence. The three CcP triple mutants constructed for this study are CcP(triAla) with mutations R48A/W51A/H52A, CcP(triVal) with mutations R48V/W51V/H52V, and CcP(triLeu) with mutations R48L/W51L/H52L.

Recombinant CcP and the CcP mutants were expressed in *E. coli* strain BL21(DE3) using published procedures [18,19]. The N-terminal methionine is removed from the rCcP produced in this expression system [18]. Cells were harvested by centrifugation and stored at -20°C if not used immediately for protein isolation. CcP and its mutants were isolated by suspending the cells from 1 L of culture in ~15 mL lysis buffer containing lysozyme, DNase, and RNase, incubated on ice for 15-20 minutes, then ruptured by sonication. Soluble proteins were separated from cell debris by centrifugation, a four-fold excess of hemin was

added to the supernatant and incubated at 4°C for at least an hour before dialyzing against 50 mM potassium phosphate buffer, pH 6.0, overnight. Insoluble material was removed from the dialysate by centrifugation and the supernatant passed through a Sephadex G-75 column (2.5×90 cm) equilibrated with the pH 6.0, 50 mM, phosphate buffer. Fractions containing CcP were pooled and applied to a diethylaminoethyl (DEAE)-Sephacel column (2.5×30 cm) equilibrated in 50 mM potassium phosphate buffer, pH 6.0. CcP was eluted using a linear gradient consisting of 200 mL 50 mM potassium phosphate and 200 mL of 1.0 M potassium phosphate, both at pH 6.0. Fractions were monitored by UV-vis spectroscopy and fractions with absorbance ratios (408nm/280nm) >1 were pooled. The sample was concentrated on a small DEAE-Sephacel column and dialyzed against several changes of deionized distilled water to induce crystallization. On occasion, CcP samples were lyophilized after dialysis against deionized distilled water.

2.2. Other Materials

Yeast iso-1 cytochrome α (C102T) was purified as described previously [20,21]. Potassium cyanide, potassium phosphate salts, and hydrogen peroxide (30%) were obtained from Fisher Scientific. Hydrogen peroxide solutions were standardized by titration with Ce(IV) [22].

2.3. Buffers

Between pH 4.0 and 5.5, buffers were 0.010 M acetate with sufficient KH_2PO_4 to adjust the ionic strength to 0.100 M. Between pH 5.5 and 8.0, the buffers were mixtures of KH_2PO_4 and K_2HPO_4 with a total ionic strength of 0.100 M.

2.4. Spectroscopic Measurements and Protein Concentration Determination

Electronic absorption spectra of protein solutions were determined using either a Varian/Cary Model 3E or a Hewlett Packard Model 8452A spectrophotometer. The extinction coefficients of the three triple mutants were determined using the pyridine hemochromogen method of Berry and Trumpower [23].

2.5 Circular Dichroism (CD) Spectroscopy

An AVIV Model 215 circular dichroism spectrophotometer was used to determine the circular dichroism (CD) spectra of rCcP and the triple mutants. Data were collected between 450 and 195 nm at 1 nm intervals. Protein concentrations were 1.0 μM in a 0.10 M ionic strength potassium phosphate buffer, pH 6.0. The temperature of the samples was maintained at 25 °C.

2.6. Equilibrium Constant Determinations

Spectroscopic changes associated with formation of the cyanide complex enabled monitoring of complex formation. Determination of the equilibrium constants was done by titrating ~10 μM protein with increasing concentrations of buffered cyanide solution until saturation with cyanide was reached. The spectrum of the solution was determined after each cyanide addition. Equilibrium constants were determined from changes in absorbance as a function of the cyanide concentration. Equilibrium studies were carried out at 0.5 pH intervals between 4 and 8, 25°C.

2.7. Steady-State Kinetic Measurements

Initial velocities for the enzyme-catalyzed oxidation of yeast iso-1 ferrocycytochrome α (C102T) by hydrogen peroxide were determined at pH 6.0, 25 °C by monitoring the change in absorbance during the conversion of reduced to oxidized cytochrome c , generally at 550 nm [24]. The initial velocities were corrected for the oxidation of ferrocycytochrome c in the

absence of enzyme and determined as a function of hydrogen peroxide (0.02 to 1.0 mM) and cytochrome *c* (3 to 25 μ M) concentration. CcP triple mutant concentrations in the μ M region were required in order to observe the catalyzed rates.

2.8. Transient-State Kinetic Measurements

The reaction between CcP(triAla) and hydrogen peroxide was investigated by stopped-flow techniques using an Applied Photophysics Ltd. Model DX.17MV stopped-flow instrument. The spectra-kinetic mode was used to determine if any spectroscopically-distinct intermediates were detectable between 10 ms and 10 s after mixing the reactants.

The rates of cyanide binding were determined using the Applied Photophysics Ltd. stopped-flow instrument. Reactions were carried out under pseudo-first order conditions with excess cyanide. Protein concentrations were typically ~ 1 μ M. Observed rate constants were determined at a minimum of five different cyanide concentrations at each pH with the cyanide concentrations varying by at least a factor of five for each experiment. A minimum of 10 individual traces of absorbance change versus time were acquired at each cyanide concentration allowing the mean value of the observed rate constant and its standard deviation to be determined. Kinetic studies were carried out at every half pH between pH 4 and 8 at 0.10 M ionic strength, 25 $^{\circ}$ C.

3. Results

3.1. Spectroscopic Properties of CcP Triple Mutants

CcP and its mutants are generally stable between pH 4 and 8 and spectra for the triple mutants were determined at each half pH over this range. The spectra of CcP(triAla), CcP(triVal), and CcP(triLeu) at pH 6.0 are shown in Fig. 1. The Soret maxima occur at 406, 406, and 400 nm for the triAla, triVal, and triLeu mutants, respectively. Spectroscopic parameters at pH 6.0 are included in Table 1. The spectra of all three mutants depend on pH. Spectra of the triple mutants at pH 4.0, 6.0, and 8.0 are shown in the supplementary data accompanying this article. At pH 4, the Soret bands occur at 403, 391, and 396 nm for CcP(triAla), CcP(triVal), and CcP(triLeu), respectively, blue-shifted compared to their positions at pH 6.0. At pH 8, the Soret maxima are red-shifted compared to the position at pH 6, with maxima occurring at 410, 408, and 409 nm for CcP(triAla), CcP(triVal), and CcP(triLeu), respectively. Fig. 2 shows the extinction coefficient near the Soret maximum as a function of pH for the three triple mutants. The extinction coefficients have a bell-shaped dependence on pH indicating that the spectra are influenced by a minimum of two acid/base transitions. The low pH transition, which is evident below pH 5 for these CcP mutants, causes a blue-shift and reduced absorptivity in the Soret band, which we attribute to the onset of acid denaturation. The pK_A for this transition, pK_{A1} , can only be estimated since the low pH limit of the absorptivity has not been determined. We estimate pK_{A1} by assuming that the absorptivity of the acid denatured form is similar to that of free heme in acidic solution [25]. These estimates are collected in Table 2. The transition near neutral pH, designated pK_{A2} in Table 2, is due to a change in heme ligation, with the heme switching from a five-coordinate, high-spin form at low pH to a six-coordinate low-spin form at high pH.

3.2 CD Spectra

CD spectra of the triple mutants was determined at pH 6.0 and compared to that of rCcP. The CD spectra of the triple mutants are essentially identical to the CD spectrum of rCcP (supplementary data).

3.3. Catalytic Activity of the Triple Mutants

All three triple mutants catalyze the oxidation of ferrocycytochrome *c* by hydrogen peroxide. Fig. 3 shows plots of the initial velocity as a function of enzyme concentration for the three mutants at pH 6.0, 0.100 M ionic strength. The initial velocity increases linearly with increasing enzyme concentration for all three mutants, with CcP(triAla) the most active of the three. The slopes of the plots give the enzyme turnover number (TN), which are collected in Table 3 along with turnover numbers of authentic yCcP and two recombinant CcP's under similar, although not identical, experimental conditions. Although catalytically active, the activities of the triple mutants are significantly less than those of yCcP, rCcP, and CcP(MI). CcP(triAla) is about 16 times more active than the triVal and triLeu mutants but only 0.014% as active as CcP(MI) at pH 6.0, 0.10 M ionic strength, 25°C, Table 3.

The dependence of the initial velocity on the concentrations of hydrogen peroxide and cytochrome *c* were determined at pH 6.0, 0.10 M ionic strength. The catalytic activities of CcP(triVal) and CcP(triLeu) are independent of the cytochrome *c* concentration between 3 and 25 μ M and linearly dependent upon the hydrogen peroxide concentrations between 0.020 and 1.0 mM. These data indicate that under the conditions of the kinetic measurements, the reaction between hydrogen peroxide and the triVal and triLeu mutants is rate limiting and that k_I , Eq. (2), can be determined from the initial velocity measurements. The values of k_I , determined from the slope of the plots of v_0/e_0 versus the hydrogen peroxide concentration are 11 ± 1 and $4.6 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ for CcP(triVal) and CcP(triLeu), respectively (supplementary data). The values for k_I are included in Table 3.

In contrast to the data for the triVal and triLeu mutants, the initial velocity for CcP(triAla) is a hyperbolic function of both the hydrogen peroxide and cytochrome *c* concentrations. These data suggest that oxidation of ferrocycytochrome *c* limits the overall rate of reaction above 0.20 mM hydrogen peroxide. Below 0.20 mM hydrogen peroxide, the initial velocity does decrease with decreasing hydrogen peroxide, allowing k_I to be determined from the limiting slope of the hyperbola at low hydrogen peroxide (supplementary data). The value of k_I for the reaction of hydrogen peroxide and CcP(triAla) is $(7.8 \pm 1.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, some 3 orders of magnitude larger than for the triVal and triLeu mutants, Table 3, but still 4 orders of magnitude slower than the reactions with yCcP and rCcP [26,27].

The maximum turnover number and Michaelis Constant (K_M) for the interaction of cytochrome *c* and CcP(triAla) can be determined from the hyperbolic dependence of the initial velocity on cytochrome *c* concentration at high hydrogen peroxide concentrations. At pH 6.0, 0.10 M ionic strength, the maximum TN and K_M are $0.20 \pm 0.02 \text{ s}^{-1}$ and $4.8 \pm 1.6 \mu\text{M}$, respectively, for the CcP(triAla) catalyzed oxidation of ferrocycytochrome *c* by hydrogen peroxide. These parameters are included in Table 3 and compared to those of yCcP, rCcP, and CcP(MI) under similar experimental conditions [28-30]. The K_M values are similar but the maximum turnover number is 3.5 to 4.5 orders of magnitude slower than those for yCcP, rCcP, and CcP(MI).

3.4. Reaction of the Triple Mutants with Hydrogen Peroxide

Although CcP(triAla) is the most active of the three mutants under study, there is no spectroscopic evidence that CcP(triAla) forms a CcP compound I-like intermediate upon reaction with hydrogen peroxide in the absence of cytochrome *c*. Fig. 4A shows an experiment in which CcP(triAla) and hydrogen peroxide are manually mixed in a standard 10 mm cuvette and spectra obtained at timed intervals using the HP diode-array spectrophotometer. The first spectrum was determined within 10 s of mixing and is essentially identical to the Fe(III) form of the enzyme. This is an interesting observation since the steady-state kinetics indicates that hydrogen peroxide reacts with CcP(triAla) with

a bimolecular rate constant of $7.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ to form a catalytic intermediate capable of oxidizing ferrocycochrome *c*. Under the conditions used for the experiment shown in Fig. 4A, the pseudo-first order rate constant for formation of the catalytic intermediate is 0.78 s^{-1} giving a half-time for the reaction of 0.89 s. In the absence of competing reactions, the catalytic intermediate should be completely formed within 10 s. The data presented in Fig. 4A suggest that either the spectrum of the catalytic intermediate is identical to the Fe(III) form of the enzyme or that the intermediate is rapidly reduced such that its steady-state concentration is very low, below our detectability limits. We have used stopped-flow techniques to look for a spectroscopically-distinct intermediate that might form on a faster time scale than that shown in Fig. 4A but have found no evidence for such an intermediate between 10 ms and 10 s (supplementary data).

The spectroscopic changes shown in Fig. 4A suggest that the heme undergoes irreversible oxidative degradation when hydrogen peroxide is added to CcP(triAla). The rate of degradation can be monitored by observing the decrease in heme absorbance as a function of time. Fig. 4B shows the change in absorbance at 424 nm for the reaction between CcP(triAla) and a 10-fold excess of hydrogen peroxide. The absorbance change is biphasic and characterized by two first-order rate constants designated k_{ox1} and k_{ox2} . Values of k_{ox1} and k_{ox2} for the CcP(triAla) reaction are independent of the hydrogen peroxide concentration (0.030 to 1.0 mM) and the average rates are included in Table 3.

In contrast to CcP(triAla), when hydrogen peroxide is added to either CcP(triVal) or CcP(triLeu), there is an initial, small increase in the absorbance at 424 nm, Fig. 4B, which we take as evidence for CcP compound I formation [1,26]. Unfortunately, the amplitude of the absorbance at 424 nm is so small and the rate of compound I formation (estimated from the steady-state kinetic results) is very similar to the rate of heme degradation that it has been impossible to extract an accurate value of k_I from the transient-state studies. Values of the heme oxidative degradation rate constants in the presence of hydrogen peroxide, k_{ox1} and k_{ox2} , for the triVal and triLeu mutants can be determined and these are included in Table 3.

3.5. Cyanide Binding to the CcP Triple Mutants

3.5.1. Spectroscopic Properties—In a previous communication [11] we have shown a strong correlation between the rate of cyanide binding and the rate of the hydrogen peroxide reaction for CcP and a number of CcP heme pocket mutants. In light of the difficulty in determining the value of k_I for CcP(triAla), CcP(triVal), and CcP(triLeu) from the direct reaction of the mutants with hydrogen peroxide, we decided to investigate the rate and equilibrium properties of cyanide binding to the triple mutants. Cyanide should form stable complexes with the mutants and the data will not be influenced by heme degradation as is the hydrogen peroxide reaction.

Addition of buffered cyanide solutions to the three triple mutants causes large changes in the absorption spectrum of the enzyme as cyanide binds. Spectral changes associated with cyanide binding to CcP(triLeu) at pH 7.0 are shown in Fig. 5A. The Soret maximum shifts from 400 nm to 419 nm and increases in absorptivity as cyanide binds to the mutant. Fig. 5B shows a difference spectrum between CcP(triLeu) in the presence and absence of 97.5 mM cyanide, with maximum and minimum in the difference spectrum occurring at 424 nm and 392 nm, respectively. Fig. 6 shows a plot of the difference in absorbance at 424 and 392 nm, $\Delta(A_{424} - A_{392})$, as a function of the total cyanide concentration at both pH 5.0 and 7.0. Using a difference in two absorbance values rather than the absolute absorbance minimizes the effect of baseline shifts during the titration since some of the equilibrium titrations were monitored for up to 24 hours using a single-beam diode-array spectrophotometer. At pH 7.0,

cyanide binding fits a simple hyperbolic binding equation where the absorbance change is given by Eq. (3). In Eq.

$$\Delta A_{obs} = \Delta A_0 + \Delta A_{max} \frac{[L]}{K_D + [L]} \quad (3)$$

(3), ΔA_{obs} is the observed absorbance change, ΔA_0 is the difference in absorbance at zero cyanide concentration, ΔA_{max} is the maximum absorbance change in the presence of infinite cyanide, K_D is the equilibrium dissociation constant, and $[L]$ is the total cyanide concentration. For the pH 7.0 data shown in Fig. 6, best-fit values for ΔA_{max} and K_D are 0.667 ± 0.003 and 0.84 ± 0.02 mM, respectively.

The spectroscopic changes induced by binding cyanide to CcP(triAla) and CcP(triVal) are similar to those for CcP(triLeu). Fig. 5B shows difference spectra of the three mutants at saturating concentrations of cyanide and zero cyanide. Spectroscopic parameters for the three cyano complexes are collected in Table 1. The spectra of the cyano complexes are similar with the Soret maxima occur at 418 ± 1 nm with the maximum extinction coefficient varying between 99 and 121 $\text{mM}^{-1} \text{cm}^{-1}$. The visible region of the spectrum is dominated by the β band, which occurs at 541 ± 1 nm. The α band occurs as a shoulder near 570 nm. The spectrum of the cyano complex for each of the three triple mutants is independent of pH between pH 4 and 8.

3.5.2. Cyanide Binding to the CcP Triple Mutants – Equilibrium Dissociation Constants—The cyanide titration curves for CcP(triLeu) are monophasic between pH 7 and 8. However, below pH 7 cyanide binding is weaker and the titration curves become biphasic. Fig. 6 shows a cyanide titration curve for CcP(triLeu) at pH 5.0. The pH 5.0 data were fit to Eq. (4) where K_{DA} and K_{DB} are apparent equilibrium dissociation constants for the two cyanide binding phases

$$\Delta A_{obs} = \Delta A_0 + \Delta A_A \frac{[L]}{K_{DA} + [L]} + \Delta A_B \frac{[L]}{K_{DB} + [L]} \quad (4)$$

while ΔA_A and ΔA_B are the maximum absorbance changes associated with each phase of the reaction. The second phase of cyanide binding becomes quite weak at low pH and fitting the data to Eq. (4) required constraining the absorbance changes to those predicted for 100% complex formation.

Cyanide binding to the three triple mutants was investigated as a function of pH. Cyanide binding to CcP(triAla) is biphasic over the entire pH region, 4 to 8. The amplitudes of the two phases appear to be relatively constant with pH, with the high-affinity phase accounting for 45 ± 9 % of the total absorbance change. Cyanide binding to CcP(triVal) is biphasic between pH 4 and 6 and monophasic between pH 6.5 and 8. The high-affinity phase accounts for 80% of the absorbance change upon cyanide binding at pH 4, increasing to 100% at pH 6.5 and above. Cyanide binding to CcP(triLeu) is biphasic between pH 4 and 6.5 and monophasic between pH 7 and 8. The amplitude of the high-affinity phase increases from about 20% at pH 4.0 to 100% at pH 7.0 and above. Values of K_{DA} and K_{DB} are collected in the supplementary data, along with the relative amplitudes of the two binding phases.

Plots of the negative logarithm of the equilibrium dissociation constant, $-\log(K_{DA})$, as a function of pH for each of the triple mutants are shown in Fig. 7. Both the high-affinity and low-affinity binding phases have similar pH dependencies, increasing with increasing pH. The cyanide binding appears to be influenced by a single ionization in the neutral pH region

for both the high- and low-affinity phases for all three mutants. The data were fit to a titration curve involving a single ionization in the protein, Eq. (5). The low and high pH limits for K_{DA}

$$-\log(K_{DA}) = \log\left(\frac{1}{K_{DA}^{acid}} + \frac{1}{K_{DA}^{base}} \frac{K_{A2}}{[H^+]}\right) - \log\left(1 + \frac{K_{A2}}{[H^+]}\right) \quad (5)$$

are designated K_{DA}^{acid} and K_{DA}^{base} , respectively, and the apparent acid dissociation constant for the ionizable group that affects cyanide binding is designated K_{A2} since it may be related to the ionization that affects the electronic absorption spectrum, Fig. 2. Values of K_{DA}^{acid} , K_{DA}^{base} and pK_{A2} are collected in Table 4. The cyanide binding is strongest at alkaline pH, where K_{DA}^{base} varies between 0.15 and 0.26 mM for the three mutants.

Plots of the negative logarithm of K_{DB} as a function of pH for the triple mutants are also shown in Fig. 7. K_{DB} values for the CcP(triAla)/cyanide reaction were fit to an equation similar to Eq. (5). The high pH limit is not observed for the CcP(triVal) and CcP(triLeu) data and best-fit values for K_{DB}^{acid} and the ratio K_{DB}^{base}/K_{A2} are included in Table 4.

3.5.3. Kinetics of Cyanide Binding to the CcP Triple Mutants—The binding of cyanide to the CcP triple mutants was sufficiently fast that the reaction was monitored by stopped-flow techniques. The reactions of cyanide with all three mutants are biphasic and the observed pseudo-first-order rate constants are defined as k_{fast} and k_{slow} for the fast and slow phases, respectively. For CcP(triAla) and CcP(triLeu), k_{fast} is linearly dependent upon the cyanide concentration, Fig. 8A, while k_{slow} is independent of cyanide concentration. For CcP(triVal), both k_{fast} and k_{slow} are hyperbolic functions of the cyanide concentration, Fig. 8B.

The observed pseudo-first order rate constants for both phases of cyanide binding to all three mutants can be fit to the empirical equation shown in Eq. (6) (or simplifications of Eq. (6)). In

$$k_{obs} = \frac{P_1 [L] + P_2}{P_3 [L] + 1} \quad (6)$$

Eq. (6), k_{obs} can be either k_{fast} or k_{slow} and P_1 , P_2 , and P_3 are empirical parameters whose interpretation depends upon the binding mechanism. We will give a mechanistic interpretation of the kinetic parameters in Section 4. The observed rate constants for the fast phases of cyanide binding to CcP(triAla) and CcP(triLeu) were fit to Eq. (6) using the assumption that $P_3[L] \ll 1$. In this case, Eq. (6) becomes linear and the parameter P_1 can be identified as the association rate constant for cyanide binding, k_a , and P_2 can be identified as the rate constant for dissociation of cyanide from the cyano complex, k_d . The association and dissociation rate constants can be obtained from the slope and intercept of plots such as those shown in Fig. 8A. Best-fit values for k_a and k_d from the fast phases of cyanide binding to CcP(triAla) are plotted as a function of pH in Fig. 9

The values of k_a for cyanide binding to CcP(triAla), CcP(triLeu), and the fast and slow phases of cyanide binding to CcP(triVal) are 4.0 ± 0.6 , 5.1 ± 1.0 , 11 ± 2 , and $1.2 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$, respectively at pH 6, similar to each other but at least four-orders of magnitude slower than the rate of cyanide binding to yCcP ($1.1 \pm 0.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) [15].

On the other hand, the cyanide dissociation rate constants for the three mutants are very similar to that of the yCcP/cyanide complex. The values of k_d for the cyano complexes of

CcP(triAla), CcP(triLeu), and the high- and low-affinity phases of the CcP(triVal) complex are 0.25 ± 0.03 , 0.41 ± 0.04 , 0.072 ± 0.031 , and $0.39 \pm 0.01 \text{ s}^{-1}$, respectively at pH 6, while that of the yCcP/cyanide complex is $0.39 \pm 0.05 \text{ s}^{-1}$ [15].

The slow phases of the CcP(triAla) and CcP(triLeu)/cyanide reactions are independent of both the cyanide concentration and pH. The average values of k_{slow} are 0.05 ± 0.01 and $0.04 \pm 0.01 \text{ s}^{-1}$ for the triAla and triLeu mutants, respectively. The limiting values for k_{fast} and k_{slow} for cyanide binding to CcP(triVal) at high cyanide concentrations are also independent of pH, averaging 1.7 ± 0.8 and $0.12 \pm 0.05 \text{ s}^{-1}$, respectively.

4. Discussion

Heme protein perform a variety of functions in living organisms, with the reactivity of the heme modulated by amino acid residues surrounding the heme. As a class, the peroxidases have polar distal and proximal heme pockets and a distal histidine residue is important for hydrogen peroxide activation. On the other hand, the distal heme pocket of the cytochrome P450s are relatively nonpolar to promote binding of apolar organic substrates near the heme iron, facilitating oxygen transfer from the ferryl heme to the organic substrate. In this study we report on the initial characterization of peroxidase variants in which all of the amino acid residues forming the distal heme pocket have been converted to apolar residues. We have constructed three triple mutants of CcP, CcP(triAla), CcP(triVal), and CcP(triLeu), all of which retain detectable catalytic activity toward yeast iso-1 ferrocyclochrome *c* oxidation and have detectable reactivity with hydrogen peroxide. Preliminary experiments (J. Erman, unpublished data) indicate that these mutants have enhanced peroxygenase activity, similar to that of the cytochrome P450s, toward hydroxylation of naphthalene derivatives. The apolar CcP triple mutants have lowered affinity for cyanide (this work) but increased affinity for imidazole and imidazole derivatives (J. Erman, unpublished data). Spectroscopic studies including both UV-visible absorption spectra and CD spectra indicate that the structures of the triple mutants are not significantly altered from that of the wild-type enzyme.

As will be discussed below, there is a good correlation between the rate of reaction of hydrogen peroxide with CcP(triVal) and CcP(triLeu) and the rate of binding of cyanide to these two mutants as well as with nine other distal heme pocket mutants suggesting a common mechanistic component in these two reactions. However, the hydrogen peroxide and cyanide binding reactions of CcP(triAla) are not correlated as for the other mutants, suggesting a change in mechanism for the CcP(triAla)/hydrogen peroxide reaction.

4.1. Spectroscopic Properties of CcP Triple Mutants

All heme proteins have four basic absorption bands defined as the α , β , γ (Soret), and Δ bands in the visible and near UV region of the electronic absorption spectrum [31,32]. High-spin ferric heme proteins have two additional ligand-to-metal charge transfer bands designated CT1 and CT2 [31,32]. Both the band position and the extinction coefficient are sensitive to the spin and coordination state of the ferric heme group, providing information about the nature of heme ligation. Heme protein spectra are often pH dependent indicating changes in heme coordination. CcP and its mutants are generally stable between pH 4 and 8 and spectra for rCcP and the CcP triple mutants were determined over this region.

The spectra of rCcP and all three triple mutants are dependent upon the pH, shifting from predominantly high-spin ferric heme spectra at pH 5 to spectra that are characteristic of predominantly six-coordinate, low-spin ferric heme at pH 8. The most probable distal heme ligand in the six-coordinate high-spin form at low pH is a water molecule while hydroxide is the likely ligand at high pH. If this interpretation is correct, pK_{A2} (Table 2) is a measure of the ionization of the heme-bound water. The pK_{A2} values for ionization of the heme bound

water are substantially smaller in the CcP triple mutants compared to wild-type CcP, reflecting the more hydrophobic character of the distal heme pocket in the triple mutants.

As the pH is decreased from 5 to 4, the Soret band for all three mutants undergoes a blue-shift with loss of absorptivity at the Soret maximum suggesting the beginning of acid denaturation. At pH 4, the Soret band of CcP(triVal) occurs at 391 nm, closer to that of free heme [25] than to that of CcP(triVal) at pH 5. At pH 5.0, prior to the onset of denaturation, Fig. 2, the spectra of the three triple mutants are characteristic of high-spin hemes with dominant charge transfer bands in the visible region. In the past we have used the absorbance ratio of the Soret maximum to that in the Δ band near 380 nm to monitor changes in the amounts of five- and six-coordinate high-spin heme in various CcP variants [8,33,34]. Wild-type yeast CcP, CcP(MI), and rCcP have A_{Soret}/A_{380} values of 1.51, 1.53, and 1.54, respectively, at pH 6.0 and all are thought to be essentially 100% five-coordinate. However, the residues in the distal heme pocket affect the correlation between the A_{Soret}/A_{380} value and the amount of five- and six-coordinate heme. CcP(MI,H52L) has the lowest A_{Soret}/A_{380} value previously observed among CcP mutants and has a value of 1.16 ± 0.02 in potassium phosphate buffer at pH 6.0 [8]. The A_{Soret}/A_{380} values for CcP(triAla), CcP(triVal) and CcP(triLeu) are 1.54, 1.12, and 1.16, respectively. The latter two mutants have absorbance ratios similar to that of CcP(MI,H52L) and are most likely five-coordinate at pH 5. The higher value of the ratio for CcP(triAla) suggests that CcP(triAla) may have some six-coordinate high-spin heme with a water bound to the heme iron, even though its A_{Soret}/A_{380} value is similar to that of wild-type CcP.

Although crystal structures of the triple mutants are not available at this time, all evidence suggests that the structures of the mutants are similar to that of wild-type CcP. CD spectra indicate that the secondary structure of the mutants is essentially identical to that of rCcP. In addition, the electronic absorption spectra of the mutants indicate that the heme binds in an apolar pocket, protected from solvent, and the pH stability regions of the mutants are similar to that of rCcP, suggesting that the three-dimensional fold is intact.

4.2. Reaction with Hydrogen Peroxide

Yeast CcP, CcP(MI) and rCcP all react rapidly with CcP, with apparent bimolecular rate constants, k_f , of $(4.5 \pm 0.3) \times 10^7$, $(4.7 \pm 0.4) \times 10^7$, and $(4.8 \pm 0.2) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively [27]. His-52 is the most critical residue for facilitating the reaction with hydrogen peroxide and an H52L mutation of CcP(MI) reduces the rate by five orders of magnitude to $(7.3 \pm 0.4) \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ [7,8]. A second single site mutant, CcP(MI,R48L) has a more complex reaction with hydrogen peroxide [35]. Plots of the observed pseudo-first order rate constants as a function of the hydrogen peroxide concentration shows saturation at high hydrogen peroxide between pH 5 and 8. The limiting value of k_f at low hydrogen peroxide is $(1.3 \pm 0.1) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for the R48L mutant at pH 6.0, about 2.5 orders of magnitude slower than the wild-type enzyme. However, Arg-48 is critical for stabilizing compound I. CcP(MI,R48L) Compound I is very unstable, decaying within seconds of formation. Trp-51 has very little influence on the rate of compound I formation.

When we replace all three distal residues with apolar groups, both the rate of formation and stability of compound I is affected. Each of the triple mutants reacts uniquely with hydrogen peroxide. No compound I-like intermediate is observed upon addition of hydrogen peroxide to CcP(triAla). Either compound I is not formed or else the rate of formation is so slow and the rate of decay so fast, that the amount of compound I formed is negligible. Another consideration is that without the polar residues in the distal heme pocket, activation of hydrogen peroxide by the heme iron may involve homolytic cleavage of the peroxide oxygen-oxygen bond generating two hydroxyl radicals, which may decay quickly and also lead to rapid oxidative heme degradation. Interestingly though, CcP(triAla) has the highest

catalytic activity of the three triple mutants and the steady-state analysis indicates that hydrogen peroxide reacts with CcP(triAla) with a bimolecular rate constant of $7.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ to generate a catalytic intermediate capable of oxidizing ferrocycytochrome *c*.

The rates of reaction of CcP(triVal) and CcP(triLeu) with hydrogen peroxide are much slower than the reaction between CcP(triAla) and hydrogen peroxide. The bimolecular rate constants for the hydrogen peroxide reaction are 11 and $4.6 \text{ M}^{-1} \text{ s}^{-1}$ for the triVal and triLeu mutants, respectively. These are essentially identical to the rate of binding of cyanide to these two mutants and slower than the rate of reaction between CcP(H52L) and hydrogen peroxide, $(7.3 \pm 0.4) \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ [8]. Interestingly, the values of k_I for triVal and triLeu mutants are slower than those for the reaction of hydrogen peroxide with a water-soluble model heme, tetrakis (2,6-dimethyl-3-sulfonatophenyl)-porphinato iron(III), which is $343 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.18 [36]. The polar nature of the solvent surrounding the model heme is most likely responsible for enhancing the rate of proton removal from H_2O_2 as the peroxide anion binds to the heme iron in comparison to the reaction in the very nonpolar distal heme pockets of the CcP triple mutants.

4.3. Cyanide Binding

The binding of cyanide to the triple mutants is complex. All three mutants have biphasic equilibrium titration curves, Fig. 6 and supplementary data, although at more alkaline pH the binding of cyanide to two of the mutants, CcP(triVal) and CcP(triLeu), becomes monophasic. The biphasic nature of the equilibrium binding curves suggests two independent conformers of the proteins that have different cyanide affinities. These conformers would have to exchange slowly on the time scale of cyanide binding experiments. A plausible mechanism for cyanide binding to all three mutants is given by Scheme 1. In Scheme 1, A and B represent two conformers of the mutants, each of which binds cyanide independently. We will assume that conformer A gives rise to the high-affinity phase of cyanide binding, K_{DA} , and the fast kinetic phase of the cyanide binding, k_{fast} . Conformer B gives rise to the slow, low-affinity phase of cyanide binding, K_{DB} and k_{slow} . The subscripts U and R represent unreactive and reactive conformations, respectively for each independent conformer. The observed equilibrium and kinetic properties for the three CcP triple mutants depends upon the relative rates of the isomerization and the cyanide binding steps for each of the conformers and these may be different for each of the triple mutants.

The easiest way to explain the hyperbolic dependence of both k_{fast} and k_{slow} on the cyanide concentration for CcP(triVal) and the biphasic cyanide equilibrium titration curves is to assume that both conformer A and B are predominantly in their unreactive forms in the absence of ligand, *i.e.*, the isomerization equilibrium lies toward A_U and B_U for each of the two conformers. If one assumes that reactive forms are present at very low concentrations and maintain a steady-state during cyanide binding, the equilibrium dissociation constants and the observed rate constant for cyanide binding will be given by Eqs. (7) and (8) for conformer A and an equivalent

$$K_{DA} = K_{DA}^R / K_{iA} \quad (7)$$

$$k_{obs} = \frac{K_{iA} k_{aA} [L] + k_{dA}}{\frac{k_{aA} [L]}{k_{-iA}} + 1} \quad (8)$$

set of equations for conformer B. In Eqs. (7) and (8), K_{iA} is the isomerization equilibrium constant, defined as k_{iA}/k_{-iA} , and K_{DA}^R is the equilibrium dissociation constant for the cyano

complex of the reactive conformation of conformer A, k_{dA}/k_{aA} . Eq. (8) predicts the hyperbolic dependence of the observed rate constants for cyanide binding to both conformers of the mutants as observed for CcP(triVal) and provides a mechanistic interpretation to the empirical parameters used in Eq. (6). For the A conformer in Scheme 1, P_1 is identified as $K_{iA}k_{aA}$, P_2 is k_{dA} , and P_3 is k_{aA}/k_{-iA} . Equivalent relationships are defined for the B conformer. Under the assumptions used to derive Eqs. (7) and (8), K_{iA} is much less than 1 and the parameter P_1 provides a lower limit for the association rate constant for cyanide binding to the reactive conformation of conformer A of CcP(triVal). The value of k_{iA} , which can be found from the ratio of P_1/P_3 , gives the maximum rate of cyanide binding at infinitely high cyanide concentrations with the reaction being limited by the rate of conversion of the unreactive conformer to the reactive form of CcP(triVal).

Neither k_{fast} nor k_{slow} for CcP(triAla) and CcP(triLeu) are hyperbolic functions of the cyanide concentration but this can be explained based on the relative rates of isomerization between reactive and unreactive conformations and the apparent rate of cyanide binding to the reactive forms in Scheme 1. For the fast phase of cyanide binding to CcP(triAla) and CcP(triLeu), if $K_{iA} \gg 1$, then the reactive conformer, A_R , is dominant in solution and one observes direct binding of cyanide to A_R without being limited by the rate of the A_U to A_R conversion. On the other hand, for the low-affinity, slow binding phase of the triAla and triLeu reaction, if $K_{iB} \ll 1$ and k_{aB} are sufficiently large, then the slow phase of cyanide binding will always be limited by k_{iB} and k_{slow} for the triAla and triLeu mutants will be independent of the cyanide concentration as observed.

4.4. pH Dependence of Cyanide Binding

Cyanide binding to the CcP triple mutants has a relatively small dependence upon pH suggesting that HCN, rather than the cyanide anion, is the reactive form of the ligand in this pH region. The cyanide binding process can be viewed as a diffusion-limited equilibration of HCN within the distal heme pocket, followed by ionization of HCN within the pocket, and binding of the cyanide anion to a penta-coordinate heme iron. If the equilibrium state of the heme is predominantly six-coordinate, the equilibrium and rate of the hexa-to-penta-coordination can be an important factors in determining the overall rate of cyanide binding. For many heme proteins, ionization of HCN within the distal heme pocket and the small steady-state concentration of anion within the heme pocket appears to control the rate for cyanide binding [37,38] and this also appears to be the case for the CcP triple mutants.

The small pH dependence of the rate and affinity for cyanide binding to the CcP triple mutants is most likely related to changes in heme ligation. The small increases in binding parameters above pH 6 may be attributed to a greater reactivity of CcP with hydroxide ion bound to the heme iron than to penta-coordinate CcP, Scheme 2. We assume that HCN ionization is very small within hydrophobic heme pocket, limiting the rate of cyanide binding as discussed above. In the penta-coordinate, acidic form of the triple mutants, the rate of cyanide binding is basically the product of the HCN concentration within the distal heme pocket times the unimolecular rate for cyanide anion binding. In alkaline CcP, the hydroxy ligand needs to dissociate prior to cyanide binding and normally this would slow down the cyanide binding reaction. However, in the CcP triple mutants, hydroxide dissociation is not rate-limiting and can facilitate cyanide binding. When the hydroxide ion dissociates from the heme iron, if there is an HCN nearby, the released hydroxide can accept a proton from HCN, acting as a base catalyst and increasing the effective concentration of the cyanide anion within the distal heme pocket. Catalysis by the dissociated hydroxide makes k_{a2} about 10 times larger than k_{a1} for the fast phase of cyanide binding to the CcP triple mutants, Fig. 9.

4.5 Correlation of the Rates of Cyanide Binding and the Reaction of H₂O₂ with CcP and CcP Variants

Fig. 10 shows a comparison of the rate constants for the hydrogen peroxide reaction, k_f , and the binding of HCN, k_b , to wild-type CcP and 12 distal pocket mutants of CcP [7,8,11,15,26,33-35,39,40]. There is a good correlation between the two bimolecular rate constants suggesting a common mechanistic element for the two reactions. It is well established that the role of the distal histidine in CcP, His52, is to serve as a base catalyst to facilitate ionization of hydrogen peroxide and allowing the peroxide anion to bind rapidly to the heme iron. We conclude also, that the distal histidine promotes HCN ionization within the distal pocket of wild-type CcP as a key step in cyanide binding. When the distal histidine is replaced by residues with weaker side-chain bases such as CcP(H52D) and CcP(H52E) or with less optimal basic residue placement such as the CcP(W51H/H52W) and CcP(W51H/H52L) mutants, the rates of both cyanide binding and compound I formation are reduced, Fig. 10. Ionization of either HCN or of H₂O₂ is difficult in the distal pockets of the CcP triple mutants. These mutants lack a basic residue in the distal pocket and the very nonpolar environment inhibits ionization. Never-the-less, the reactions of CcP(triVal) and CcP(triLeu) show the same correlation as yCcP, rCcP, and the nine mutants previously investigated. The rates of the cyanide binding and H₂O₂ reactions of CcP(triAla) are not correlated as those of the other mutants.

The anomalous CcP(triAla) reaction appears to be the reaction with H₂O₂ since this rate is about three-orders of magnitude faster than the H₂O₂ reactions for the triVal and triLeu mutants while the rates of the cyanide binding reactions are about the same for all three triple mutants. A reasonable explanation is that neutral H₂O₂ binds to the heme iron of CcP(triAla) faster than does the very low levels of any H₂O₂ anion that may be present. This is consistent with the UV-visible spectra of the triple mutants, which suggest that the CcP(triVal) and CcP(triLeu) mutants are essentially 100% five-coordinate at low pH while CcP(triAla) has some six-coordinate, high-spin character, with water the most likely sixth ligand. If neutral water binds to the heme iron in CcP(triAla), binding of neutral H₂O₂ is also reasonable. Another anomaly with CcP(triAla) is that we have not been able to detect any compound I-like intermediate in the CcP(triAla) reaction with H₂O₂ although there is evidence that the other two triple mutants do form compound I, Fig. 4. The lack of a compound I-like intermediate in the CcP(triAla)/H₂O₂ reaction may be due to homolytic cleavage of the bound neutral H₂O₂ rather than the heterolytic cleavage that leads to the ferryl form of compound I.

4.6. Conclusions

Replacing Arg48, Trp51, and His52 in CcP by all alanine residues, all valine residues, or all leucine residues creates CcP variants with very hydrophobic distal heme pockets. The spectroscopic properties of the CcP triple mutants indicate a change in heme ligation over the pH range 4 to 8 with the low pH form of the hemes predominantly five-coordinate, high-spin and the high pH forms six-coordinate, low-spin with a hydroxide bound to the heme. The apparent pK_A for the hydroxide ion binding varies between 6.2 for both CcP(triAla) and CcP(triVal) to pH 7.1 for CcP(triLeu). Compared to wild-type CcP, the CcP triple mutants have substantially reduced rates of reaction with hydrogen peroxide and with cyanide. The rate of the hydrogen peroxide reaction is reduced by six to seven orders of magnitude and the cyanide binding reaction by four to five orders of magnitude. Correlation of the apparent bimolecular rate constants for the hydrogen peroxide and cyanide binding reactions for CcP(triVal) and CcP(triLeu), as well as with data from nine other distal pocket mutants of CcP obtained from the literature, indicate a common mechanistic feature, which we attribute to ionization of the two reactants within the distal heme pocket. In wild-type CcP, His52 catalyzes the ionization of both H₂O₂ and HCN to enhance the rates of reaction. In the CcP

triple mutants, there is no catalytic amino acid residue to promote H₂O₂ or HCN ionization and the reaction of these ligands with the heme iron in the triple mutants is very slow. Of all the mutants, CcP(triAla) falls the greatest distance from the correlation line, reacting more rapidly with hydrogen peroxide than predicted from the HCN data suggesting a unique reaction between CcP(triAla) and hydrogen peroxide, perhaps binding of neutral H₂O₂ to the heme iron followed by homolytic cleavage of the peroxide bond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CcP mutants with apolar heme pockets retain detectable catalytic activity.
- CcP mutants react with H_2O_2 10^4 - to 10^7 -times more slowly than wild-type CcP.
- Cyanide binding affinity is 10^3 -fold less than wild-type CcP.
- Rates of the H_2O_2 and HCN reactions correlate over 7 orders of magnitude for 11 CcP mutants.

Fig. 1. The absorption spectra of CcP(triAla) (thick solid line), CcP(triVal) (thin solid line), and CcP(triLeu) (dashed-double dotted line) are shown at pH 6.0 in a 0.10 M ionic strength potassium phosphate buffer, 25 °C. The visible regions of the spectra are expanded by a factor of 5.

Fig. 2. Plot of the extinction coefficient in the Soret region as a function of pH for CcP(triAla), CcP(triVal), and CcP(triLeu). The wavelength for each plot is the wavelength of the Soret maximum at pH 4.0 for each of the mutants: CcP(triAla) 403 nm (filled circles); CcP(triVal) 391 nm (filled triangles); and CcP(triLeu) 396 nm (open circles). The solid lines were calculated assuming that two proton binding/dissociation steps in the CcP mutants affect the electronic absorption spectrum. The low pH transition is attributed to the onset of acid denaturation and the transition near neutral pH affects heme ligation.

Fig. 3. Catalytic activity of the three triple mutants. Plot of the initial velocity (v_0), corrected for the blank rate, for the oxidation of yeast iso-1 ferrocycytochrome α (C102T) by hydrogen peroxide as a function of enzyme concentration: CcP(triAla)-filled circles, CcP(triVal)-filled triangles, and CcP(triLeu)-open circles. Experimental conditions: $[\text{yCyt}c] = 9.0 \mu\text{M}$, $[\text{H}_2\text{O}_2] = 1.00 \text{ mM}$, pH 6.0, 0.10 M ionic strength, potassium phosphate buffer, 25°C.

Fig. 4.

Spectroscopic changes associated with the reaction between H₂O₂ and the CcP triple mutants. Panel A – Spectroscopic changes observed upon mixing 9.9 μ M CcP(triAla) and 100 μ M H₂O₂. Spectra are shown at 1 min intervals for the first 20 min, 2 min intervals between 20 and 60 min, and 5 min intervals between 60 and 120 min. Panel B – Absorbance at 424 nm as a function of time for the reaction between 9.9 μ M CcP(triAla) and 100 μ M H₂O₂, between 9.3 μ M CcP(triVal) and 94 μ M H₂O₂, and between 7.5 μ M CcP(triLeu) and 75 μ M H₂O₂. The traces are labeled for each mutant. Experimental conditions: pH 6.0, 0.10 M ionic strength potassium phosphate buffer, 25 °C.

Fig. 5.

Spectroscopic changes associated with binding of cyanide to the CcP triple mutants. Panel A – Spectrophotometric titration of 7.15 μM CcP(triLeu) between 0 and 97.5 mM cyanide at pH 7.0. The Soret maximum shifts from 408 nm in the absence of cyanide to 419 nm in the presence of 97.5 mM cyanide. Panel B – Difference spectra of the triple mutants in the presence and absence of saturating concentrations of cyanide. CcP(triLeu) in the presence and absence of 97.5 mM cyanide (solid line), CcP(triVal) in the presence and absence of 97.5 mM cyanide (dashed line), and CcP(triAla) in the presence and absence of 48.8 mM cyanide (dashed-double dotted line).

Fig. 6. Titration of 7.15 μM CcP(triLeu) with cyanide at pH 5.0 and 7.0. The difference in absorbance near 424 and 390 nm is plotted as a function of the cyanide concentration. Actual wavelengths are 424/392 nm for the pH 7.0 data (see Panel B, Figure 5) and 423/386 for the pH 5.0 data. The data were fit either to a single hyperbolic titration equation (pH 7.0) or to a double hyperbolic function (pH 5.0). The best-fit curves are shown by solid lines. The dashed line shows the best-fit to a single hyperbolic titration equation for the pH 5.0 data.

Fig. 7. pH dependence of the equilibrium constant for cyanide binding to the CcP triple mutants. The upper set of curves is for the high-affinity binding phase (K_{DA}) and the lower set of curves is for the low-affinity binding phase (K_{DB}). CcP(triAla) – filled circles, CcP(triVal) – open squares, and CcP(triLeu) – filled triangles. All six data sets were fit to a titration curve involving the ionization of a single acidic group. Best-fit parameters are collected in Table 4.

Fig. 8.

Dependence of the observed rate constants for cyanide binding to the CcP triple mutants as functions of the cyanide concentration. Panel A - The observed rate constant for the fast cyanide binding phase, k_{fast} to CcP(triAla) (solid circles) and CcP(triLeu) (open circles) as a function of the cyanide concentration. The solid lines are calculated from linear least squares regression. Panel B - Cyanide dependence of the observed rate constants for the two binding phases of CcP(triVal). The data for k_{slow} have been multiplied by a factor of 10 in the plot. The solid lines are calculated from nonlinear least squares fitting to Eq. (6) in the text. Experimental conditions: pH 8.0, 0.10 M ionic strength potassium phosphate buffer, 25 °C.

Fig. 9. pH dependence of the association and dissociation rate constants for the fast phase of cyanide binding to CcP(triAla) (k_a – solid circles, k_d – open circles) and the observed rate constant for the slow phase of the cyanide binding reaction (k_{slow} – solid triangles). The solid lines for k_a and k_d are nonlinear least squares fits to a titration curve assuming that a single acid/base group affects cyanide binding. The solid line through k_{slow} data represents the average value over the pH range 4 to 8.

Fig. 10.

Correlation between the bimolecular rate constant (k_1) for the reaction of hydrogen peroxide with wild-type CcP and several CcP distal pocket mutants and the apparent association constant (k_a) for HCN binding to these same CcP variants. Experimental conditions: 0.100 M ionic strength potassium phosphate buffer, pH 6.0, 25 °C. Data from this study and references [7, 8, 11, 15, 26, 33-35, 39, 40].

Scheme 1.

Scheme 2.

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Table 1

Spectroscopic parameters for the CcP triple mutants and their cyanide complexes at pH 6.

Protein	protein λ (e) b	δ band λ (e) b	Soret band λ (e) b	CT2 band ^a λ (e) b	β band λ (e) b	α band λ (e) b	CT1 band ^a λ (e) b
rCcP	282 (76)	374 (60sh)	408 (101)	504 (11.2)	542 (9.2sh)	590 (3.7sh)	641 (3.5)
CcP(triAla)	282 (72)	382 (74sh)	406 (110)	502 (11.0)	540 (8.2sh)	596 (2.9sh)	642 (3.5)
CcP(trnVal)	280 (68)	378 (59sh)	406 (76)	515 (9.2)	550 (5.9sh)	590 (3.7sh)	640 (2.7sh)
CcP(triLeu)	282 (78)	382 (85sh)	400 (93)	504 (12.1)	538 (9.4sh)	596 (3.4sh)	646 (4.0)
CcP(triAla)CN		357 (42)	418 (121)		541 (16)	~570 (11.4sh)	
CcP(trnVal)CN		358 (31)	418 (102)		541 (12)	~570 (8.7sh)	
CcP(triLeu)CN		358 (37)	419 (99)		542 (13)	~570 (9.1sh)	

^a CT1 and CT2 represent charge transfer bands.

^b λ , wavelength in nm. e, extinction coefficient in $\text{mM}^{-1} \text{cm}^{-1}$; sh follows the value of e if the band appears as a shoulder.

Table 2

pH dependence of the CcP triple mutant spectra: apparent pK_A values for the transitions.

Protein	pK_{A1}^a	pK_{A2}^b
yCcP ^c	< 3.5	8.7 ± 0.2
CcP(triAla)	~3.7	6.2 ± 0.2
CcP(triVal)	~3.5	6.2 ± 0.3
CcP(triLeu)	~3.3	7.1 ± 0.2

^aEstimated pK_A for acid denaturation.

^b pK_A for the high-spin to low-spin transition.

^cData from references [26].

Table 3
Steady-state and transient-state kinetics of the triple mutant reactions with H₂O₂ and yeast cytochrome *c*^a

Protein	steady-state kinetics		transient-state kinetics				
	TN ^b (s ⁻¹)	TN _{max} (s ⁻¹)	K _M (μM)	k _f ^c (M ⁻¹ s ⁻¹)	k _f ^c (M ⁻¹ s ⁻¹)	k _{oxf} ^d (s ⁻¹)	k _{ox2} ^d (s ⁻¹)
CcP(triAla)	0.091 ± 0.003	0.20 ± 0.02	4.8 ± 1.6	7,800 ± 1,300	ND ^e	(2.9 ± 0.8) × 10 ⁻³	(3.8 ± 0.8) × 10 ⁻⁴
CcP(triVal)	0.0058 ± 0.0003			11 ± 1		(2.5 ± 2.4) × 10 ⁻³	(2.3 ± 0.9) × 10 ⁻⁴
CcP(triLeu)	0.0057 ± 0.0002			4.6 ± 0.2		(2.1 ± 1.0) × 10 ⁻³	(3.8 ± 1.7) × 10 ⁻⁴
yCcP	1,900 ^f 680 ^g	7,000 ^f 880 ^g	25 ^f 2.6 ^g	6 × 10 ⁷ ^f	(4.5 ± 0.3) × 10 ⁷ ^h	(1.3 ± 0.3) × 10 ⁻³ ⁱ	(2.9 ± 0.6) × 10 ⁻⁵ ⁱ
rCcP	520 ^j	640 ^j	2.1 ^j	(4.8 ± 0.2) × 10 ⁷ ^j			
CcP(MI)	640 ^M	900 ^M	3.7 ^M	(4.7 ± 0.4) × 10 ⁷ ^l			~6 × 10 ⁻⁵ ^m

^aExperimental Conditions: pH 6.0, 0.100 M ionic strength, potassium phosphate buffer.

^bat [H₂O₂] = 1.0 mM. [yCytC] = 9.0 μM.

^cRate of CcPI formation, eq 2.

^dRate of oxidative heme degradation.

^eReaction not detected.

^fpH 6.0, 0.20 M ionic strength, ref [28].

^gpH 7.5, 0.10 M ionic strength, ref [29]

^href [26].

ⁱref [41].

^jpH 7.5, 0.10

^Mionic strength, ref [27].

^kvalues interpolated from data in ref [30].

^lref [32].

^mref [42].

Table 4

pH-independent equilibrium dissociation constants for the high- and low-affinity cyanide binding phases of CcP(triAla), CcP(triVal), and CcP(triLeu).^a

Parameter	CcP(triAla)	CcP(triVal)	CcP(triLeu)
K_{DA}^{acid}	14 ± 4	3.7 ± 0.7	7.6 ± 1.4
K_{DA}^{base}	0.23 ± 0.07	0.15 ± 0.03	0.26 ± 0.08
pKA2	6.9 ± 0.2	7.0 ± 0.2	7.2 ± 0.2
K_{DB}^{acid}	860 ± 360	290 ± 90	360 ± 170
K_{DB}^{base}	1.3 ± 0.1	ND	ND
pKA2	7.4 ± 0.3	ND	ND
K_{DB}^{base} / K_{A2}	$(3.4 \pm 2.5) 10^4$	$(4.7 \pm 1.8) 10^4$	$(5.1 \pm 2.2) 10^4$

^aThe parameters for the high-affinity binding phase are defined in Eq. 5 of the text with analogous parameters for the low-affinity binding phase. Experimental conditions: pH 4 to 8,