Oxidation of the His-52 → Leu Mutant of Cytochrome *c* Peroxidase by *p*-Nitroperoxybenzoic Acid: Role of the Distal Histidine in Hydroperoxide Activation†

Amitha H. Palamakumbura, Lidia B. Vitello, and James E. Erman*

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

Received June 29, 1999; Revised Manuscript Received September 16, 1999

ABSTRACT: Both cytochrome *c* peroxidase (CcP) and a mutant cytochrome *c* peroxidase in which the distal histidine has been replaced by leucine, CcP(H52L), are converted to hydroxy-ligated derivatives at alkaline pH. In CcP, the hydroxy-ligated derivative is subsequently converted to a bis-imidazole species prior to protein denaturation while the initial hydroxy-ligated CcP(H52L) is converted to a second, spectroscopically distinct hydroxy-ligated species prior to denaturation. The spectra of the alkaline forms of CcP and CcP(H52L) have been determined between 310 and 700 nm. The pH dependence of the rate of reaction between CcP(H52L) and hydrogen peroxide has been extended to pH 10. The hydroxy-ligated form of CcP(H52L) reacts with hydrogen peroxide 4 times more rapidly than the pentacoordinate, high-spin form of CcP(H52L) that exists at neutral pH. The rate of the reaction between *p*-nitroperoxybenzoic acid and CcP(H52L) has been measured between pH 4 and pH 8. Neutral *p*-nitroperoxybenzoic acid reacts with CcP(H52L) 10^5 times more slowly than with CcP while the negatively charged *p*-nitroperoxybenzoate reacts with CcP(H52L) 10^3 times more slowly than with CcP. These data indicate that the role of the distal histidine during the initial formation of the peroxy anion/heme iron complex is not simply base catalysis.

Heme proteins perform many functions within living organisms including transport and storage of oxygen; sensing of small diatomic molecules such as O_2, CO, and NO; and oxidation of a wide variety of organic and inorganic compounds utilizing either oxygen or hydrogen peroxide as oxidants. The differential reactivity of heme proteins has been attributed to the amino acid residues surrounding the heme binding site. Crystallographic structures show that many heme proteins contain a histidine residue within the distal heme pocket (*1*, *2*), and this residue has been implicated in the reactivity of various heme proteins. Postulated roles of the distal histidine range from stabilization of heme-bound ligands to base catalysis in promoting reaction of hydroperoxides (*3*, *4*). With the development of site-directed mutagenesis, numerous studies of distal histidine mutants have been undertaken in efforts to clarify the role of the distal histidine.

The reaction that distinguishes the peroxidases from other heme proteins is the very rapid oxidation of the ferric state of the enzyme to an enzyme intermediate called compound I (*5*). Replacement of the distal histidine in the peroxidases with a nonpolar amino acid residue decreases the rate of reaction with hydrogen peroxide by 5 orders of magnitude (*6*, *7*). Poulos and Kraut have postulated a mechanism for the reaction between cytochrome *c* peroxidase (CcP)[1] and hydrogen peroxide based on the differences in the active site structures of CcP and metmyoglobin (*3*). The mechanism involves base catalysis by the distal histidine promoting the binding of the peroxy anion to the heme iron, followed by heterolytic oxygen−oxygen bond cleavage of the bound peroxide.

If the primary role of the distal histidine in CcP is to assist in the removal of a proton from the reacting hydrogen peroxide to promote binding of the peroxide anion to the heme iron, then a reactant that does not require proton removal to bind to the heme iron should not need the distal histidine for rapid reaction with the heme iron. Frew and Jones found that both peroxybenzoic acids and their anions oxidized CcP to compound I (*8*). The rate of reaction of the neutral peroxyacids and the peroxyacid anions with CcP were about 2 and 20 times slower than the reaction of CcP with hydrogen peroxide. In the preceding paper (*9*), we have shown that the most probable cause for the differential CcP oxidation rates for *p*-nitroperoxybenzoic acid (pNPBA) and its anion is diffusion through the protein matrix. In this paper, we investigate the role of the distal histidine in CcP. If the primary role of the distal histidine is base catalysis, we predict that the rate of reaction between CcP(H52L) and pNPBA will be significantly slower than the reaction between CcP and pNPBA and that the reaction rate of the pNPBA anion will be essentially the same for both CcP and CcP(H52L) since the rate of binding of the pNPBA anion to the heme iron cannot be assisted by base catalysis.

† This work was supported in part by grants from the National Science Foundation (MCB 95-13047) and from the National Institutes of Health (R15 GM54328-01).

* To whom correspondence should be addressed.

[1] Abbreviations: CcP, cytochrome *c* peroxidase; CcP(H52L), cytochrome *c* peroxidase with His-52 replaced by Leu; pNPBA, *p*-nitroperoxybenzoic acid.

10.1021/bi991498o CCC: $18.00
Published on Web 11/05/1999

Table 1: Observed Rate Constants for the Alkaline Transitions in CcP(H52L)[a]

final pH	k_A^{obs} (s^{-1})	k_B^{obs} (s^{-1})	k_C^{obs} (s^{-1})	k_D^{obs} (s^{-1})
8.0	20 ± 4	0.14 ± 0.03		
8.6	62 ± 50	0.58 ± 0.12	0.057 ± 0.011	
9.4	220 ± 50	0.71 ± 0.14	0.12 ± 0.02	
10.1	420 ± 80	2.9 ± 0.6	0.35 ± 0.07	
11.2	>500	16 ± 3	2.3 ± 0.5	0.43 ± 0.09
11.6	>500	18 ± 4	3.7 ± 0.7	2.5 ± 0.5

[a] pH-jump experiments from pH 6 to final pH in 0.10 M ionic strength buffers, 25 °C.

MATERIALS AND METHODS

Preparation of CcP, CcP(H52L), pNPBA, and buffer solutions and stopped-flow techniques have been described previously (*6*, *9*). The investigation of the alkaline transitions in CcP and CcP(H52L) were performed using pH-jump techniques with an Applied Photophysics DX.17MV stopped-flow spectrophotometer equipped with a diode-array detector.

RESULTS

Spectroscopic Properties of CcP(H52L) at Alkaline pH. The UV/visible spectrum of CcP(H52L) is dependent upon time and pH at alkaline pH. The spectroscopic properties of CcP(H52L) between pH 4 and pH 8 have been reported previously (*6*). At neutral pH, the spectrum of CcP(H52L) is characteristic of a pentacoordinate, high-spin ferric heme protein. As the pH increases to pH 8, the enzyme is partially converted to a hexacoordinate low-spin form of the enzyme. We have extended the investigation of the spectroscopic properties of CcP(H52L) into the alkaline pH region (pH 8−11.6) by using pH-jump techniques. Depending upon the final pH, up to four kinetic processes are observed. The simplest mechanism to explain these results involves the sequential conversion of the enzyme into four spectroscopically distinct species as illustrated in eq 1:

$$\mathrm{N} \underset{k_{-A}}{\overset{k_A}{\rightleftharpoons}} \mathrm{A} \underset{k_{-B}}{\overset{k_B}{\rightleftharpoons}} \mathrm{B} \underset{k_{-C}}{\overset{k_C}{\rightleftharpoons}} \mathrm{C} \underset{k_{-D}}{\overset{k_D}{\rightleftharpoons}} \mathrm{D} \qquad (1)$$

In eq 1, N represents the pentacoordinate, high-spin enzyme present at neutral pH. A and B represent two spectroscopically distinct hexacoordinate, low-spin forms of the enzyme while C and D represent denatured forms of the enzyme.

Values of the observed rate constants for formation of the alkaline species of CcP(H52L) are given in Table 1. Between pH 8 and pH 10, conversion of the native form to species A can be observed with a rate equal to k_A^{obs}. Above pH 11, A is formed within the mixing time (~2 ms) of the stopped-flow apparatus. Species A is converted to B followed by the unfolding of the enzyme to give C and D. The enzyme is completely denatured within 10 s above pH 11 to give D. The spectrum of A is that observed immediately after mixing CcP(H52L) with buffers giving a final pH of greater than 11. The spectrum of D is that after the cessation of all kinetic processes. The spectra of B and C can be calculated from the time dependence of the spectroscopic changes, assuming that the reactions are sequential and essentially irreversible above pH 11 (*10*). The spectra of A, B, and D along with that of the neutral form of CcP(H52L), N, are shown in Figure 1. To simplify Figure 1, the spectrum of C is not shown. The spectrum of C is similar to that of D but with a slightly higher absorption between 380 and 420 nm. The positions and extinction coefficients of the principal bands for N, A, and B are given in Table 2.

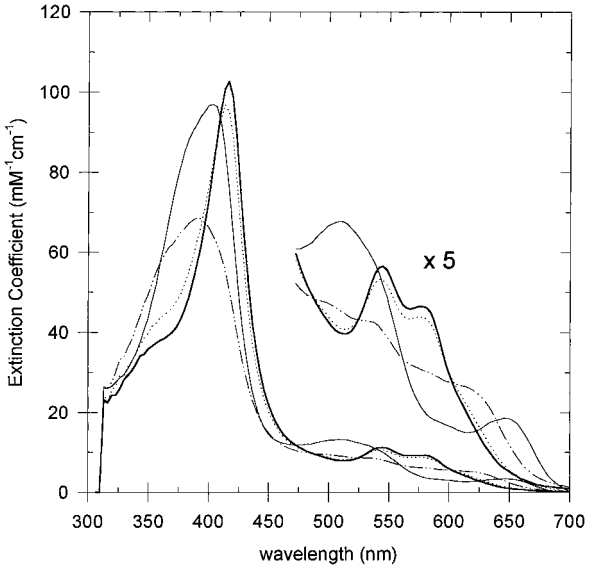

FIGURE 1: Spectra of CcP(H52L) at neutral and alkaline pH. Thin solid line, neutral form of CcP(H52L), species N. Thick solid line, initial alkaline form of CcP(H52L), species A. Dotted line, secondary alkaline form of CcP(H52L), species B. Dashed−dotted line, denatured form of CcP(H52L), species D. Spectra of species A, B, and D determined from the time dependence of the spectroscopic changes at pH 11.2 using a diode-array detector with 3.3 nm resolution. The visible region of the spectra are shown expanded 5-fold.

Wild-type yeast CcP undergoes similar alkaline transitions and has been previously investigated using stopped-flow techniques (*10*). In the previous study, rudimentary spectra of A−D between 350 and 450 nm were constructed point by point using single wavelength data. For comparison with the CcP(H52L) data, we have reinvestigated the spectroscopic properties of the alkaline denaturation of wild-type yeast CcP at pH 11.7 using the diode-array detector equipped stopped-flow spectrophotometer. Spectra of wild-type yeast CcP at pH 6 and of A, B, and D acquired at pH 11.7 for the wild-type enzyme are shown in Figure 2. The positions of the principal bands and extinction coefficients are given in Table 2.

Reaction of pNPBA with CcP(H52L). The reaction of pNPBA with CcP(H52L) is significantly slower than the reaction with CcP (*9*). The reaction between CcP(H52L) and pNPBA was investigated using manual mixing and a diode-array spectrophotometer between pH 5 and pH 7. Figure 3 shows the spectroscopic changes in the Soret region upon mixing 4.3 μM CcP(H52L) and 143 μM pNPBA at pH 6. Compound I is formed over the first 60 s (Figure 3A) followed by significant bleaching of the Soret band over the succeeding 20 min (Figure 3B). The bleaching of the Soret band is due to the oxidative degradation of the heme by pNPBA. pNPBA appears to degrade the heme group in CcP(H52L) to a much greater extent than in wild-type CcP (*9*). Below pH 5 and above pH 7, stopped-flow techniques were used to measure the rate of reaction between CcP(H52L) and pNPBA.

The pseudo-first-order rate constant for the reaction between pNPBA and CcP(H52L) is linearly dependent upon

Table 2: Spectroscopic Properties of the Neutral and Alkaline Forms of CcP and CcP(H52L)[a]

protein	species	Soret	CT1	β	α	CT2
CcP	N	408 (98)	507 (13.0)			643 (4.7)
CcP(H52L)	N	403 (97)	509 (13.2)			647 (3.4)
CcP	A	419 (101)		545 (11.3)	578 (9.5)	
CcP(H52L)	A	416 (103)		545 (11.3)	576 (9.3)	
CcP	B	413 (117)		537 (10.1)	575 (7.9) sh	
CcP(H52L)	B	414 (97)		543 (10.7)	576 (8.8)	

[a] Data obtained by pH-jump studies using a stopped-flow spectrometer equipped with a diode-array detector with 3.3 nm resolution. Values in parentheses are millimolar extinction coefficients.

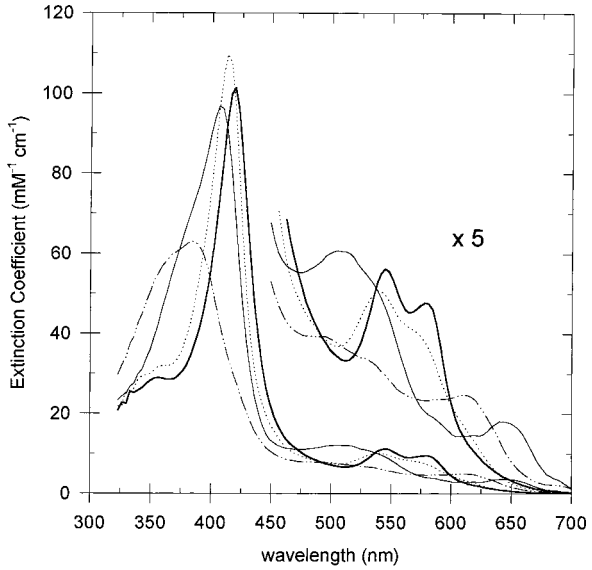

FIGURE 2: Spectra of CcP at neutral and alkaline pH. Thin solid line, neutral form of CcP, species N. Thick solid line, initial alkaline form of CcP, species A. Dotted line, secondary alkaline form of CcP, species B. Dashed−dotted line, denatured form of CcP, species D. Spectra of species A, B, and D determined from the time dependence of the spectroscopic changes at pH 11.7 using a diode-array detector with 3.3 nm resolution. The visible region of the spectra are shown expanded 5-fold.

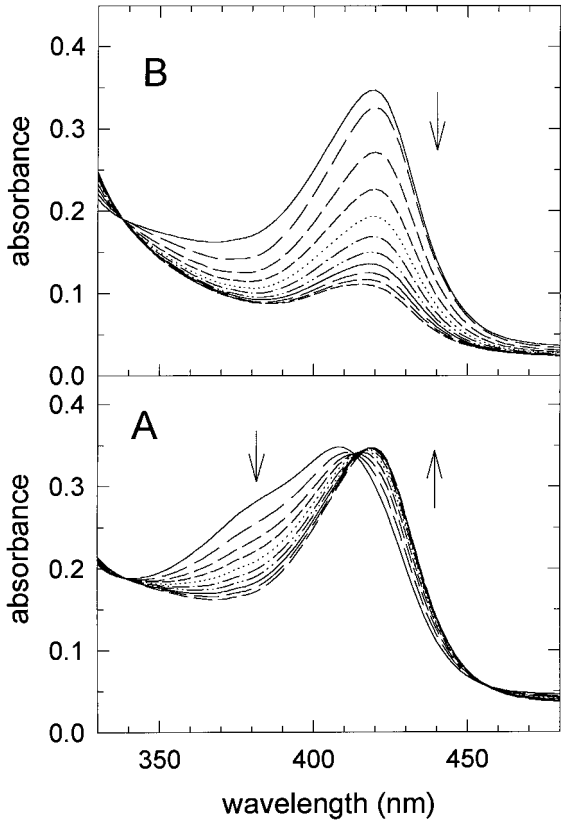

FIGURE 3: Spectroscopic changes upon mixing CcP(H52L) with *p*-nitroperoxybenzoic acid at pH 6.0. (A) Formation of compound I. Spectra were collected at 10-s intervals for the first 90 s after mixing 4.3 μM CcP(H52L) with 143 μM pNPBA. The spectrum of the high-spin Fe(III) form of the enzyme (Soret maximum 403 nm) is converted to compound I (Soret maximum 420 nm). (B) Oxidative degradation of the heme in CcP(H52L) compound I by pNPBA. Following formation of compound I, the Soret band is bleached due oxidation of the heme group. Spectra were taken at 100-s intervals for about 18 min.

the pNPBA concentration, and apparent bimolecular rate constants were determined from slopes of plots of the observed rate constant versus the pNPBA concentration. The intercepts of plots of k^{obs} versus pNPBA were zero within experimental error between pH 7 and pH 8 but had finite positive intercepts between pH 4 and pH 6.5. The pH dependence of the apparent bimolecular rate constant for the reaction of CcP(H52L) and pNPBA between pH 4 and pH 8 is shown in Figure 4 along with similar data for the reaction of pNPBA with yeast CcP (*9*).

Attempts to measure the rate of the reaction between CcP(H52L) and pNPBA above pH 8 were unsuccessful. When CcP(H52L), in a weakly buffered solution at pH 8, is rapidly mixed with highly buffered alkaline solutions containing between 33 and 520 μM pNPBA, an initial increase in absorbance at 424 nm was observed followed by a decrease in absorbance. The initial rate was independent of the pNPBA concentration and was identical to k_A^{obs} for the conversion of neutral CcP(H52L) to the hydroxy-ligated form, the N to A transition described in eq 1 above. The decrease in absorbance is due to the oxidative degradation of the heme by pNPBA.

Reaction of H_2O_2 with CcP(H52L). We have been able to extend the previous investigation of the reaction of hydrogen peroxide with CcP(H52L) (*6*) to about pH 10 using pH-jump techniques. Rapid mixing of CcP(H52L) with millimolar concentrations of hydrogen peroxide above pH 8.5 leads to a rapid conversion of N to A (eq 1) followed by oxidation of the enzyme to compound I and the subsequent decay of compound I. Using between 0.6 and 3.5 mM hydrogen peroxide, the pseudo-first-order rate constant for formation of compound I is faster than the conversion of species A to B between pH 8.5 and pH 10. The apparent bimolecular rate constant for the CcP(H52L)/hydrogen peroxide as a function of pH is shown in Figure 5 along with the data for yeast CcP for comparison.

DISCUSSION

Spectroscopic Properties of CcP and CcP(H52L) at Alkaline pH. Yeast CcP is stable between pH 4 and pH 8.

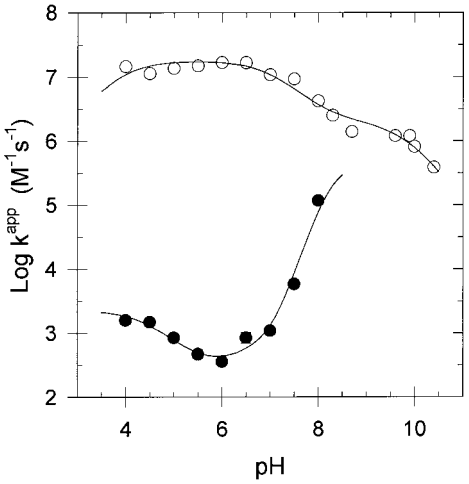

FIGURE 4: pH dependence of the apparent bimolecular rate constants for the oxidation of CcP and CcP(H52L) by *p*-nitroperoxybenzoic acid. ●, CcP(H52L). ○, CcP, data from ref *9*. The ionic strength of the buffers were adjusted to 0.1 M using potassium phosphate salts, 25 °C. Solid lines are calculated from mechanisms discussed in the text.

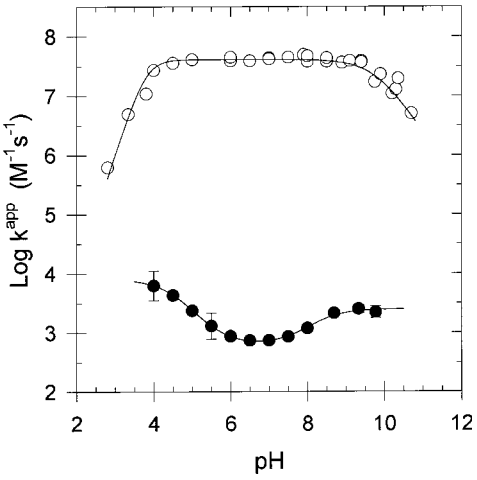

FIGURE 5: pH dependence of the apparent bimolecular rate constants for the oxidation of CcP and CcP(H52L) by hydrogen peroxide. ●, CcP(H52L), data between pH 4 and pH 8 from ref *6*. ○, CcP, data from this study and from ref *6*. The ionic strength of the buffers were adjusted to 0.1 M using potassium phosphate salts, 25 °C. Solid lines are calculated from mechanisms discussed in the text.

Above pH 8, the enzyme undergoes a complex series of spectroscopic transformations as it ultimately denatures. Denaturation of the enzyme is dependent upon time and pH. Physicochemical measurements on CcP samples incubated for 24 h at various pH values indicate that the midpoint for loss of tertiary structure is pH 10.3 ± 0.1 (*11*). Spectroscopic studies of the alkaline denaturation of CcP indicate that, as CcP unfolds, the spectrum of the enzyme is ultimately converted to one characteristic of free heme in solution (*10*). The midpoint for conversion of the spectrum to that of free heme is 10.8 ± 0.3 when the spectrum is measured within 3 min of exposure to alkaline pH.

In this study, we have used a stopped-flow spectrophotometer equipped with a diode-array detector in order to evaluate the spectroscopic changes that occur in alkaline solution for both CcP and CcP(H52L). Up to four kinetic processes are observed within the first minute of exposure of both CcP and CcP(H52L) to alkaline pH. There are additional slow spectroscopic changes that can occur over the period of hours depending upon the final pH (*10*). The simplest mechanism to explain the fast (less than 1 min) spectroscopic changes is that shown in eq 1. The reactions are reversible with the equilibrium lying toward the neutral form of the enzyme, N, at low pH and toward the denatured enzyme, D, at high pH. Above pH 11, all four processes are complete within 10 s and both CcP and CcP(H52L) are completely denatured. Assuming that each step in the kinetic process is essentially irreversible above pH 11, one can solve the kinetic equations and calculate the spectrum of each species as it evolves in time. The spectra of N, A, B, and D are shown in Figure 1 for CcP(H52L) and in Figure 2 for yeast CcP.

The spectroscopic properties of the neutral forms, N, of both CcP and CcP(H52L) indicate that the heme in these species is pentacoordinate and high-spin (Figures 1 and 2; Table 2). The high-spin forms are characterized by a Soret maximum less than 410 nm and charge-transfer bands (CT1 and CT2) near 510 and 650 nm (Figures 1 and 2; Table 2).

Species A and B for both CcP and CcP(H52L) are hexacoordinate, low-spin forms of the enzyme as characterized by the Soret maximum above 410 nm, α and *β* bands near 570 and 540 nm, respectively, and the lack of charge-transfer bands (Figures 1 and 2; Table 2). The spectroscopic properties of A for both yeast CcP and CcP(H52L) are similar to each other, both in terms of the band positions and extinction coefficients. Comparing the positions of the α and *β* bands of the two A species with those of authentic hydroxy-ligated ferric heme protein strongly suggest that these are both hydroxy-ligated heme species (*12*).

Conversion of A to B in CcP causes significant changes in the spectrum. Both the Soret and *β* bands shift to the blue, 6 and 8 nm, respectively, and the extinction coefficient of the Soret band increases by 16%. The α band shifts about 3 nm to the blue with a decrease in extinction so that it appears as a shoulder on the *β* band rather than a distinct peak. The spectroscopic properties of B are those characteristic of bis-imidazole forms of ferric heme proteins (*12*).

Species B for CcP(H52L) is quite different than B for CcP. In fact, B for CcP(H52L) is almost identical to the initial hydroxy-ligated form of CcP(H52L). There is a small blue shift of the Soret and *β* bands and a small decrease in the extinction coefficient of all three principal bands during the conversion of CcP(H52L) A to B. We conclude that CcP(H52L) species B is a second hydroxy-ligated form of the enzyme, one which has a different conformation within the heme binding site. The small spectroscopic differences between CcP(H52L) species A and B may be due to changes in solvation of the heme group. CcP(H52L) lacks the distal histidine that coordinates to the heme iron in yeast CcP species B and so cannot form the bis-imidazole species seen in yeast CcP.

Conversion of N to A is pH dependent. An apparent pK_A for the transition can be calculated from the spectroscopic data. The apparent pK_A for the N to A transition in CcP(H52L) is 9.4 ± 0.1 (this study) and 9.7 ± 0.2 in yeast CcP (*10*). In yeast CcP, the distal histidine displaces the hydroxide ligand, converting A to B. In CcP(H52L), the A to B conversion stabilizes the hydroxy-ligated form by a factor of about 20, and the apparent pK_A for conversion of pentacoordinate CcP(H52L) to B is 8.2 ± 0.2. Note that

Table 3: Kinetic Parameters for the Reaction of Hydrogen Peroxide with CcP and CcP(H52L)[a]

parameter	CcP	CcP(H52L)
k_1 (M^{-1} s^{-1})	NA[b]	$(8.1 \pm 0.7) \times 10^3$
k_2 (M^{-1} s^{-1})	$(4.1 \pm 0.2) \times 10^7$	$(6.3 \pm 0.3) \times 10^2$
k_3 (M^{-1} s^{-1})	NA	$(2.5 \pm 0.1) \times 10^3$
n	2	1
pK_{E1}	3.8 ± 0.1	4.5 ± 0.1
pK_{E2}	9.8 ± 0.1	8.3 ± 0.1

[a] Parameters are defined in eq 2 of the text. [b] Parameter does not make a contribution to the observed rate constant.

species A in both CcP and CcP(H52L) is transitory, giving rise to the more stable B before undergoing denaturation.

pH Dependence of the Reaction of H_2O_2 with CcP and CcP(H52L). The pH dependence of the reaction of hydrogen peroxide with yeast CcP and with CcP(H52L) is shown in Figure 5. Two apparent ionizations in the enzyme influence the rate of reaction for both CcP and CcP(H52L) with hydrogen peroxide. The pH dependence of the observed rate constant for the reaction of both CcP and CcP(H52L) with hydrogen peroxide can be fit to

$$k^{app} = \frac{k_1\dfrac{[H^+]^n}{K_{E1}^n} + k_2 + k_3\dfrac{K_{E2}}{[H^+]}}{\dfrac{[H^+]^n}{K_{E1}^n} + 1 + \dfrac{K_{E2}}{[H^+]}} \quad (2)$$

Values of the best-fit parameters are shown in Table 3.

The reaction between hydrogen peroxide and CcP is dependent upon two apparent ionizations in the protein. Yeast CcP is most reactive between pH 4 and pH 9 with a second-order rate constant of $(4.1 \pm 0.2) \times 10^7$ M^{-1} s^{-1}. CcP has no detectable reactivity when the group associated with pK_{E1} is protonated and when the group associated with pK_{E2} is unprotonated. The apparent ionization that occurs at low pH, pK_{E1}, has been associated with the distal histidine (*6*). Yeast CcP also undergoes rapid denaturation in this pH region, and the data below pH 4 were obtained using pH-jump techniques. To fit the apparent second-order rate constant below pH 4, a cooperative two-proton binding step must be associated with the pK_{E1} transition. In eq 2, the value of n is equal to 2 for CcP. The second protein ionization occurs at alkaline pH and has a pK_a value of 9.8 (*13*). This second ionization correlates with conversion of CcP to the hydroxy-ligated species, A, indicating that hydroxy-CcP is much less reactive than the pentacoordinate form that exists at neutral pH.

The pH dependence of the reaction between CcP(H52L) and hydrogen peroxide is quite different than that of CcP and hydrogen peroxide. In the neutral pH region, CcP(H52L) is about 5 orders of magnitude less reactive than CcP indicating the importance of the distal histidine in catalyzing the reaction between CcP and hydrogen peroxide (*6*). Protonation of a group with pK_{E1} of 4.5 ± 0.1 in CcP(H52L) increases the rate by a factor of 13. We have speculated that this group may be Asp-235 or a buffer anion bound in the heme pocket (*6*). At alkaline pH, a group with pK_{E2} of 8.3 $\pm$ 0.1 increases the rate by a factor of 4. The apparent ionization that increases the rate of the hydrogen peroxide reaction is identical to the apparent pK_A for conversion of CcP(H52L) N to B. This suggests that, in the absence of the distal histidine, the hydroxide ion ligated to the ferric heme iron can assist in the reaction between hydrogen peroxide and the mutant enzyme.

Table 4: Kinetic Parameters for the Reaction of *p*-Nitroperoxybenzoic Acid with CcP and CcP(H52L)[a]

parameter	CcP	CcP(H52L)
k_1 (M^{-1} s^{-1})	NA[b]	$(2.3 \pm 0.6) \times 10^3$
$(k_2 + k_4K_S/K_{E1})$ (M^{-1} s^{-1})	$(1.6 \pm 0.2) \times 10^7$	$(2.9 \pm 1.6) \times 10^2$
$(k_3K_{E2}/K_S + k_5)$ (M^{-1} s^{-1})	$(1.6 \pm 0.2) \times 10^6$	$(1.6 \pm 1.0) \times 10^3$
k_6 (M^{-1} s^{-1})	NA	$(4.3 \pm 1.2) \times 10^5$
n	1	2
pK_{E1}	3.8	4.5
pK_{E2}	9.8	8.3

[a] Parameters are defined in eq 3 of the text. [b] Parameter does not make a contribution to the observed rate constant.

pH Dependence of the Reaction of pNPBA with CcP and CcP(H52L). The pH dependence of the reaction between pNPBA and both CcP and CcP(H52L) are show in Figure 4. The data for the CcP/pNPBA reaction is from the accompanying paper (*9*). CcP is only reactive when the pK_{E1} group is unprotonated and the pK_{E2} group is protonated. The inflection near pH 7 is due to the ionization of pNPBA to the peroxybenzoate anion.

The pH dependence of the reaction between pNPBA and CcP(H52L) (Figure 4) is similar to the pH dependence of the reaction between hydrogen peroxide and CcP(H52L) (Figure 5) in that the rate has a minimum rate near neutral pH and becomes faster at the pH extremes. The pH dependence of the pNPBA reaction is more complex than the reaction with hydrogen peroxide because there are two forms of the substrate that can react with three forms of the enzyme, giving six potential reactions. The pH dependence of the reaction of pNPBA with both CcP and CcP(H52L) was fit to eq 3 (*9*).

$$k^{app} = \frac{k_1\dfrac{[H^+]}{K_{E1}} + \left(k_2 + k_4\dfrac{K_S}{K_{E1}}\right) + \left(k_3\dfrac{K_{E2}}{K_S} + k_5\right)\dfrac{K_S}{[H^+]} + k_6\dfrac{K_{E2}^nK_S}{[H^+]^{n+1}}}{\left(\dfrac{[H^+]}{K_{E1}} + 1 + \dfrac{K_{E2}^n}{[H^+]^n}\right)\left(\dfrac{K_S}{[H^+]} + 1\right)} \quad (3)$$

The rate constants k_1, k_2, and k_3 refer to the reaction of neutral pNPBA with the acidic, neutral, and alkaline forms of the enzyme, respectively, and rate constants k_4, k_5, and k_6 refer to the reaction of the peroxybenzoate anion with the acidic, neutral, and alkaline forms of the enzyme, respectively. We assume that the same two protein ionizations in CcP and CcP(H52L) that influence the hydrogen peroxide reaction also influence the reaction with pNPBA. Values for pK_{E1} and pK_{E2} were fixed at the values obtained for the hydrogen peroxide reaction (Table 3), and pK_S was fixed at its literature value of 7.1 (*8*). The best-fit values for the remaining four parameters are collected in Table 4.

For the pNPBA/CcP reaction, k_1 and k_6 do not contribute to the observed rate constant. If we assume that the acid and alkaline forms of CcP have negligible reactivity toward both pNPBA and its anion just as for hydrogen peroxide, then k_3 and k_4 will also be negligible. Under these conditions

only k_2 and k_5 contribute to the observed rate constant, and their values are $(1.6 \pm 0.2) \times 10^7$ and $(1.6 \pm 0.2) \times 10^6$ M^{-1} s^{-1}, respectively.

For the pNPBA/CcP(H52L) reaction, all four rate parameters contribute to the observed rate constant. In fitting the CcP(H52L) data, the ionization specified by pK_{E2} is a cooperative two-proton ionization, and the value of n in eq 3 is equal to 2 for these data (Table 4).

Mechanism of Compound I Formation. The preponderance of evidence indicates that oxidation of peroxidases to compound I is a sequential two-step mechanism in which the peroxide anion binds to the heme iron followed by O−O bond cleavage to form compound I (eq 4):

$$\mathrm{CcP} + \mathrm{ROOH} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{HCcP{\cdot}OOR} \xrightarrow{k_c} \mathrm{CcP{\cdot}I} + \mathrm{ROH} \quad (4)$$

Using excess hydroperoxide, the observed pseudo-first-order rate constant is given by eq 5 (15).

$$k^{obs} = \frac{k_c k_a[\mathrm{ROOH}]}{k_a[\mathrm{ROOH}] + k_d + k_c} \quad (5)$$

The O−O bond cleavage step is very fast, and saturation in not observed using standard stopped-flow techniques with wild-type proteins. The limiting slope at low substrate concentrations gives the apparent bimolecular rate constant (eq 6).

$$k^{app} = k_a \frac{k_c}{(k_c + k_d)} \quad (6)$$

Interpretation of the data obtained in this study depends critically upon the relative values of k_c and k_d.

The best estimate of k_c is $\sim 10^5$ s^{-1} based on extrapolation of the cryogenic studies of Balny et al. on the horseradish peroxidase/hydrogen peroxide reaction (*16*). Dissociation of anions from CcP is slow in comparison to 10^5 s^{-1} (*17, 18*) leading to the conclusion that $k_d \ll k_c$ and that k^{app} is a true measure of k_a, the bimolecular rate constant for the binding of hydroperoxides to CcP (eq 6). The rate of O−O bond cleavage, k_c, has no effect on k^{app}.

Effect of His-52 on k_a. The pH dependence of k^{app} allows determination of the rate constants for the reaction of the neutral forms of CcP and CcP(H52L) with both peroxybenzoic acid and its anion, k_2 and k_5. In eq 3, both k_2 and k_5 are part of composite parameters involving two rate and two acid dissociation constants. However, maximum values for the rate constants can be determined. The maximum value of k_2 is 290 M^{-1} s^{-1} (Table 4). Comparing the maximum value of k_2 to the value of 1.6×10^7 M^{-1} s^{-1} for the reaction of pNPBA with CcP (*9*) demonstrates that CcP(H52L) reacts about 5 orders of magnitude more slowly with pNPBA than does CcP and that this decrease is of the same order of magnitude as the decrease observed for the reaction with hydrogen peroxide.

The maximum value of k_5 is 1.6×10^3 M^{-1} s^{-1} (Table 4). Comparing the maximum value of k_5 to the value of 1.6×10^6 M^{-1} s^{-1} observed for the reaction of the *p*-nitroperoxybenzoate anion with CcP (*9*) indicates that the anion reacts at least 3 orders of magnitude more slowly with CcP(H52L) than with CcP. The substantial decrease in rate for the reaction of the peroxybenzoate anion with CcP(H52L) in comparison to CcP suggests that the distal histidine has a more complex role than simple base catalysis to promote binding of hydroperoxides to the heme iron.

Another intriguing aspect of the reactivity of CcP(H52L) is that the hydroxy-ligated form is 4 times more reactive toward hydrogen peroxide and at least 300 times more reactive toward the peroxybenzoate anion than is the neutral pentacoordinate form of CcP(H52L). We have speculated that the increased hydrogen peroxide reactivity is due to base catalysis by the released hydroxide ion (*6*), but this cannot be the case for the peroxybenzoate anion.

REFERENCES

1. Takano, T. (1977) *J. Mol. Biol. 110*, 537−568.
2. Finzel, B. C., Poulos, T. L., and Kraut, J. (1984) *J. Biol. Chem. 259*, 13027−13036.
3. Poulos, T. L., and Kraut, J. (1980) *J. Biol. Chem. 255*, 8199−8205.
4. Poulos, T. L., and Finzel, B. C. (1984) *Peptide Protein Rev. 4*, 115−171.
5. Bosshard, H. R., Anni, A., and Yonetani, T. (1991) in *Peroxidases in Chemistry and Biology, Vol. II* (Everse, J., Everse, K. E., and Grisham, M. B., Eds.) pp 51−84, CRC Press, Boca Raton, FL.
6. Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A., and Kraut, J. (1993) *Biochemistry 32*, 9798−9806.
7. Newmyer, S. L., and Ortiz de Montellano, P. R. (1995) *J. Biol. Chem. 270*, 19430−19438.
8. Frew, J. E., and Jones, P. (1983) *Biochim. Biophys. Acta 742*, 1−8.
9. Palamakumbura, A. H., Foshay, M. C., Vitello, L. B., and Erman, J. E. (1999) *Biochemistry 38*, 15647−15652.
10. Dhaliwal, B. K., and Erman, J. E. (1985) *Biochim. Biophys. Acta 827*, 174−182.
11. Dowe, R. J., and Erman, J. E. (1985) *Biochim. Biophys. Acta 827*, 183−189.
12. Vitello, L. B., Erman, J. E., Miller, M. A., Mauro, J. M., and Kraut, J. (1992) *Biochemistry 31*, 11524−11535.
13. Loo, S., and Erman, J. E. (1975) *Biochemistry 14*, 3467−3470.
14. Erman, J. E., and Vitello, L. B. (1998) *J. Biochem. Mol. Biol. 31*, 307−327.
15. Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J., and Kraut, J. (1993) *Biochemistry 32*, 9807−9818.
16. Balny, C., Travers, F., Barman, T., and Douzou, P. (1987) *Eur. Biophys. J. 14*, 374−378.
17. Erman, J. E. (1974) *Biochemistry 13*, 34−39.
18. Erman, J. E. (1974) *Biochemistry 13*, 39−44.

BI991498O