Oxidation of Cytochrome *c* Peroxidase to Compound I by Peroxyacids: Evidence for Rate-Limiting Diffusion through the Protein Matrix†

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ABSTRACT: The rate of the reaction between *p*-nitroperoxybenzoic acid and cytochrome *c* peroxidase (CcP) has been investigated as a function of pH and ionic strength. The pH dependence of the reaction between CcP and peracetic acid has also been determined. The rate of the reactions are influenced by two heme-linked ionizations in the protein. The enzyme is active when His-52 (pK_a 3.8 ± 0.1) is unprotonated and an unknown group with a pK_a of 9.8 ± 0.1 is protonated. The bimolecular rate constant for the reaction between peracetic acid and CcP and between *p*-nitroperoxybenzoic acid and CcP are (1.8 ± 0.1) × 10^7 and (1.6 ± 0.2) × 10^7 M^{-1} s^{-1}, respectively. These rates are about 60% slower than the reaction between hydrogen peroxide and CcP. A critical comparison of the pH dependence of the reactions of hydrogen peroxide, peracetic acid, and *p*-nitroperoxybenzoic acid with CcP provides evidence that both the neutral and anionic forms of the two peroxyacids react directly with the enzyme. The peracetate and *p*-nitroperoxybenzoate anions react with CcP with rates of (1.5 ± 0.1) × 10^6 and (1.6 ± 0.2) × 10^6 M^{-1} s^{-1}, respectively, about 10 times slower than the neutral peroxyacids. These data indicate that CcP discriminates between the neutral peroxyacids and their negatively charged ions. However, the apparent bimolecular rate constant for reaction between *p*-nitroperoxybenzoate and CcP is independent of ionic strength in the range of 0.01−1.0 M, suggesting that electrostatic repulsion between the anion and CcP is not the cause of the lower reactivity for the peroxybenzoate anion. The data are consistent with the hypothesis that the rate-limiting step for the oxidation of CcP to compound I by both neutral peroxyacid and the negatively charged peroxide ion is diffusion of the reactants through the protein matrix, from the surface of the protein to the distal heme pocket.

One of the reactions that distinguish the major classes of heme proteins is their reaction with hydrogen peroxide. Hydrogen peroxides oxidize peroxidases to an intermediate state called compound I with bimolecular rate constants on the order of 10^7−10^8 M^{-1} s^{-1} (*1*). Metmyoglobin, the ferric form of the oxygen transport/storage heme protein myoglobin, reacts with hydrogen peroxide about 10^5 times more slowly than the peroxidases (*2*). In both instances, hydrogen peroxide oxidizes the ferric heme protein 2 equiv to an oxidized intermediate that contains an oxyferryl, Fe(IV), species and an organic free radical. In the classical peroxidases, such as horseradish peroxidase, the organic radical is a porphyrin π-cation radical (*3*), while in cytochrome *c* peroxidase (CcP)[1] it is an amino acid free radical localized on Trp-191 (*4*). The organic free radical in the oxidized form of metmyoglobin decays significantly more rapidly than those in the peroxidases and is generally thought to be tyrosine radicals, although that is not universally accepted (*5*).

Despite the detailed structural data available for both the peroxidases and the globins (*6, 7*) the reason for the very large difference in reactivity with hydrogen peroxide is still unclear. Both the peroxidases and metmyoglobin have histidine residues in the distal heme pocket that can interact with reactants approaching the heme iron. A major difference between the distal heme pockets of peroxidases and metmyoglobin is the presence of an arginine residue in the peroxidases. Poulos and Kraut (*8*) have postulated a mechanism for the reaction between CcP and hydrogen peroxide based on the differences in the active site structures of CcP and metmyoglobin. 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 (O−O) cleavage of the bound peroxide. The positively charged arginine residue promotes O−O bond cleavage by stabilizing the transition state during the heterolytic cleavage as negative charge develops on the distal oxygen of the bound peroxide. Studies of the reaction between CcP and hydrogen peroxide using CcP variants with distal pocket mutations largely confirm the Poulos/Kraut mechanism (*3, 9, 10*). Replacement of the distal histidine in CcP with a nonpolar leucine residue, CcP(H52L), decreases the rate of reaction with hydrogen peroxide by 5 orders of magnitude to 3 × 10^2 M^{-1} s^{-1} (*9*), about the same rate as that between metmyoglobin and hydrogen peroxide (*2*). On the other hand, replacement of the distal arginine in CcP with a leucine, CcP(R48L), only decreases the observed bimolecular rate constant for the reaction by about 2 orders of magnitude (*10*).

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; PAA, peracetic acid; pNPBA, *p*-nitroperoxybenzoic acid.

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Investigations of the reaction between metmyoglobin and hydrogen peroxide using distal pocket mutations show significantly different behavior (*11*). Replacement of the distal histidine in metmyoglobin with glycine, alanine, threonine, or valine decreases the rate of reaction with hydrogen peroxide by factors of 5, 7.5, 28, and 78, respectively. However, replacement of the distal histidine with a glutamine in metmyoglobin actually increases the rate of reaction with hydrogen peroxide by a factor of 3. Comparison of the mutant studies in CcP and metmyoglobin suggest that the distal histidine in these two heme proteins have very different roles in the reaction with hydrogen peroxide. In CcP, the distal histidine appears to be essential for rapid reaction with hydrogen peroxide, while in metmyoglobin the distal histidine has only a moderate effect.

If the only role of the distal histidine in CcP is to serve as a base catalyst, 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. Peroxyacids provide a system in which the effects of protonation and deprotonation of the reactant can be investigated. Frew and Jones (*12*) have investigated the pH dependence of the reaction between CcP and a series of substituted peroxybenzoic acids. The pK_a values of the substituted peroxybenzoic acids used in the Frew and Jones study vary between 7.10 and 7.91, allowing the investigators to evaluate the reactivity of both the neutral peroxybenzoic acids and the negatively charged peroxybenzoate anions with CcP. Frew and Jones found that neutral substituted peroxybenzoic acids oxidized CcP to compound I at rates that were about a factor of 2 slower than the reaction between CcP and hydrogen peroxide. The substituted peroxybenzoate anions also reacted with CcP, but with rates that were about 10 times slower than the neutral peroxybenzoic acids. Frew and Jones attributed the discrimination between the neutral and charged reactants to an "electrostatic gate" that slowed the rate of access of the anions to the distal heme pocket.

In this paper, we investigate the pH dependence of the reaction of *p*-nitroperoxybenzoic acid (pNPBA) and peracetic acid (PAA) with CcP and the ionic strength dependence of the pNPBA/CcP reaction in order to characterize the importance of electrostatic effects on the rate of reaction between CcP and the negatively charged peroxybenzoate anion. In an accompanying paper (*13*), we investigate the influence of the distal histidine in CcP on the reaction between CcP and both neutral pNPBA and the pNPBA anion.

MATERIALS AND METHODS

Yeast CcP was isolated as previously described (*14*). CcP solutions were prepared by dissolving the crystalline enzyme into appropriate buffers and centrifuged for 20 min at 12000*g* to remove any insoluble material. CcP concentrations were determined spectroscopically using an absorptivity of 98 mM^{-1} cm^{-1} at 408 nm (*14*). PZ values for the samples used in this study ranged between 1.25 and 1.30. Absorbance ratios at 408/380 and 620/647 nm were 1.50 ± 0.01 and 0.74 ± 0.05, respectively, indicating that the CcP preparations were five-coordinate, high-spin Fe(III) forms (*14*).

p-Nitroperoxybenzoic acid was purchased from K & K Laboratories (Cleveland, OH). pNPBA has limited solubility in aqueous solution. Stock solutions were prepared by dissolving the solid material in up to 10% methanol/water and then diluting with water to a final concentration of 3.00 mM. Stock solutions of pNPBA prepared in this manner were stable for at least 4 h. Stability of pNPBA was monitored by titration with CcP. pNPBA is unstable above pH 6, with up to 50% loss within 2 h at pH 7.0.

Peracetic acid (PAA) was purchased from Aldrich (Milwaukee, WI). PAA is in equilibrium with acetic acid and hydrogen peroxide. The rate of equilibration is quite slow (*15*), and hydrogen peroxide can be removed by adding catalase to buffered solutions. PAA solutions (before and after treatment with catalase) were characterized by pH titrations, titrations with CcP, and redox titrations using permanganate and thiosulfate. The pK_a of PAA was determined to be 8.18 ± 0.02 at 25 °C, 0.1 M ionic strength. The stability of PAA was determined at pH values of 5, 7, and 9 with initial rates of decay of 2.5, 22, and 46% h^{-1}, respectively.

The rates of reaction of both pNPBA and PAA with CcP were determined using stopped-flow techniques. Experiments were performed with an Applied Photophysics DX.17MV sequential-mixing stopped-flow spectrophotometer or a Hi-Tech Scientific model SPQ-53 stopped-flow spectrophotometer. For kinetic experiments between pH 4 and pH 8, where CcP is stable, aliquots of a stock solution of pNPBA in water were diluted into an appropriate buffer just before placement into one of the reservoir syringes of the stopped-flow instrument. These samples were mixed with CcP, and the reaction was observed within 2 min of the initial dilution of pNPBA into buffer. Aliquots of PAA were diluted into an appropriate buffer containing between 20 and 25 nM catalase, placed in the stopped-flow, incubated for approximately 1 min to remove hydrogen peroxide, and then mixed with CcP to observe the reaction. All kinetic studies were performed at 25 °C.

For experiments above pH 8, where CcP begins to undergo slow denaturation, double-mixing experiments were performed with the Applied Photophysics sequential-mixing stopped-flow instrument. An appropriate dilution of either pNPBA (in water) or PAA (in 2 mM phosphate buffer, pH 6.2, with catalase) was placed in one of four syringes. The peroxyacid was mixed with an equal volume of an alkaline buffer (pH 8.5−12 at twice the ionic strength of the final solution) contained in a second syringe and incubated for between 0.2 and 1.0 s. After incubation, the alkaline peroxyacid solution was mixed with a pH 8 solution of CcP at an appropriate ionic strength contained in a third syringe, and the reaction was observed. The fourth syringe contained a flushing solution. The pH of the solution after mixing all components was determined experimentally.

All reactions were carried out under pseudo-first-order conditions with the peroxyacid in excess. The observed pseudo-first-order rate constants, k^{obs}, increased linearly with increasing peroxyacid concentration, and the apparent second-order rate constants, k^{app}, were determined from the slope of plots of k^{obs} versus peroxyacid concentration.

RESULTS

pH Dependence of the Reaction of pNPBA and PAA with CcP. Both pNPBA and PAA rapidly oxidize CcP to CcP

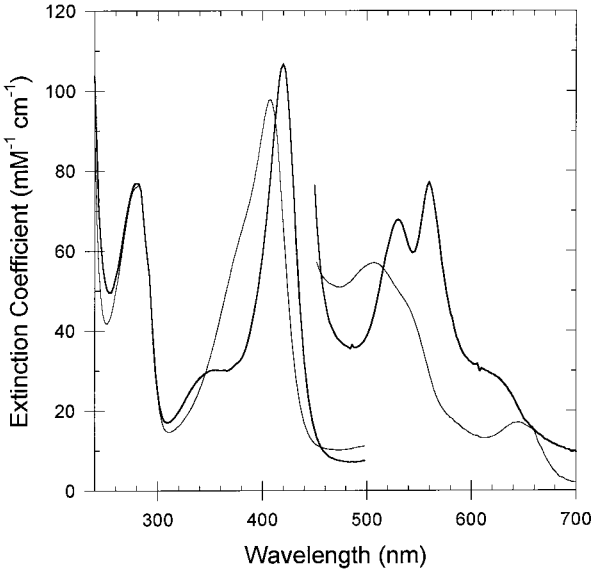

FIGURE 1: Spectra of CcP (thin line) and CcP compound I (thick line). CcP compound I was formed by the oxidation of CcP with *p*-nitroperoxybenzoic acid in phosphate buffer, pH 6.0, and 0.1 M ionic strength. The visible region is expanded a factor of 5. The α, *β*, and Soret bands of CcP compound I occur ar 560, 530, and 420 nm, respectively, with extinction coefficient of 15.2, 13.6, and 107 mM^{-1} cm^{-1}, respectively.

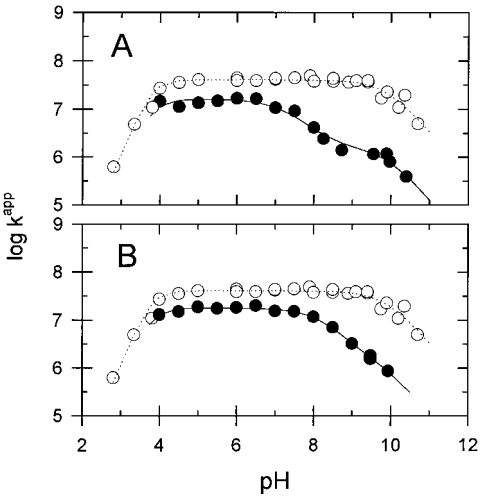

FIGURE 2: pH dependence of the apparent rate constant for the oxidation of CcP to compound I. (A) ●, oxidation by *p*-nitroperoxybenzoic acid; ○, oxidation by hydrogen peroxide (*9, 16*). (B) ●, oxidation by peracetic acid; ○, same as in panel A. All data collected at 0.10 M ionic strength.

compound I (eq 1), causing large changes in the absorption spectrum of the enzyme (Figure 1):

$$\text{CcP} + \text{ROOH} \xrightarrow{k^{app}} \text{CcP-I} + \text{ROH} \qquad (1)$$

In eq 1, ROOH represents the peroxy acid, ROH represents the carboxylic acid product, and k^{app} is the apparent bimolecular rate constant. Values of k^{app} as a function of pH for the reaction between CcP and pNPBA are shown in Figure 2A, and those for the reaction between CcP and PAA are shown in Figure 2B. The ionic strength of the buffers used for the studies shown in Figure 2 was 0.10 M. For comparison purposes, values of the apparent bimolecular rate constant for the reaction between CcP and hydrogen peroxide as a function of pH at 0.10 M ionic strength are shown in each panel (*9, 16*).

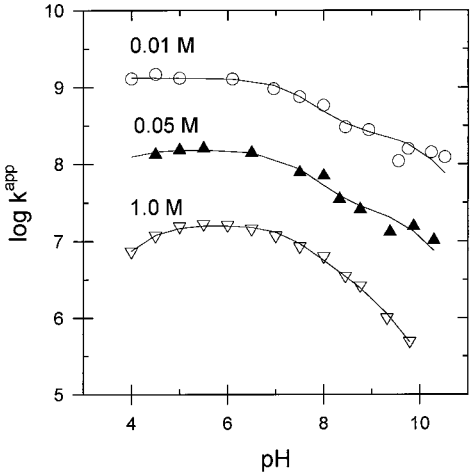

FIGURE 3: pH dependence of the rate of oxidation of CcP by *p*-nitroperoxybenzoic acid at three values of ionic strength. The data at 0.05 M ionic strength have been offset by adding 1 to the experimental value of log k^{app}, and the data at 0.01 M ionic strength have been offset by adding 2 to the experimental value of log k^{app}.

Ionic Strength Dependence of the Reaction between pNPBA and CcP. The pH dependence of the rate of the reaction between pNPBA and CcP was determined at four different ionic strengths. The data for 0.010, 0.050, and 1.00 M ionic strengths are shown in Figure 3. The data for 0.1 M ionic strength are shown in Figure 2A. Values of the rate constants at different ionic strengths have been offset from one another to simplify the figure.

DISCUSSION

pH Dependence of the Reaction between Peroxyacids and CcP. The reaction between hydrogen peroxide and CcP is dependent upon two apparent ionizations in the protein, one with a pK_a of 3.8, which has been attributed to the distal histidine, and the second with a pK_a of 9.8 (*16*). The second ionization correlates with conversion of CcP to a transient, hexacoordinated, low-spin alkaline form of CcP that is observed prior to CcP denaturation (*17*). Ionization of the group with pK_a of 9.8 produces an alkaline form of CcP, which has a hydroxide ligated to the heme iron. The hydroxide-ligated form is subsequently converted to a bis-imidazole form with the distal histidine displacing the hydroxide ion (*13*). The alkaline forms of CcP are unreactive toward hydrogen peroxide (Figure 2).

We assume that the two enzyme ionizations that influence the hydrogen peroxide reaction also influence the reactions with peroxyacids and their anions. In addition, the ionization of the peroxyacid has been shown to influence the reaction between these substrates and CcP (*12*). A minimal mechanism to explain the pH dependence of the reaction between peroxyacids and CcP is shown in Figure 4. The apparent bimolecular rate constant for the mechanism shown in Figure 4 is given in

$$k^{app} = \frac{k_1\frac{[H^+]}{K_{E1}} + \left(k_2 + k_4\frac{K_S}{K_{E1}}\right) + \left(k_3\frac{K_{E2}}{K_S} + k_5\right)\frac{K_S}{[H^+]} + k_6\frac{K_{E2}K_S}{[H^+]^2}}{\left(\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}\right)\left(\frac{K_S}{[H^+]} + 1\right)} \qquad (2)$$

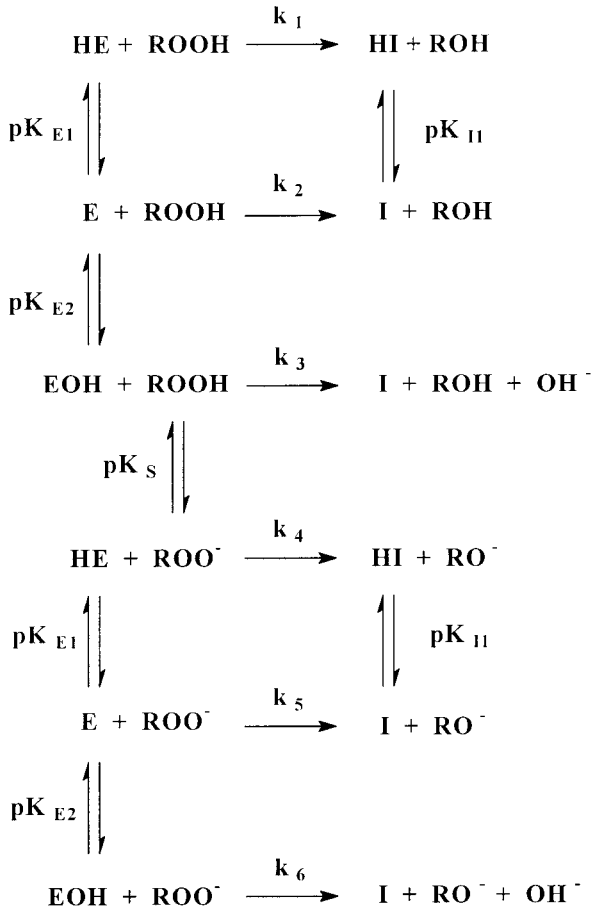

FIGURE 4: Mechanism for the reaction between hydroperoxides and CcP. Three different protonated forms of CcP have different reactivities toward hydroperoxides and their anions. HE and E represent CcP with the distal histidine protonated and unprotonated, respectively. EOH represents the hexacoordinated alkaline form of CcP. The pK_a values for the enzyme ionizations are designated pK_{E1} and pK_{E2}. The pK_a value for the hydroperoxide substrate is designated pK_S.

We can simplify the mechanism shown in Figure 4 in the case of hydrogen peroxide. The value of pK_S for hydrogen peroxide is 11.6, and the reaction between CcP and the hydrogen peroxide anion makes negligible contributions to the apparent bimolecular rate constant (*16*). This means that k_4, k_5, and k_6 can be effectively set equal to zero for the hydrogen peroxide reaction. It can be shown that both k_1 and k_3 are also negligible. The dashed line through the hydrogen peroxide data in Figure 2 is calculated assuming k_1 and k_3 are zero. With all of the rate constants negligible except k_2, eq 2 reduces to eq 3 for the reaction between CcP and hydrogen peroxide:

$$k^{app} = \frac{k_2}{\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}} \quad (3)$$

On the basis of an analogy with the hydrogen peroxide/CcP reaction, we assume that k_1, k_3, and k_6 are negligible for the reaction between peroxyacids and CcP. If the distal histidine is protonated, it cannot promote binding of the peroxyacids to the heme iron through base-catalyzed deprotonation of the substrate; therefore, k_1 is negligible. Likewise, the reaction between alkaline CcP, where the sixth coordination site of the heme iron is occupied, and exogenous substrates is significantly slower than the reaction with native, pentacoordinated CcP, and both k_3 and k_6 in Figure 4, are negligible. We can further simplify eq 2 by noting that the values of k_4 and k_5 will be similar. The reactions characterized by k_4 and k_5 are those of the peroxy anion with CcP when the distal histidine is either protonated or deprotonated. The anions do not need assistance from the distal histidine to bind to the heme iron, and it should be immaterial to the reaction between the peroxy anions and CcP whether the distal histidine is protonated. If k_4 has a value similar to that of k_5, which is about 10 times slower than the reaction with the neutral peroxyacid, k_2, it can be shown that the term involving k_4K_S/K_{E1} in eq 2 is negligible as compared to k_2 and that eq 2 reduces to

$$k^{app} = \frac{k_2 + k_5\frac{K_S}{[H^+]}}{\left(\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}\right)\left(\frac{K_S}{[H^+]} + 1\right)} \quad (4)$$

Table 1: Kinetic Parameters for Oxidation of Cytochrome *c* Peroxidase by Hydrogen Peroxide, Peracetic Acid (PAA), and *p*-Nitroperoxybenzoic Acid (pNPBA)[a]

	H_2O_2	PAA	pNPBA
pK_S	11.6	8.2	7.1
$10^{-7} \times k_2$ (M^{-1} s^{-1})	4.1 ± 0.2	1.8 ± 0.1	1.6 ± 0.2
$10^{-6} \times k_5$ (M^{-1} s^{-1})	NA[b]	1.5 ± 0.1	1.6 ± 0.2

[a] The data in Figure 2 were fit to the mechanism shown in Figure 4. All rate constants defined in Figure 4 are negligible except k_2 and k_5. Equation 3 of the text was used to fit the H_2O_2 data, and eq 4 was used to fit the PAA and pNPBA data. During the fitting, only k_2 and k_5 were allowed to vary. Values of pK_{E1} and pK_{E2}, defined in Figure 4, were fixed at their experimental values of 3.8 and 9.8, respectively (*16*). The pK_S values for each substrate were fixed at their experimental values during the fitting procedure and are shown in the table (PAA, this work; pNPBA, ref *12*). Experimental conditions were 0.10 M ionic strength, 25 ± 1 °C. [b] The parameter made negligible contributions to the apparent rate constant.

The data in Figure 2A for the reaction between pNPBA and CcP and the data in Figure 2B for the reaction between PAA and CcP were fit to equation 4. Best-fit parameters are given in Table 1. The solid lines in Figure 2, panels A and B, were calculated using eq 4 and the parameters given in Table 1.

Ionic Strength Dependence of the Reaction between pNPBA and CcP. Frew and Jones suggest that the discrimination between neutral and charged substrates could be due to an "electrostatic gate" formed by negatively charged residues on the enzyme surface near the entrance to the heme site (*12*). Charge−charge repulsion between CcP and the peroxyacid anions slows the rate of reaction on the anion as compared to that of the neutral acid. To test this model, we have investigated the ionic strength dependence of the reaction (Figure 3). If electrostatic repulsion of the peroxy anion by the surface of CcP is the principal cause of the slower anionic reaction rate, then the reaction between the peroxyacid anions and CcP should be influenced by the ionic strength of the buffer. The value of k_5 should increase

Table 2: Ionic Strength Dependence of the Kinetic Parameters for the Reaction between *p*-Nitroperoxybenzoic Acid and Cytochrome *c* Peroxidase

	ionic strength (M)			
	0.01	0.05	0.10[a]	1.00
pK_{E1}		3.4 ± 0.6	3.8	4.1 ± 0.1
pK_{E2}[b]	10.2	9.9	9.8	9.1
pK_S	7.5 ± 0.3	7.5 ± 0.2	7.1	7.5 ± 0.1
$10^{-7} \times k_2$ (M^{-1} s^{-1})	1.3 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.1
$10^{-6} \times k_5$ (M^{-1} s^{-1})	2.4 ± 0.3	2.4 ± 0.2	1.6 ± 0.2	3.1 ± 0.2

[a] pK_a values fixed at literature values (*12, 16*). [b] pK_{E2} values fixed at literature values (*12*).

significantly with increasing ionic strength as the electrostatic repulsion is mitigated by ionic shielding. In contrast, the value of k_2 should be independent of ionic strength since this is the rate constant for the reaction of the neutral peroxyacid with CcP. The ionic strength dependence of the reaction was investigated between 0.01 and 1.00 M ionic strength (Figures 2 and 3). The data were fit to eq 4, and the best-fit parameters are collected in Table 2. Both k_2 and k_5 are essentially independent of ionic strength. The ionic strength dependence of the reaction between pNPBA and CcP does not support the electrostatic gate model of Frew and Jones (*12*).

Microscopic Mechanism for the Reaction of Peroxyacids with CcP. The reaction between CcP and a peroxide substrate to form compound I can be envisioned as the result of four microscopic steps: (i) diffusion of the substrate to the surface of the protein; (ii) diffusion of the substrate within the protein matrix to the heme pocket; (iii) binding of the substrate to the heme iron; and (iv) cleavage of the O−O bond of the bound peroxide to form compound I. Under our experimental conditions, these four microscopic steps result in the observation of a single apparent bimolecular rate constant, k_2 for hydrogen peroxide and peroxyacids and k_5 for the peroxyacid anions. The most probable cause for discrimination between neutral and charged substrates is that diffusion within the protein matrix is the rate-limiting process for both charged and uncharged reactants.

We have previously shown that the reaction between CcP and hydrogen peroxide is not diffusion-limited and that the apparent bimolecular rate constant of 4.1×10^7 M^{-1} s^{-1} (Table 1) is about 2 orders of magnitude slower than the diffusion-limited rate (*18*). The similarity of the values of k_2 for hydrogen peroxide and pNPBA (Table 1) and the observation that k_5 is smaller than k_2 indicate that the reaction for the peroxyacids and their anions with CcP is not diffusion-limited. The reaction is strictly bimolecular up to the limits of measurement by stopped-flow techniques, which indicates that the O−O bond cleavage is not rate-limiting under our experimental conditions. Estimates of the rate of O−O cleavage are on the order of 10^5 s^{-1} (*10*). With the elimination of step i, diffusion of the reactants to the surface of the protein, and step iv, O−O bond cleavage as rate-limiting steps, we need only consider step ii, diffusion within the protein matrix, and step iii, binding of the substrate within the heme pocket to the heme iron, to form a peroxy complex as potential rate-limiting processes.

Step iii should be faster for the peroxy anion than for the peroxyacid. First, proton dissociation from the substrate is not required for the peroxybenzoate ion as it is for the peroxyacid; second, electrostatic attraction between the positively charged heme iron and the negatively charged peroxybenzoate ion will promote anion binding as compared to the neutral peroxyacid. Step iii cannot be rate limiting since if it were, k_5 would be larger than k_2, contrary to observation (Table 1).

If step iii is not rate limiting, then diffusion within the protein matrix from the surface entrance site to the distal heme pocket has to be the rate-limiting step. This is consistent with the studies of Lakowicz and Weber (*19*), who demonstrate that small cations and anions have diffusion rates within the interiors of a number of proteins that are about an order of magnitude slower than small neutral molecules. The relative independence of k_2 and k_5 on the size of the oxidizing substrate can be rationalized on the basis of a gating mechanism for diffusion within the protein matrix.

The importance of this study is the demonstration that both the neutral peroxyacid and the negatively charged peroxy anion can diffuse rapidly into the heme pocket of CcP, bind to the heme iron, and undergo O−O bond cleavage. In an accompanying paper (*13*), we investigate the role of the distal histidine in compound I formation. If the dominant role of the distal histidine is to promote proton dissociation from incoming hydroperoxide to facilitate binding of the peroxide anion to the heme iron, then the CcP mutant in which the distal histidine is replaced by a leucine residue, CcP(H52L), should have a significantly diminished rate for peroxyacids just as observed for the reaction with hydrogen peroxide (*9*). However, CcP(H52L) should react just as rapidly with the peroxybenzoate anion as wild-type CcP.

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