Kinetic Studies of Fluoride Binding by Cytochrome *c* Peroxidase†

James E. Erman

ABSTRACT: The association and dissociation rate constants for the binding of fluoride by cytochrome *c* peroxidase have been determined over the pH range 2.5–9.8 at 25° and 0.15 M ionic strength by the stopped-flow technique. The pH dependence of the association rate constant indicates that a protein group with pK_a of 5.5 and the ionization of hydrofluoric acid strongly influence the rate of fluoride binding. Two mechanisms are consistent with the binding data. The fluoride anion could bind to the enzyme when the group with pK_a of 5.5 is protonated or hydrofluoric acid could bind to the basic form of the enzyme. Binding of hydrofluoric acid is the favored interpretation, giving a pH-independent rate of 5.1×10^7 M^{-1} sec^{-1}. The pH dependence of the dissociation rate constant indicates at least two ionizable groups on the enzyme–fluoride complex influences the dissociation of fluoride from the complex. The pK_a's of the two groups are 6.0 and 8.5.

The peroxidases are a group of heme-containing enzymes which can catalyze the oxidation of a variety of organic and inorganic compounds by hydrogen peroxide (Saunders *et al.*, 1964). The hydrogen peroxide interacts with the enzyme at the heme, oxidizing the enzyme to a higher oxidation state in a two-electron process (George, 1952; Chance, 1952a; Yonetani, 1965). The heme groups of the enzymes also interact with a number of inorganic ligands (Chance, 1943, 1952b) and these ligand binding reactions can be used to probe the environment of the heme group. This paper reports kinetic studies of fluoride binding to ferric cytochrome *c* peroxidase as a function of pH in order to determine if there are any ionizable groups in cytochrome *c* peroxidase which can influence reactions at the heme site.

Experimental Procedure

Cytochrome *c* peroxidase was isolated from baker's yeast and crystallized by dialyzing against distilled water according to the method of Yonetani (Yonetani and Ray, 1965; Yonetani *et al.*, 1966a). The purity index (ratio of absorbance at 408 and 282 nm) of the enzyme preparation was 1.18. Enzyme concentrations varied from 9×10^{-7} to 5×10^{-6} M for various experiments and were determined spectrophotometrically using an extinction coefficient of 95 mM^{-1} cm^{-1} at 408 nm (Coulson *et al.*, 1971). Reagent grade potassium fluoride was used without further purification. Fluoride concentrations ranged from 2×10^{-6} to 1×10^{-1} M depending upon pH.

Absorption spectra were obtained on a Cary Model 14 or a Model 1501 recording spectrophotometer with cell compartment thermostatted at 25°. The maximum difference in the absorption spectrum between the fluoride complex and the native enzyme occurred at 406 nm. All kinetic experiments were carried out at this wavelength.

The kinetics of the fluoride–cytochrome *c* peroxidase complex formation were determined by the stopped-flow method using a Durrum-Gibson stopped-flow spectrophotometer with 20-mm light-path thermostatted at 25°. The enzyme is stable in the pH region from 4 to 7.5. The stopped-flow kinetic studies were extended to include the range between pH 2.5 and 10 by maintaining the enzyme in an unbuffered solution near pH 5 in one drive syringe and having fluoride in the second drive syringe buffered at the desired final pH. The pH of the final solution was checked by measurement on a pH meter after the solutions had been mixed and the fluoride complex formed. Phthalate buffers were used between pH 2.5 and 5.5, acetate, buffers between pH 4 and 6, phosphate buffers between pH 5.5 and 8, and borate buffers between pH 8 and 10. Results were independent of the type of buffer used in overlapping regions. In all cases total buffer concentration was 1×10^{-2} M and the ionic strength was adjusted to 0.15 M with potassium nitrate in all cases.

In all kinetic studies, the fluoride concentration was at least ten times greater than the enzyme concentration in order to make all observed reactions first order. The enzyme concentration was dilute enough to keep changes in transmission less than 5%. Under these conditions the voltage changes observed on the oscilloscope of the stopped-flow instrument are directly proportional to concentration changes.

Results

Soret Band of the Enzyme and Enzyme–Fluoride Complex at Different Values of pH. The absorption spectrum of cytochrome *c* peroxidase and its fluoride complex are shown in Figure 1A and are nearly identical with the previously reported spectra (Yonetani *et al.*, 1966b). Figure 1B shows the difference spectrum between the fluoride complex and the enzyme. The spectra of both the enzyme and the fluoride complex show small changes as a function of pH between pH 4 and 8. The differences between the enzyme spectrum at pH 4.5, 6.5, and 7.5 relative to the enzyme spectrum at pH 6.5 are shown in Figure 2A. The variation of the extinction coefficient at 406 nm at different pH values relative to pH 6 are shown as a function of pH in Figure 3. At pH 7.5 and above, the enzyme begins to undergo irreversible denaturation with the rate of the denaturation increasing with increasing pH.

The spectrum of the enzyme–fluoride complex is invariant within experimental error between pH 4.5 and 7.5. At pH 4 and 8 there are significant changes. The spectral shifts at pH 8 for the enzyme–fluoride complex do not vary with time as do

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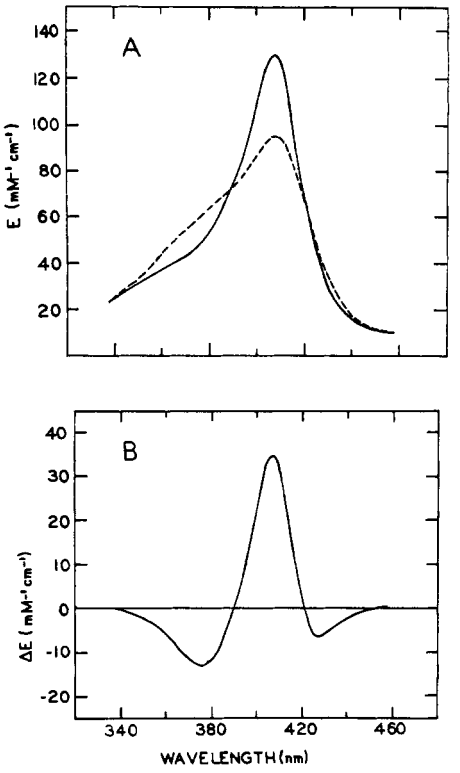


FIGURE 1: (A) Absorption spectra of cytochrome *c* peroxidase (dashed line) and its fluoride complex (solid line) in the Soret region at pH 6. (B) Difference spectrum between the fluoride complex and the native enzyme at pH 6.

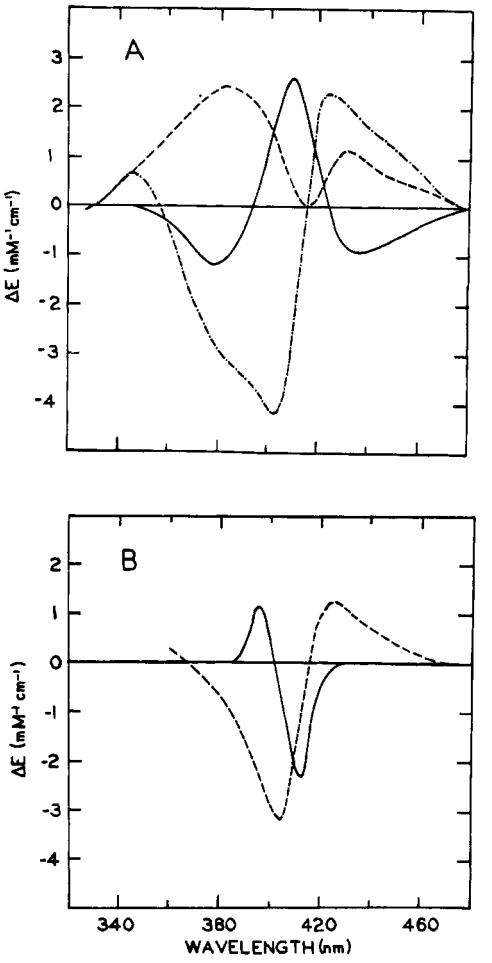


FIGURE 2: (A) Difference spectra between cytochrome *c* peroxidase at pH 4.5 (solid line), pH 6.5 (dashed line), and pH 7.5 (dashed dotted line), and the enzyme at pH 5.5. (B) Difference spectra between the enzyme fluoride complex at pH 4 (solid line) and at pH 8 (dashed line) and the enzyme–fluoride complex at pH 6.

those for the free enzyme. The difference spectrum between the fluoride complex at pH 4 and 6 and between the complex at pH 8 and 6 are shown in Figure 2B. At pH 4 there is a slight blue shift of the Soret maximum and at pH 8 there is a slight red shift. The differences in the extinction coefficient of the fluoride complex at pH 6 and the fluoride complex at different pH values at 406 nm are shown as a function of pH in Figure 3.

Kinetics of the Enzyme–Fluoride Complex Formation. Between pH 3.5 and 8.0, only a single reaction was observed upon mixing cytochrome *c* peroxidase and fluoride in the stopped-flow instrument. For reversible complex formation between enzyme and fluoride, both the association and dissociations rates must be taken into account.

$$E + F \underset{k_d}{\overset{k_a}{\rightleftharpoons}} EF \qquad (1)$$

The differential rate equation for the formation of the enzyme–fluoride complex is given by equation 2. Under the

$$d(EF)/dt = k_a(E)(F) - k_d(EF) \qquad (2)$$

conditions of the kinetic experiments, the concentration of unbound fluoride is much greater than the enzyme concentration and remains essentially constant with time. A pseudo-first-order rate constant can be defined.

$$k_a' = k_a[F] \qquad (3)$$

Making use of eq 3, the conservation of mass applied to the enzyme species, and the equilibrium condition, eq 4 can be

$$\ln \{[(\overline{EF}) - (EF)]/(\overline{EF})\} = -(k_a' + k_d)t \qquad (4)$$

derived. In eq 4, $(\overline{EF})$ represents the equilibrium concentration of the enzyme–fluoride complex and (EF) represents the concentration of the enzyme–fluoride complex at time *t*. Equation 4 is the equation for a simple first-order process with A plot of the left-hand side of eq 4, which can be calculated

$$k^{obsd} = k_a' + k_d \qquad (5)$$

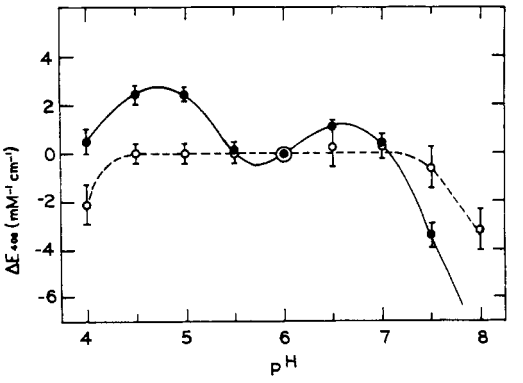


FIGURE 3: Variation in the difference of the extinction coefficient at 406 nm at various pH values and pH 6 for cytochrome *c* peroxidase (solid line) and the enzyme–fluoride complex (dashed line).

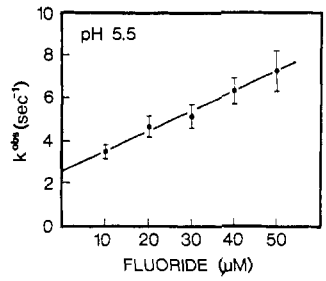


FIGURE 4: Plot of k^{obsd} as a function of total fluoride concentration at pH 5.5.

from the experimental data, as a function of time, is linear with the slope equal to k^{obsd}. Combining eq 3 and 5 yields

$$k^{obsd} = k_a[F] + k_d \quad (6)$$

According to eq 6, k^{obsd} is a linear function of fluoride concentration and a plot of k^{obsd} *vs.* fluoride concentration will give a straight line with intercept equal to k_d and slope equal to k_a. Such a plot is shown in Figure 4 for data obtained at pH 5.5. The value of k^{obsd} at each fluoride concentration is the average of at least four determinations with an average standard deviation of about 15%. At each pH, k^{obsd} was determined at a minimum of five different fluoride concentrations. Values of k_a and k_d were obtained by fitting k^{obsd} as a function of fluoride concentration to eq 6, using a general purpose curve-fitting program, KINET, developed at Michigan State University (Dye and Nicely, 1971). These values of k_a and k_d along with estimates of their standard error are given in Table I for different pH values.

TABLE I: Rate and Equilibrium Constants for Binding of Fluoride to Cytochrome *c* Peroxidase.[a]

pH	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	$K_D = k_d/k_a$ (M)
2.5	$(4.8 \pm 0.7) \times 10^4$	37 ± 5	$(0.8 \pm 0.1) \times 10^{-3}$
3.0	$(8.3 \pm 1.2) \times 10^4$	5.4 ± 0.6	$(0.7 \pm 0.2) \times 10^{-4}$
3.5	$(1.5 \pm 0.2) \times 10^5$	1.4 ± 0.5	$(1.0 \pm 0.4) \times 10^{-5}$
4.1	$(1.4 \pm 0.1) \times 10^5$	1.1 ± 0.4	$(0.8 \pm 0.3) \times 10^{-5}$
4.2	$(1.1 \pm 0.1) \times 10^5$	1.7 ± 0.4	$(1.6 \pm 0.5) \times 10^{-5}$
4.5	$(1.4 \pm 0.1) \times 10^5$	1.6 ± 0.2	$(1.2 \pm 0.2) \times 10^{-5}$
5.0	$(1.2 \pm 0.1) \times 10^5$	1.5 ± 0.2	$(1.3 \pm 0.2) \times 10^{-5}$
5.5	$(9.6 \pm 0.7) \times 10^4$	2.4 ± 0.2	$(2.4 \pm 0.3) \times 10^{-5}$
6.0	$(4.3 \pm 0.5) \times 10^4$	2.6 ± 1.5	$(0.6 \pm 0.4) \times 10^{-4}$
6.5	$(2.0 \pm 0.2) \times 10^4$	2.2 ± 0.4	$(1.1 \pm 0.3) \times 10^{-4}$
7.0	$(5.2 \pm 0.2) \times 10^3$	3.1 ± 0.7	$(0.6 \pm 0.2) \times 10^{-3}$
7.4	$(2.2 \pm 0.1) \times 10^3$	3.3 ± 0.2	$(1.5 \pm 0.1) \times 10^{-3}$
7.8	$(9.8 \pm 0.8) \times 10^2$	3.2 ± 0.5	$(3.3 \pm 0.6) \times 10^{-3}$
8.4	$(1.7 \pm 0.2) \times 10^2$	2.5 ± 0.1	$(1.5 \pm 0.1) \times 10^{-2}$
8.9	$(9.1 \pm 1.2) \times 10^1$	2.0 ± 0.1	$(2.2 \pm 0.3) \times 10^{-2}$
9.4	$(1.5 \pm 0.1) \times 10^2$	1.6 ± 0.1	$(1.1 \pm 0.1) \times 10^{-2}$
9.8	$(2.2 \pm 0.6) \times 10^2$	2.2 ± 0.4	$(1.0 \pm 0.3) \times 10^{-2}$

[a] 25°, 0.15 M ionic strength.

Dependence of k_a and k_d on pH. Only a single reaction, attributable to complex formation, was observed on mixing the fluoride and enzyme solutions in the stopped-flow apparatus between pH 3.5 and 8.0. At lower and higher pH values, denaturation of the enzyme occurred in addition to complex formation. The denaturation rates differed for the free enzyme and the enzyme–fluoride complex and both were multiphasic processes. Corrections were made for the amplitude of the denaturation reactions in order to determine k^{obsd} for fluoride binding.

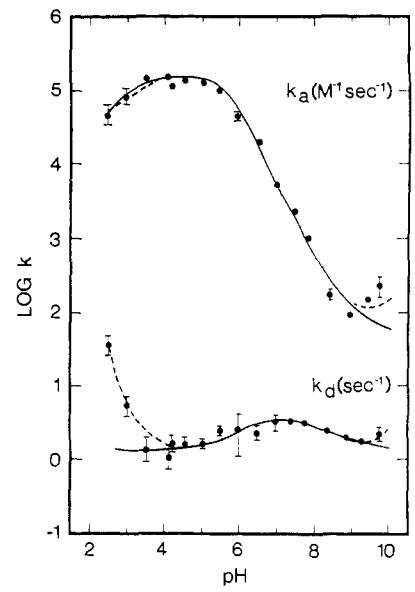


FIGURE 5: Plot of log k_a (upper curve) and log k_d (lower curve) as a function of pH. The solid and dashed lines are calculated from mechanisms discussed in the text.

Values of log k_a and log k_d as a function of pH are shown in Figure 5. Error bars are included where the standard error exceeds the size of the symbols in the figure. Log k_a has a maximum between pH 3.5 and 5.0. Log k_d has a maximum between pH 7.0 and 8.0, decreases to minima at about pH 4 and 9.5, then increases at the pH extremes.

K_D as a Function of pH. Values of the equilibrium dissociation constant, K_D, calculated from the ratio of k_d to k_a are included in Table I. Values of log K_D as a function of pH are shown in Figure 6. The strongest binding occurs at pH 4 where the dissociation constant has a value of 8×10^{-6} M. The dissociation constant increases with increasing pH to a value of about 2×10^{-2} M at pH 9.

Values of K_D were determined from equilibrium binding studies on the Cary spectrophotometer between pH 4 and 8 at 0.1 M ionic strength and 25°. The values were two to three times smaller than those calculated from the kinetic parameters determined at 0.15 M ionic strength. Values of K_D determined from the equilibrium experiments had standard deviations of about 50%. The pH dependence of the equilibrium dissociation constant paralleled that calculated from the kinetic rate parameters.

Discussion

The pH dependence of log k_a observed in Figure 6 indicates that two ionizable groups strongly influence the association rate constant between pH 2.5 and 9. The two groups have pK_a's of 3.0 and 5.5. The pK_a of 3.0 is attributed to ligand ionization and the pK_a of 5.5 is assigned to a protein group. The assignments are confirmed in an accompanying paper (Erman, 1974) where cyanide binding has been investigated. For cyanide binding, the association rate constant is influenced by two groups, one with a pK_a of 5.4 and the second with a pK_a of 9.0. The group with pK_a of 5.5 ± 0.1 is common to

both reactions indicating a common reactant, the enzyme, while the group with pK_a of 3.0 for fluoride binding is certainly due to the ionization of hydrofluoric acid which has a pK_a of 3.0 at 25° and 0.15 M ionic strength, calculated from the data in the literature (Vanderborgh, 1968). The pK_a of 9.0 for cyanide binding must be due to hydrocyanic acid dissociation which has a value of 9.0 at 25° and 0.15 M ionic strength (Izatt *et al.*, 1962).

Since the ligand ionization strongly influences the rate of binding, one form of the ligand binds much faster than the other form. The observed pH dependence of k_a between pH 2.5 and 9 can be explained on the basis of two simple mechanisms. The acid form of the ligand could bind to the enzyme when the enzyme group with a pK_a of 5.5 is unprotonated as shown in the following

$$\begin{array}{ccc} & \mathrm{HE} & \\ & K_E \uparrow\downarrow & \\ \mathrm{HL} + \mathrm{E} & \xrightarrow{k_1} & \mathrm{EL} + \mathrm{H^+} \\ K_L \uparrow\downarrow & & \\ \mathrm{L} & & \end{array} \qquad (7)$$

The observed association rate constant for this mechanism is given by eq 8. The value for k_1 is 5.1×10^7 M^{-1} sec^{-1}, for K_L is

$$k_a = \frac{k_1}{\left(1 + \frac{K_L}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_E}\right)} \qquad (8)$$

1.00×10^{-3} M, and for K_E is 3.2×10^{-6} M, determined from a best fit of the k_a *vs.* pH data.

The second mechanism involves the anionic form of the ligand binding to the enzyme when the group with pK_a of 5.5 is protonated according to

$$\begin{array}{ccc} \mathrm{HL} & & \\ K_L \uparrow\downarrow & & \\ \mathrm{L} + \mathrm{HE} & \xrightarrow{k_2} & \mathrm{HEL} \\ & K_E \uparrow\downarrow & \\ & \mathrm{L} & \end{array} \qquad (9)$$

For this mechanism

$$k_a = \frac{k_2}{(1 + [H^+]/K_L)(1 + K_E/[H^+])} \qquad (10)$$

The best-fit values are the same for K_L and K_E as for eq 8 and k_2 equals 1.6×10^5 M^{-1} sec^{-1}. These two mechanisms are kinetically indistinguishable and

$$k_1 = k_2 K_E / K_L \qquad (11)$$

The question of whether the neutral or anionic form of moderately basic ligands such as fluoride, azide, and cyanide bind to ferric heme proteins has been discussed many times but an unequivocal answer is not possible (George and Lyster, 1958; Goldsack *et al.*, 1966; Ellis and Dunford, 1968). Even in the simpler inorganic systems, the protonated state of these ligands which react with ferric iron is ambiguous (Seewald and Sutin, 1965). However, there are several facts which seem to favor binding of the protonated form of the ligands to cytochrome *c* peroxidase.

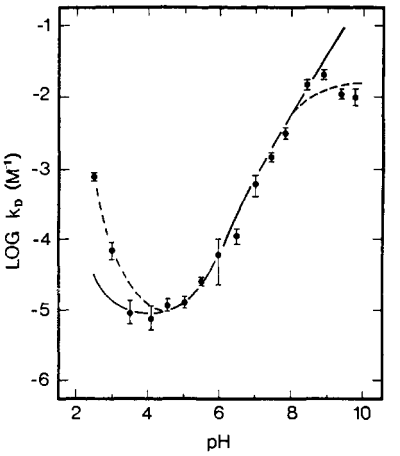


FIGURE 6: Plot of log K_D as a function of pH. The solid and dashed lines are calculated from the same mechanisms as those in Figure 5.

The ligand binding reactions to form simple ferric iron complexes seems to be resolved on the basis of a mechanism in which the rate-controlling step is the dissociation of water from the inner coordination sphere of the metal ion (Wilkens and Eigen, 1965). This mechanism makes the rate of ligand binding insensitive to the nature of the ligands and, as a consequence, the pH-independent rate constants for all ligands are relatively constant.

In the neutral pH region, water occupies the sixth coordination site of ferric heme proteins (Kendrew *et al.*, 1960), and it is conceivable that the rate of water dissociation could be the rate-determining step in the ligand binding reactions. Using the criterion of constancy of the ligand binding rates, the binding of the protonated forms of fluoride and cyanide to both cytochrome *c* peroxidase and horseradish peroxidase is favored (Dunford and Alberty, 1967; Ellis and Dunford, 1968; Erman, 1974). The pH-independent rate constants for the protonated form of the ligand binding varies by about 2.5 orders of magnitude in the four cases mentioned above, while the pH-independent rate constants, assuming anion binding, vary by nearly five orders of magnitude.

Recently Schonbaum has reported on a series of complexes between horseradish peroxidase and hydroxamic acids, hydrazides, and amides (Schonbaum, 1973). The ligands bind very rapidly and although they are apparently not directly coordinated to the iron, the ligands are probably close to the heme, since the spin state of the iron is perturbed and the ligands compete with hydrogen donors such as phenols and amines which are substrates for the enzyme. The dependence of the dissociation constant on the structural variations of several ligands suggest the binding site is a large apolar region of the enzyme. If this apolar region is in fact the region surrounding the sixth coordination site of the iron, it would explain the discrimination between the rate of binding of the neutral and charged forms of the ligands that do bind at the iron and favor binding of the neutral species.

Additional evidence in support of the neutral ligands as the species which binds preferentially is the reaction between the peroxidases and hydrogen peroxide. Although these are not ligand binding reactions, the bimolecular rate constants are large, 2×10^8 M^{-1} sec^{-1} for hydrogen peroxide and cytochrome *c* peroxidase at pH 6 (Yonetani and Ray, 1966). In this case it is certain that the neutral form of hydrogen peroxide interacts with the heme site. Preliminary experiments on the pH dependence of the hydrogen peroxide reaction with

cytochrome *c* peroxidase indicate that hydrogen peroxide reacts with the enzyme when the group with pK_a of 5.5 is unprotonated (H. Hordi and J. E. Erman, unpublished results) consistent with the interpretation that the neutral form of the ligand binds to the unprotonated form of the enzyme.

To reiterate, although it cannot be proven with certainty, the evidence cited above suggests that it is the neutral form of weakly basic ligands such as fluoride which bind preferentially to the peroxidases. Upon binding, the proton would be released into solution or picked up by an exposed group on the protein (George and Lyster, 1958).

Between pH 3.5 and 10, the dissociation rate constant varies by only a factor of three, with a maximum near pH 7.3. The rather small pH dependence of the dissociation rate constant could be due to the total ionization state of the enzyme. For example, the increase in k_d between pH 3.5 and 7.5 could be due to the effect of the net charge on the protein as the pH is varied through pH 5.2, the isoelectric point of the enzyme. The net negative charge on the protein above pH 5.2 should make it easier for the ligand anion to dissociate. Likewise, the decrease in k_d between pH 7.5 and 9.5 could be due to changes in the conformation of the protein since the native enzyme begins to undergo irreversible denaturation above pH 7.5. In the absence of additional information is is difficult to assess the contribution of effects such as the total ionization state of the enzyme on the apparent dissociation rate constant. As a consequence, to quantitatively account for the pH variation of the dissociation rate constant between pH 3.5 and 9.0, it is assumed that acid and base groups in the vicinity of the heme group specifically influence the dissociation rate.

The simplest mechanism, assuming the binding of hydrofluoric acid with release of the proton into solution, which accounts for the pH variation in both the association and dissociation rate constants and satisfys microscopic reversibility is given in Scheme I and eq 12 and 13. The best-fit parameters are given in Table II. Three ionizable groups on the protein are required to fit the data, although one, represented by K_{E3} and K_{C3}, ionizes outside the pH region studied. The values of K_{E3} and K_{C3} along with the rate constants, k_6 and k_{-6}, are not specifically determined from fitting the rate data and have a range of interdependent values. The values used to calculate the solid lines in Figures 5 and 6 are given in parentheses in Table II. The ionizable group specified by K_{E1} is the group which strongly influences the association rate constant. The group K_{E2} does not influence the association rate constant but its value is specified by microscopic reversibility. The rate constants k_3 and k_{-3} do not contribute significantly to the observed rate constants in the pH range studied.

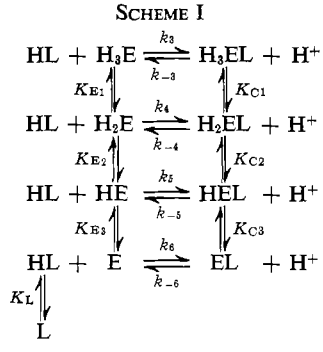
TABLE II: Rate and Equilibrium Constants for Mechanism to Account for pH Variation of k_a and k_d.[a]

k_3		k_{-3}	
k_4	5.1×10^7	k_{-4}	1.3×10^6
k_5	5.1×10^7	k_{-5}	1.1×10^9
k_6	(6.0×10^{10})	k_{-6}	(1.3×10^{12})
K_{E1}	3.2×10^{-6}	K_{C1}	1.0×10^{-6}
K_{E2}	2.7×10^{-6}	K_{C2}	3.2×10^{-9}
K_{E3}	(1.0×10^{-12})	K_{C3}	(1.0×10^{-12})

[a] All k values are in M^{-1} sec^{-1}. All K values are in M.

In order to account for the pH variation of k_a and k_d below pH 3.5 and above pH 9, additional groups which ionize outside the range of this study must be postulated. A mechanism involving five ionizable groups gives the dashed lines shown in Figures 5 and 6. However, it is more likely that the pH variation of the rate constants at the pH extremes reflect the incipient denaturation of the enzyme.

SCHEME I



$$k_a = \frac{k_3 + k_4\frac{K_{E1}}{[H^+]} + k_5\frac{K_{E1}K_{E2}}{[H^+]^2} + k_6\frac{K_{E1}K_{E2}K_{E3}}{[H^+]^3}}{\left(1 + \frac{K_L}{[H^+]}\right)\left(1 + \frac{K_{E1}}{[H^+]} + \frac{K_{E1}K_{E2}}{[H^+]^2} + \frac{K_{E1}K_{E2}K_{E3}}{[H^+]^3}\right)} \quad (12)$$

$$k_d = \frac{[H^+]\left(k_{-3} + k_{-4}\frac{K_{C1}}{[H^+]} + k_{-5}\frac{K_{C1}K_{C2}}{[H^+]^2} + k_{-6}\frac{K_{C1}K_{C2}K_{C3}}{[H^+]^3}\right)}{1 + \frac{K_{C1}}{[H^+]} + \frac{K_{C1}K_{C2}}{[H^+]^2} + \frac{K_{C1}K_{C2}K_{C3}}{[H^+]^3}} \quad (13)$$

Acknowledgment

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Kinetic and Equilibrium Studies of Cyanide Binding by Cytochrome *c* Peroxidase†

James E. Erman

ABSTRACT: The association and dissociation rate constants and the equilibrium dissociation constant for cyanide binding to cytochrome *c* peroxidase have been determined as a function of pH from pH 4 to 10.5 at 25° and 0.15 M ionic strength. The pH dependence of the association rate constant indicates the ionization of hydrocyanic acid and the ionization of a group on the protein, with a pK_a of 5.4, strongly influence the rate of cyanide binding. The association rate constant has a maximum value of 1.1×10^5 M^{-1} sec^{-1} between pH 6 and 8. Between pH 4 and 8, the dissociation rate constant varies between 0.1 and 1.0 sec^{-1}. The pH dependence of the dissociation rate constant in this pH region can be interpreted on the basis of two ionizable groups with pK_a values of 6.5 and 7.3. Above pH 7, the enzyme–cyanide complex undergoes two isomerization reactions, both of which are pH dependent.

The study of fluoride binding to cytochrome *c* peroxidase indicates there is an ionizable group in the native enzyme which strongly influences the binding reaction and perhaps two ionizable groups on the enzyme–fluoride complex which effect the dissociation rate (Erman, 1974). These ionizable groups may play an important role in the mechanism of cytochrome *c* peroxidase catalyzed oxidations. The present study of the reaction between cyanide and cytochrome *c* peroxidase was initiated to confirm the results of the fluoride binding reaction, to determine the influence of the ligand on the apparent pK_a values of the ionizable groups in the complex, and since cyanide forms a much stronger complex at a higher pH than fluoride, to extend the ligand binding studies to higher pH values.

Experimental Procedure

The experimental procedures were essentially the same as for the fluoride binding studies (Erman, 1974). Cytochrome *c* peroxidase was isolated from baker's yeast and crystallized by dialyzing against distilled water (Yonetani *et al.*, 1966a). Enzyme concentrations ranged from 1×10^{-7} to 5×10^{-6} M. Reagent grade potassium cyanide was used without further purification. Cyanide solutions were adjusted to the proper pH immediately prior to each experiment with small amounts of HNO_3 or KOH and kept stoppered with a minimum of air space above the solution to minimize loss of HCN. Cyanide concentrations ranged from 1×10^{-6} to 6.7×10^{-3} M. All solutions were 0.15 M ionic strength, adjusted with KNO_3, and total buffer concentration was 0.01 M, with acetate, phosphate, and borate buffers used in the appropriate pH region. Stopped-flow studies were carried out in a Durrum-Gibson stopped-flow spectrophotometer thermostatted at 25°. Equilibrium binding studies, slow isomerization kinetics, and spectra were obtained on either a Cary Model 14 or a Cary Model 1501 spectrophotometer with cell compartments thermostatted at 25°.

Results

Soret Band of Cytochrome c Peroxidase–Cyanide Complex as a Function of pH. The absorption spectra of cytochrome *c* peroxidase and its cyanide complex in the Soret region at pH 6 are shown in Figure 1A. The spectrum of the cytochrome *c* peroxidase–cyanide complex has a maximum at 426 nm and an extinction coefficient of 103 mM^{-1} cm^{-1}, nearly identical with that previously reported (Yonetani *et al.*, 1966b). The difference spectrum between the cyanide complex and the free enzyme at pH 6 is shown in Figure 1B. The maximum difference occurs at 428 nm and this wavelength was used to monitor the binding reaction in most cases.

Upon addition of unbuffered cytochrome *c* peroxidase to a cyanide solution at pH 8 or higher, there is a rapid formation of the enzyme–cyanide complex, followed by an isomerization reaction, characterized by a shift of the Soret maximum

† From the Department of Chemistry, Northern Illinois University, DeKalb, Illinois 60115. *Received July 30, 1973.* This investigation was supported by Public Health Service Research Grant GM 18648 from the National Institute of General Medical Sciences.