The Kinetics of Fluoride Binding by Ferric Horse Radish Peroxidase*

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ABSTRACT: The kinetics of the reversible binding of fluoride by ferric horse radish peroxidase have been studied over the pH range from 4.1 to 7.9 by the temperature-jump method. The apparent bimolecular rate constant, k_{1app}, decreases from 2.0×10^6 to 9.0×10^2 over the pH range studied, whereas the apparent dissociation rate constant shows little pH dependence. Analysis of the pH dependence of k_{1app} is consistent with the presence of one heme-linked ionizable group with a pK of 4.1 ± 0.3 and suggests that fluoride ion is the only binding species of importance. The binding constants of fluoride ion to peroxidase are $(4.2 \pm 2.5) \times 10^6$ and $(3.2 \pm 1.3) \times 10^2$ M^{-1} sec^{-1} when the heme-linked group is protonated and unprotonated, respectively.

A study of the kinetics of ligand binding to horse radish peroxidase[1] (Chance, 1943; Kertesz *et al.*, 1965), can yield fundamental information about the nature of the active site in the peroxidase molecule. We report here on the use of the temperature-jump technique (Eigen and de Maeyer, 1963; Czerlinski and Eigen, 1959) to study the kinetics of fluoride binding by horse radish peroxidase.

Experimental Section

Pure HRP (RZ 3.00, lot no. 6485206) and crude HRP (RZ 0.70) were obtained from Boehringer-Mannheim Corp., N. Y. The pure sample, precipitated under ammonium sulfate, was dissolved in water and dialyzed exhaustively against water. Reagent grade sodium fluoride (Merck and Co.) was used without further purification.

Solutions for the temperature-jump experiments consisted of 4×10^{-6} M HRP, buffer of ion strength $\mu = 0.01$, plus sodium fluoride and sufficient potassium nitrate so that the total ionic strength was constant. For most experiments $\mu = 0.11$; for some experiments at pH 7 and greater, $\mu = 0.21$. The fluoride concentration varied from 10^{-4} to 0.2 M. Maleate buffer was used over the pH range 4.10–6.00 and phosphate buffer from pH 6.00 to 7.90. Care was taken to perform experiments on solutions of pH 5.00 or less within a few minutes of their preparation (Maehly, 1955; Theorell, 1943). The absorbance of the peroxidase–fluoride complex at pH 4.10 was found to decrease 0.5% in 30 min.

The temperature-jump apparatus is described elsewhere (Diven *et al.*, 1965). Light of wavelength 403 mμ was used for absorption measurements (Keilin and Hartree, 1951).

Results

A single relaxation process was observed under all experimental conditions. The concentration dependence of the relaxation time, τ, at any given pH corresponds to the following simple equation[2]

$$P + F \underset{k_{-1app}}{\overset{k_{1app}}{\rightleftarrows}} PF \qquad (1)$$

and is given by

$$1/\tau = k_{-1app} + k_{1app}[(\bar{P}) + (\bar{F})] \qquad (2)$$

(Eigen and de Maeyer, 1963). In all experiments $(\bar{F}) \gg (\bar{P})$ so that eq 2 can be simplified to

$$1/\tau = k_{-1app} + k_{1app}(F)^\circ \qquad (3)$$

The apparent rate constants, k_{1app} and k_{-1app}, are determined from the slopes and intercepts of plots of $1/\tau$ *vs.* $(F)^\circ$. The results of least-squares analyses of such plots with standard deviations for data obtained using pure HRP are shown in Table I and a corresponding plot of log k_{1app} *vs.* pH is shown in Figure 1. Results obtained at pH 6.00 using maleate and phos-

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[1] Abbreviations used: HRP or P, ferric horse radish peroxidase; RZ, purity number; μ, ionic strength; τ, relaxation time; F and PF, all ionizable species of fluoride and complex, respectively; $(\bar{P})$ and $(\bar{F})$, equilibrium concentrations of all forms of peroxidase and unbound fluoride, respectively; $(F)^\circ$, total concentration of fluorides in solution; HP, protonated form of peroxidase.

[2] We use the symbols $\rightleftarrows$ for reactions occurring at measurable rates, and $\leftrightharpoons$ for reactions which are too fast to measure (King, 1964).

TABLE I: Data for the Binding of Fluoride by Peroxidase at 25°.[a]

pH	Experimental Rate Constants with Standard Deviations		$K = (\bar{P})(\bar{F})/(\bar{PF})$	
	k_{1app} (M^{-1} sec^{-1})	k_{-1app} (sec^{-1})	From Rate Data	Titration Data (crude HRP)
4.10	$(2.0 \pm 0.3) \times 10^6$	$(2.5 \pm 0.5) \times 10^2$	$(1.2 \pm 0.4) \times 10^{-4}$	
4.25	$(1.3 \pm 0.2) \times 10^6$	$(2.5 \pm 0.5) \times 10^2$	$(1.9 \pm 0.5) \times 10^{-4}$	
4.50	$(1.2 \pm 0.2) \times 10^6$	$(2.7 \pm 0.5) \times 10^2$	$(2.4 \pm 0.7) \times 10^{-4}$	
4.75	$(6.8 \pm 0.7) \times 10^5$	$(2.6 \pm 0.5) \times 10^2$	$(3.8 \pm 1.1) \times 10^{-4}$	
5.00	$(5.4 \pm 0.4) \times 10^5$	$(3.1 \pm 0.5) \times 10^2$	$(5.7 \pm 1.4) \times 10^{-4}$	$(9 \pm 5) \times 10^{-4}$
5.50	$(1.7 \pm 0.2) \times 10^5$	$(3.1 \pm 0.5) \times 10^2$	$(1.8 \pm 0.4) \times 10^{-3}$	$(2 \pm 1) \times 10^{-3}$
6.00	$(5.2 \pm 0.4) \times 10^4$	$(4.6 \pm 0.7) \times 10^2$	$(8.8 \pm 2.2) \times 10^{-3}$	$(7 \pm 3) \times 10^{-3}$
6.25	$(2.7 \pm 0.3) \times 10^4$	$(4.2 \pm 0.6) \times 10^2$	$(1.6 \pm 0.4) \times 10^{-2}$	
6.50	$(1.4 \pm 0.1) \times 10^4$	$(3.9 \pm 0.6) \times 10^2$	$(2.8 \pm 0.7) \times 10^{-2}$	$(2 \pm 1) \times 10^{-2}$
6.75	$(8.0 \pm 0.7) \times 10^3$	$(3.8 \pm 0.6) \times 10^2$	$(4.8 \pm 1.2) \times 10^{-2}$	
7.00	$(4.6 \pm 0.5) \times 10^3$	$(4.6 \pm 0.7) \times 10^2$	$(1.0 \pm 0.3) \times 10^{-1}$	$(1.0 \pm 0.5) \times 10^{-1}$
7.25	$(2.7 \pm 0.3) \times 10^3$	$(3.7 \pm 0.6) \times 10^2$	$(1.4 \pm 0.3) \times 10^{-1}$	
7.50	$(1.8 \pm 0.2) \times 10^3$	$(4.5 \pm 0.7) \times 10^2$	$(2.5 \pm 0.6) \times 10^{-1}$	$(7 \pm 4) \times 10^{-2}$
7.75	$(1.3 \pm 0.2) \times 10^3$			
7.90	$(9.0 \pm 1.5) \times 10^2$			

[a] $\mu = 0.11$ except for pH 7.75 and 7.90 where $\mu = 0.21$.

phate buffers agreed within experimental error. The values of k_{1app} obtained at $\mu = 0.21$ with pure HRP also agreed within experimental error with those obtained at $\mu = 0.11$ for pH 7.00, 7.25, and 7.50. The values of k_{-1app} for pH $\geq$ 7.00, $\mu = 0.21$ fell within the range $(2.3 \pm 0.5) \times 10^2$ sec^{-1}. The k_{1app} values obtained using crude HRP agreed within experimental error with those for pure HRP, whereas the k_{-1app} values for crude HRP were consistently about 25% smaller than for pure HRP.

The values of the equilibrium constant, $K = (\bar{P})(\bar{F})/(\bar{PF})$, obtained from k_{-1app}/k_{1app} for pure peroxidase are shown in Table I and in Figure 2, where they are compared with results from titration data obtained at variable ion strength (Theorell, 1943). Also shown in Table I are values of K obtained for crude peroxidase by spectrophotometric titration at constant ion strength (Goldsack *et al.*, 1966).

Discussion

The pK for hydrofluoric acid ionization is 3 at 25° and the equilibrium constant for the reaction $HF + F^- = HF_2^-$ is 3.9 (Bell, 1959). At the lowest pH

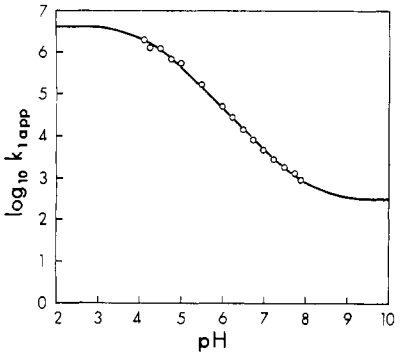

FIGURE 1: Plot of log k_{1app} *vs.* pH fluoride binding by pure peroxidase. Circles are experimental points. Solid line is calculated from eq 4 with $k_3 = 4.2 \times 10^6$ M^{-1} sec^{-1}, $k_4 = 3.2 \times 10^2$ M^{-1} sec^{-1}, and $K = 8.8 \times 10^{-5}$ M.

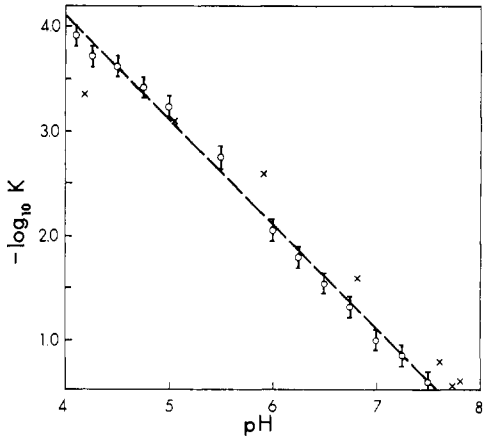

FIGURE 2: Plot of $-\log K$ *vs.* pH for the pure peroxidase–fluoride complex. Points with magnitudes of uncertainties were obtained from k_{-1app}/k_{1app} data (Table I) at constant ion strength. Crosses are titration data (Theorell, 1943).

and under the conditions used in our experiments, the concentration of fluoride ion is larger than that of bifluoride ion by a factor of at least 10^4. We shall consider henceforth only HF and F^- as possible binding species.

The simplest possible mechanism (I) to consider involves fluoride ion binding by peroxidase which has no heme-linked ionizable groups. Mechanism I, represented by eq 1, with F = fluoride ion, predicts that within experimental error there would be no pH dependence of the values of k_{1app}, and so can be eliminated. If binding by the neutral ligand is also considered, the mechanism becomes

$$\begin{array}{ccc} P + HF & \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} & PHF \\ K_L \uparrow\downarrow & & \uparrow\downarrow \\ P + F^- & \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} & PF \end{array} \qquad \text{(II)}$$

The above mechanism has been treated elsewhere (George and Tsou, 1952; Goldsack *et al.*, 1966). If it were valid, then a plot of $k_{1app}(1 + K_L/(H^+))$ *vs.* $1/(H^+)$ should be linear. Such a plot, shown in Figure 3, rules against the validity of mechanism II.

The next simplest mechanism to consider involves fluoride ion binding to peroxidase with a single heme-linked acid group. Two forms of peroxidase, P and HP, which differ by a single proton, now need to be considered. We shall ignore total charges in our representation of these species.

$$\begin{array}{ccc} HP + F^- & \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} & HPF \\ K \uparrow\downarrow & & \uparrow\downarrow K_e \\ P + F^- & \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} & PF \end{array} \qquad \text{(III)}$$

According to mechanism III, the pH dependence of

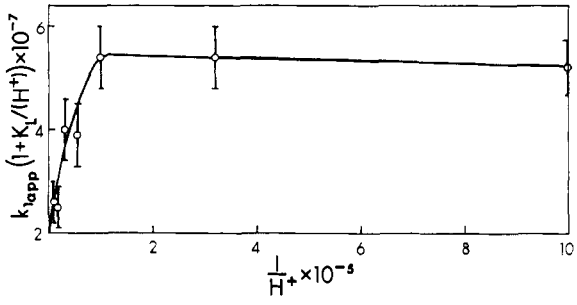

FIGURE 3: Test of mechanism for HF and F^- binding to peroxidase with no heme-linked acid groups (mechanism II). If the mechanism were valid the plot of $k_{1app}(1 + K_L/(H^+))$ *vs.* $1/(H^+)$ would be linear.

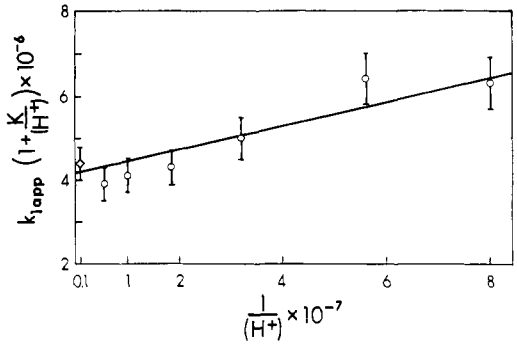

FIGURE 4: Plot of $k_{1app}(1 + K/(H^+))$ *vs.* $1/(H^+)$, where $K = 8.8 \times 10^{-5}$ M, to test mechanism for F^- binding to peroxidase with one heme-linked acid group (mechanism III). Points for pH $\leq$ 6.5, $1/(H^+) \leq 0.3 \times 10^7$, with a mean value of $(4.4 \pm 0.4) \times 10^6$, are summarized by the single point ◇. All experimental values from Table I were given equal weight in the least-squares calculation.

the bimolecular rate constants is given by

$$k_{1app} = \frac{k_3}{1 + K/(H^+)} + \frac{k_4}{1 + (H^+)/K} \qquad (4)$$

A nonlinear least-squares analysis of eq 4 was performed (University of Wisconsin Computing Center, 1966). In order to give roughly equal weight to each experimental point, both sides of eq 4 were divided by (H^+). The best-fit values obtained from the analysis are $k_3 = (4.2 \pm 2.5) \times 10^6$ M^{-1} sec^{-1}, $k_4 = (3.2 \pm 1.3) \times 10^2$ M^{-1} sec^{-1}, and $K = (8.8 \pm 6) \times 10^{-5}$ M. Errors quoted are 95% confidence limits. The solid line shown in Figure 1 is computed from eq 4 using the above values for the parameters k_3, k_4, and K. The following paragraphs discuss alternative ways of testing eq 4 in order to check the results of the nonlinear least-squares analysis.

Equation 4 can be rearranged into the form

$$k_{1app}(1 + K/(H^+)) = k_3 + k_4K/(H^+) \qquad (5)$$

A linear least-squares plot of the left-hand side of eq 5 *vs.* $1/(H^+)$, for $K = 8.8 \times 10^{-5}$ M is shown in Figure 4. Values of k_3 and k_4 obtained from the intercept and slope of this plot are identical with those quoted above. Equation 4 can be simplified under certain conditions, depending on the relative magnitudes of the various parameters. At high pH, k_{1app} should equal k_4, and at low pH it should equal k_3. From the data in Table I and Figure 1 it can be seen that these regions of pH were not studied. The limitations in the present study were the stability of the enzyme–fluoride complex at low pH and the concentration of ligand attainable at high pH. Results obtained for $\mu = 0.21$ at pH 7.75 and 7.90, where the higher ion strength permitted larger amounts of fluoride to

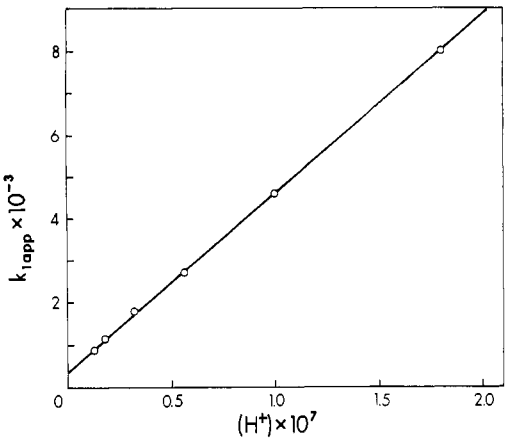

FIGURE 5: Plot of k_{1app} *vs.* (H^+). From the slope, $k_3/K = 4.3 \times 10^{10}$, and from the intercept, $k_4 = 3.5 \times 10^2$ M^{-1} sec^{-1} (eq 6).

be added, were compared directly with results obtained at lower pH values for $\mu = 0.11$ (Table I and Figure 1). Sufficient ligand could not be added to the solutions above pH 8 to attain acceptable signal:noise ratios (Diven *et al.*, 1965). At somewhat lower pH values, eq 4 can be simplified to

$$k_{1app} = k_3(H^+)/K + k_4 \quad (6)$$

A plot of k_{1app} *vs.* (H^+) for the pH range 6.75–7.90 is shown in Figure 5. From the slope and intercept it is found that $k_3/K = 4.3 \times 10^{10}$ and $k_4 = 3.5 \times 10^2$ M^{-1} sec^{-1}.

At still lower pH values, the rate data is approximated by

$$k_{1app} = k_3(H^+)/K \quad (7)$$

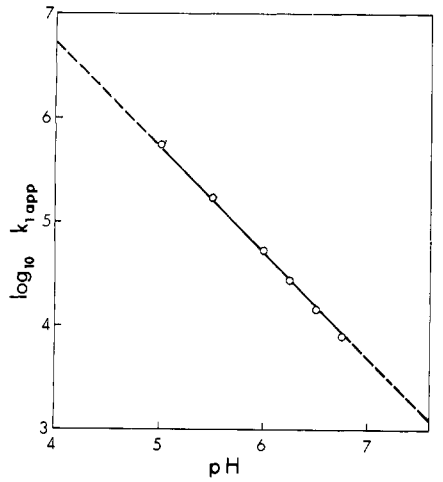

FIGURE 6: Plot of log k_{1app} *vs.* pH. Solid line has a slope of -1. From the intercept at pH 0, $k_3/K = 3.7 \times 10^{10}$ (eq 7).

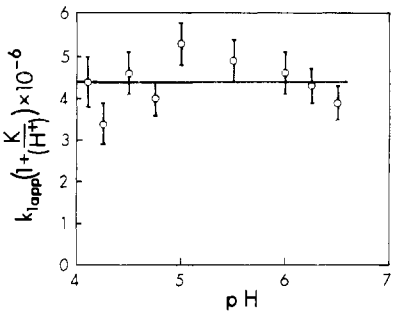

FIGURE 7: $k_{1app}(1 + K/(H^+))$ as a function of pH. The mean value of all points, $k_3 = (4.4 \pm 0.4) \times 10^6$ (eq 8).

A plot with slope of -1 of log k_{1app} *vs.* pH for the region $5.0 \leq$ pH ≤ 6.75 is shown in Figure 6. Calculation of the intercept at zero pH yields a value of $k_3/K = 3.7 \times 10^{10}$.

At the lowest pH values studied, eq 4 can be simplified to

$$k_{1app}(1 + K/(H^+)) = k_3 \quad (8)$$

By a suitable choice of K, the left-hand side of eq 8 should become pH independent. The smallest deviation in the values of k_3 for the pH range 4.1–6.5 is found for $K = 8.8 \times 10^{-5}$ M, and yields an average value of $k_3 = 4.4 \times 10^6$ M^{-1} sec^{-1} (Figure 7).

It can be seen that all of the above methods of analysis of the bimolecular rate constants give self-consistent results within the experimental error. Thus, our experimental results can be accounted for by a mechanism in which fluoride ion binds to the ferric ion of peroxidase, which has one heme-linked acid group with pK = 4.1 ± 0.3. This is consistent with part of the conclusions drawn from a titration study (Theorell and Paul, 1944). Our data do not require the postulate of a second heme-linked group, however, nor do we find it necessary to assume that the neutral ligand is the binding species (Chance, 1952).

The k_{-1app} data for $\mu = 0.11$ appear to group around two values, $(2.7 \pm 0.5) \times 10^2$ sec^{-1} for the pH range 4.1–5.5 and $(4.2 \pm 0.5) \times 10^2$ sec^{-1} for the pH range 6.0–7.5. It would appear that an inflection point may occur between pH 5 and 6. This inflection point might be attributed to the dissociation of the heme-linked group from the peroxidase–fluoride complex, HPF $\rightleftarrows$ PF + H^+. The equilibrium constant for this dissociation reaction, shown in mechanism III, has been labeled K_c. Thus pK_c may be 1.5 ± 0.5 units higher than pK because of the effect of the bound fluoride ion.

The fluoride ion is low in the "cloud-expanding" series (Orgel, 1960), which implies a minimum of covalent interaction. It would appear reasonable that the rate of dissociation of fluoride ion from the complex would be accelerated by removal of the heme-linked proton. The extent of this acceleration appears only

slightly outside the experimental error, however, whereas according to mechanism III, the presence of the heme-linked proton increases the binding specific rate constant by a factor of 10^4, a remarkable catalytic effect. Values of k_{1app} for fluoride binding by metmyoglobin are much smaller, ranging from 2×10^{-1} to 5×10^{-2} M^{-1} sec^{-1} over the pH range 6–9 (Blank *et al.*, 1961).

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Properties of Soluble Ribonucleic Acid Methylases from Rat Liver*

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ABSTRACT: The properties of a purified enzyme fraction from rat liver, which catalyzes the transfer of methyl group from *S*-adenosyl-L-methionine to soluble ribonucleic acid (sRNA), were examined. The reaction was found to be stimulated by 0.25 M ammonium ions. The RNA methylase(s) from rat liver are not active in some heterologous systems, and, therefore, seem to be highly specific in their methyl acceptor requirements. The products of the methylation reaction were identified as 5-methylcytosine, 1-methyladenine, thymine, 7-methylguanine, and N^2-methylguanine (or N^2-dimethylguanine). No differences were found in the specific activities of crude sRNA methylase(s) from normal or regenerating rat liver, nor was there any difference in the extent and pattern of methylation during liver regeneration. The RNA methylase(s) are inhibited by the presence of deoxyribonucleic acid (DNA) and synthetic polynucleotides.

Soluble ribonucleic acid from rat liver, as well as sRNA from other sources, contains a number of methylated purine and pyrimidine bases, in addition to the four common bases (Smith and Dunn, 1959; Dunn, 1959, 1961; Sluyser and Bosch, 1962; Price *et al.*, 1963). sRNA isolated from any given source is characterized by the number and type of the methylated bases it contains. The methyl groups are incorporated at the macromolecular level by enzymes which catalyze the transfer of methyl groups from SAM[1] to sRNA. These enzymes can be detected in many organisms including rat liver homogenates (Srinivasan and Borek, 1963). The sRNA methylases are species specific, and consequently, sRNA which is fully saturated with respect to its homologous enzymes offers new sites for

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[1] Abbreviations used: CTAB, cetyltrimethylammonium bromide; DTE, dithiothreitol; SAM, *S*-adenosyl-L-methionine; SAE, *S*-adenosyl-L-ethionine; TCA, trichloroacetic acid.