The Kinetics of Cyanide Binding by Lactoperoxidase*

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ABSTRACT: The kinetics of the binding reaction of cyanide by the enzyme ferric bovine lactoperoxidase at 25° have been studied over the pH range 4.55–10.60 by means of the temperature-jump technique. The pH dependence of the association rate data is most simply explained by the presence of two ionizable groups in the active site of the enzyme with molecular ionization constants of 4.9×10^{-7} and 2.4×10^{-8} M which correspond to pK values usually attributed to the imidazole group of histidine residues and to the free α-amino groups of amino acid residues. The ionizable groups are not ligands of the heme.

Kinetic studies of the association reactions between hemoproteins and ligands such as cyanide, azide, and fluoride can in principle yield information as to whether ionizable groups affect the reactivity of the heme moiety (Alberty, 1956). For this purpose we have undertaken a temperature-jump kinetic study of the binding of cyanide to lactoperoxidase over the pH range 4.55–10.60.

Experimental Section

Lactoperoxidase with a 412 mμ/280 mμ absorbancy ratio of 0.7–0.9 was isolated from cows' milk by the procedure of Morrison and Hultquist (1963) and stored as an ammonium sulfate precipitate. Stock solutions of lactoperoxidase for the experiments were prepared by dialyzing the precipitate against 0.01–0.05 M phosphate buffer of pH 7.4. The stock solutions were stored at 5° and used within 3 weeks. The absorption spectra of a solution of the most highly purified sample and of its cyanide complex at pH 7.4 were recorded on a Cary Model 14 spectrophotometer. Lactoperoxidase concentrations were determined from absorbance measurements at 412 mμ using a molar absorptivity of 1.14×10^5 M^{-1} cm^{-1} (Morrison *et al.*, 1957).

Doubly distilled water was used to prepare all solutions. Inorganic chemicals, of reagent grade, were used without further purification. Fresh potassium cyanide stock solutions (*ca.* 0.1 M) were made up weekly and standardized before or after use by titration with standard silver nitrate solution (Kolthoff and Sandell, 1952).

A Beckman expanded-scale pH meter in conjunction with a Beckman 39183 combination electrode was used for pH measurements and a Beckman DU spectrophotometer for routine absorption measurements. The temperature-jump apparatus was built in this laboratory and has been described elsewhere (B. B. Hasinoff, H. B. Dunford, and D. G. Horne, to be submitted). The discharge from a 0.1-μF capacitor charged to 22 kV was used to cause a temperature jump of about 6°. The reaction cell was maintained at 19° so that the temperature of the kinetic experiments was approximately 25°.

For each temperature-jump experiment 10 ml of a solution was used that contained 0.1 M potassium nitrate (necessary as an electrolyte to conduct the discharge current), 2.9×10^{-6}–5.6×10^{-6} M lactoperoxidase, 8×10^{-6}–1.3×10^{-3} M KCN, and a buffer of ionic strength 0.01 or 0.05. The total ionic strength was either 0.11 or 0.15. The buffers and the pH ranges covered by them were: acetate, 4.55–5.15; cacodylate, 5.80; phosphate, 6.23–8.00; borate, 8.00–9.50; and glycinate, 9.50–10.60.

Temperature-jump experiments at a given pH were conducted on six to eight solutions covering a range of cyanide concentrations varying by a factor of about 10. The relaxation to the new equilibrium after the temperature perturbation was observed spectrophotometrically at 430 mμ and the results were displayed on an oscilloscope. Photographs of the oscilloscope traces were taken and values of the voltage at a given time were read from them using a photographic enlarger. In general three of four experiments were performed on any one solution and the average of the relaxation time constants was taken.

Differences between the amplified photomultiplier voltages displayed on the oscilloscope are directly proportional to differences in the intensity of light transmitted by the reaction solution. These, in turn, because of the small over-all change (*ca.* 1–3%) in transmitted light intensity during the reaction are good approximations of absorbance differences. Consequently plots of ln $(V_\infty - V_t)$[1] *vs.* time were used to

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: LP, lactoperoxidase; CN and PCN, all forms of cyanide and complex; H_2P, HP, and P, states of ionization of lactoperoxidase; $[\overline{LP}]$ and $[\overline{CN}]$, equilibrium concentrations of all forms of lactoperoxidase and cyanide; $[CN]_0$, initial concentration of all forms of cyanide; K_L, acid dissociation constant of ligand; K_{diss}, apparent dissociation constant of lactoperoxi-

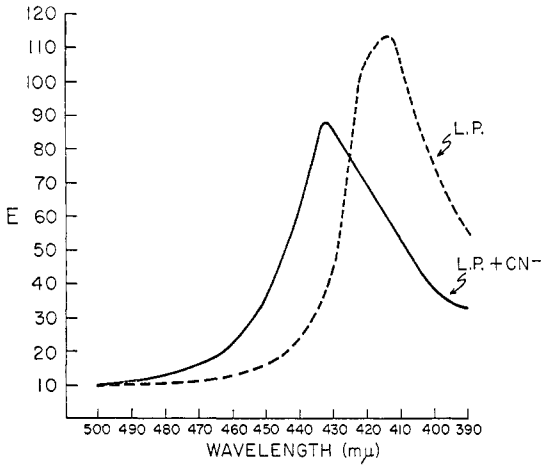

FIGURE 1: Millimolar absorptivities, E (mM^{-1} cm^{-1}), of LP and the LP–CN complex at pH 7.4 in phosphate buffer.

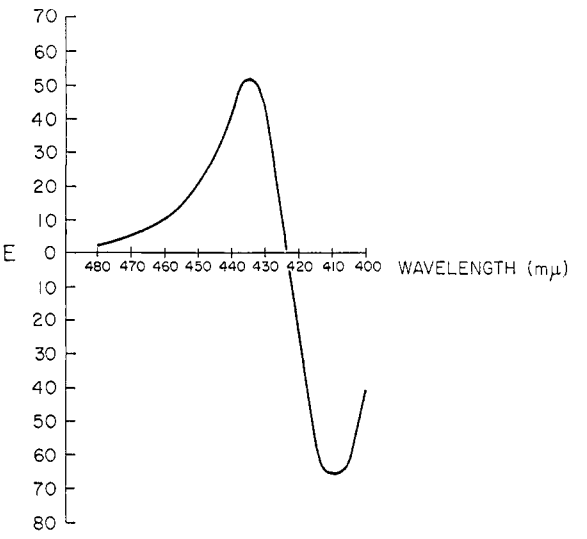

FIGURE 2: Difference spectra for the absorption curves shown in Figure 1.

determine the relaxation time constants, τ, which ranged between 5 and 100 msec.[2] Values of K_{diss} were determined at several pH values by a spectrophotometric titration method (Goldsack *et al.*, 1966).

Results

The absorption spectra of pure LP and its cyanide complex in the Soret region are shown in Figure 1 and the corresponding difference spectrum in Figure 2. The Soret maximum of the LP–CN complex occurs at 432 mμ with a molar absorptivity of 8.9×10^4 M^{-1} cm^{-1}. There are no significant spectral shifts in the LP spectrum over the pH range 4–11.

The kinetic and equilibrium behavior of the lactoperoxidase–cyanide system at a given pH is consistent with the stoichiometric equation[3]

$$LP + CN \underset{k_{-1app}}{\overset{k_{1app}}{\rightleftharpoons}} LPCN \qquad (1)$$

for which the reciprocal of the time constant describing the relaxation to equilibrium for a system near equilibrium is given by eq 2 (Eigen and de Maeyer, 1963).

$$\frac{1}{\tau} = k_{1app}([\overline{LP}] + [\overline{CN}]) + k_{-1app} \qquad (2)$$

The values of $[\overline{LP}]$ and $[\overline{CN}]$ could not always be calculated directly because the dissociation equilibrium constants for the complex were not known at all pH values for the kinetic experiments. However, the concentration of cyanide was generally much greater than that of lactoperoxidase so that the sum ($[\overline{LP}] + [\overline{CN}]$) could be approximated at first by $[CN]_0$. Approximate values for k_{1app} and k_{-1app} were obtained then from the slope and intercept of a plot of $1/\tau$ *vs.* $[CN]_0$ using least-mean-squares analysis. Next, values of the sum ($[\overline{LP}] + [\overline{CN}]$) were calculated using k_{-1app}/k_{1app} for the dissociation equilibrium constant and these values were used in a plot of $1/\tau$ *vs.* $[\overline{LP}] + [\overline{CN}]$. New values of k_{1app} and k_{-1app} were thus obtained and the whole procedure was repeated until the value of k_{1app} changed by $< 2\%$ from one cycle to the next. In no case was more than one iteration required. This procedure was adopted at every pH and the calculations were carried out by computer. Examples of plots of $1/\tau$ *vs.* $\log([\overline{LP}] + [\overline{CN}])$ are shown in Figure 3 for experiments at pH 5.80, 7.80, and 8.40.

The values of k_{1app} and k_{-1app} obtained in this manner are listed in Table I. Figure 4 is a plot of k_{1app} *vs.* pH which is a curve with a sharp maximum near pH 7.2. Because lactoperoxidase from several batches was used and because of errors inherent in the temperature-jump technique we feel that the k_{1app} and k_{-1app} values probably are accurate to within 10 and 20%.

Table I also contains the apparent dissociation equilibrium constants obtained from kinetic data as well as from equilibrium spectrophotometric measurements. Plots were made using the spectrophotometric measurements at pH 5.80 and 8.40 to determine the stoichiometry of the reaction between lactoperoxidase and cyanide. The slope of a plot of $\log((A - A_0)/(A_\infty - A))$ *vs.* $\log [\overline{CN}]$ indicates the number of cyanide molecules binding to the enzyme (Scheler, 1960). The slopes obtained at pH 5.80 and 8.40 were 1.0 and 1.1 indicating within experimental error a 1:1 ratio of cyanide to heme in the complex.

dase–cyanide complex; k_{1app} and k_{-1app}, apparent second-order association rate constant and apparent first-order dissociation rate constant; V_t and V_∞, oscilloscope display voltage at any time t during the relaxation to equilibrium and at the new equilibrium; A_0, A, and A_∞, absorbances of lactoperoxidase solutions in a 1-cm cuvet with no cyanide added, a known amount of cyanide added, and an excess of cyanide added such that complex formation is complete; HRP, horseradish peroxidase.

[2] For small temperature perturbations the relaxation process follows first-order kinetics for which the rate constant, k, is equal to the reciprocal of the relaxation time constant, τ.

[3] We use the symbols $\rightleftarrows$ for reactions occurring at measurable rates and $\rightleftharpoons$ for reactions in which equilibrium is assumed to be maintained (King, 1964).

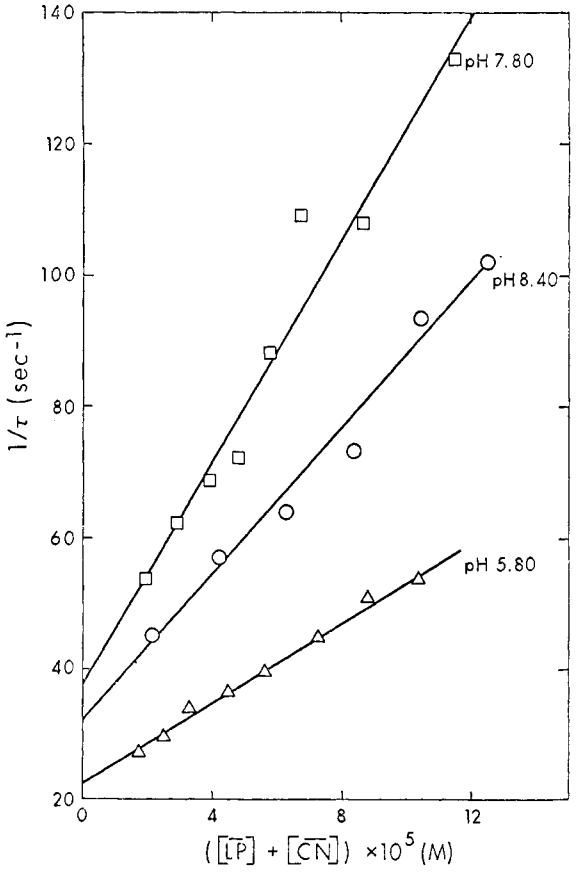

FIGURE 3: Plot of reciprocal relaxation time against equilibrium concentrations of free enzyme and cyanide at various pH values.

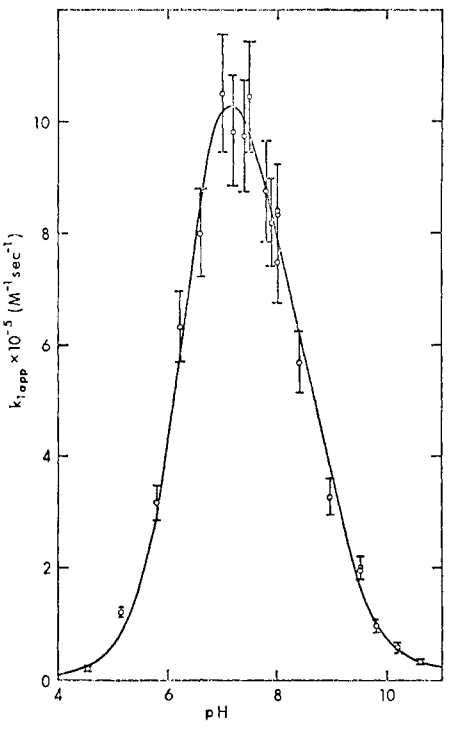

FIGURE 4: Plot of $k_{1\,app}$ *vs.* pH for the binding of cyanide to lactoperoxidase with error limits of ±10% shown for the $k_{1\,app}$ values. The solid line is calculated using the best-fit parameters of reaction II.

The kinetic and spectrophotometric determinations of the apparent dissociation equilibrium constants agree within experimental error. Similarly, the rate constants determined at the same pH but at different buffer ionic strengths, or at the same pH but using different buffers agree within experimental error indicating no buffer effects on the kinetics of the reaction.

Discussion

The spectral shift of the Soret maximum from 412 to 432 mμ upon cyanide complex formation can be compared with the corresponding shift in the HRP spectrum (Keilin and Hartree, 1951). The change in spectrum between LP and LP–CN would appear to correspond to the change from a high-spin to a low-spin complex (Brill and Williams, 1961).

The pK_a for the dissociation of HCN is 9.21 at 25° (Izatt *et al.*, 1962). Correction of this value for the effect of ionic strength by an extended Debye–Hückel equation (Davies, 1938) yields a value of 8.97 ± 0.01 for the pK of HCN at ionic strengths from 0.11 to 0.15.

From an inspection of Figure 4 it is obvious that the pH dependence of the forward rate constant, $k_{1\,app}$, can only be explained by the presence of ionizable groups on the enzyme which affect the rate of cyanide complex formation. Below pH 7 the cyanide is present almost completely as HCN and similarly above pH 11 as CN^-. If lactoperoxidase had no ionizable groups involved in cyanide complex formation then $k_{1\,app}$ should have limiting values at low and high pH and the $k_{1\,app}$ values at intermediate pH should lie between these extremes. This is not the case and therefore a mechanism capable of explaining the rate data must involve at least one ionizable group.

The next simplest possibility is reaction I, involving two species of lactoperoxidase reacting with CN^- and HCN. Protons involved in ionization equilibria have

$$\begin{array}{ccc} HP + \left\{\begin{array}{l} CN^- \xrightarrow{k_1} \\ HCN \xrightarrow{k_2} \end{array}\right\} & HP(CN) \\ \Big\downarrow\uparrow K_1 & \\ P + \left\{\begin{array}{l} CN^- \xrightarrow{k_3} \\ HCN \xrightarrow{k_4} \end{array}\right\} & P(CN) \end{array} \qquad (I)$$

been omitted from this reaction as have charges on the enzyme species since they are unknown. The form in which cyanide is bound in the complex and also the dissociation reaction paths have not been indicated since we concern ourselves here only with the association rate data. The equation for $k_{1\,app}$ in terms of this mechanism is

$$k_{1\,app} = \frac{k_1 + \dfrac{k_2(H^+)}{K_L} + \dfrac{k_3 K_1}{(H^+)} + \dfrac{k_4 K_1}{K_L}}{1 + \dfrac{K_1}{(H^+)} + \dfrac{(H^+)}{K_L} + \dfrac{K_1}{K_L}} \qquad (3)$$

where K_L is the dissociation constant of HCN corrected for ionic strength effects as outlined above. This equation takes into account the pH-dependent partitioning of cyanide between the two species HCN and CN^-.

With the aid of a nonlinear least-squares program[4] eq 3 can be used to yield the best-fit values for the rate constants, k_1 to k_4, and for the dissociation constant, K_1, from an analysis of the k_{1app} data. Because the k_{1app} values cover a range of 10^2 the program was modified to minimize the sum of the squares of the relative residuals rather than the sum of the squares of the absolute residuals. The k_{1app} data could not be fitted within experimental error using eq 3. Consequently a mechanism capable of explaining the observed forward rate data must involve more than one ionizable group in the active site of the enzyme.

The simplest mechanism that will explain the data satisfactorily involves two such groups and in its general form can be written as reaction II. From this

$$\begin{array}{l} H_2P + \left\{\begin{array}{l} CN^- \xrightarrow{k_5} \\ HCN \xrightarrow{k_6} \end{array}\right\} H_2P(CN) \\ \quad\downarrow\uparrow K_2 \\ HP + \left\{\begin{array}{l} CN^- \xrightarrow{k_7} \\ HCN \xrightarrow{k_8} \end{array}\right\} HP(CN) \\ \quad\downarrow\uparrow K_3 \\ P + \left\{\begin{array}{l} CN^- \xrightarrow{k_9} \\ HCN \xrightarrow{k_{10}} \end{array}\right\} P(CN) \end{array} \qquad \text{(II)}$$

it can be shown that

$$k_{1app} = \frac{k_5 + \dfrac{k_7K_2}{(H^+)} + \dfrac{k_9K_2K_3}{(H^+)^2}}{\left(1 + \dfrac{K_2}{(H^+)} + \dfrac{K_2K_3}{(H^+)^2}\right)\left(1 + \dfrac{(H^+)}{K_L}\right)} + \frac{k_6 + \dfrac{k_8K_2}{(H^+)} + \dfrac{k_{10}K_2K_3}{(H^+)^2}}{\left(1 + \dfrac{K_2}{(H^+)} + \dfrac{K_2K_3}{(H^+)^2}\right)\left(1 + \dfrac{K_L}{(H^+)}\right)} \qquad (4)$$

In the above scheme we have chosen to use molecular ionization constants rather than group ionization constants (Dixon and Webb, 1964) in analyzing the rate data. The group ionization constants for a mechanism involving two ionizable groups cannot be determined from an analysis of the kinetic data alone. Nevertheless the expressions for k_{1app} derived from schemes using either molecular or group ionization constants are mathematically equivalenet with the result that the best-fit parameters for either version would predict identical values of k_{1app} at a given pH.

There exist four simplified forms of reaction II (reactions IIa–d) which will reproduce the forward rate data within experimental error and which in fact predict exactly the same values of k_{1app} at any given pH. Rate constants for reaction paths that have been

$$\begin{array}{l} H_2P + CN^- \xrightarrow{k_5} H_2P(CN) \\ \quad\downarrow\uparrow K_2 \\ HP + CN^- \xrightarrow{k_7} HP(CN) \\ \quad\downarrow\uparrow K_3 \\ P \quad + CN^- \xrightarrow{k_9} P(CN) \end{array} \qquad \text{(IIa)}$$

$$\begin{array}{l} H_2P + CN^- \xrightarrow{k_5} H_2P(CN) \\ \quad\downarrow\uparrow K_2 \\ HP \\ \quad\downarrow\uparrow K_3 \left\{\begin{array}{l} CN^- \xrightarrow{k_6} \\ HCN \xrightarrow{k_{10}} \end{array}\right\} P(CN) \\ P \end{array} \qquad \text{(IIb)}$$

$$\begin{array}{l} H\ P \\ \downarrow\uparrow K_2 \quad \downarrow\uparrow K_3 \\ HP + \left\{\begin{array}{l} CN^- \xrightarrow{k_7} \\ HCN \xrightarrow{k_8} \end{array}\right\} HP(CN) \\ P + CN^- \xrightarrow{k_9} P(CN) \end{array} \qquad \text{(IIc)}$$

$$\begin{array}{l} H_2P \\ \quad\downarrow\uparrow K_2 \\ HP + HCN \xrightarrow{k_8} HP(CN) \\ \quad\downarrow\uparrow K_3 \\ P + \left\{\begin{array}{l} CN^- \xrightarrow{k_9} \\ HCN \xrightarrow{k_{10}} \end{array}\right\} P(CN) \end{array} \qquad \text{(IId)}$$

omitted in each scheme have effective values of zero. The best-fit values for the rate and equilibrium constants for each simplified mechanism obtained using the nonlinear least-squares program and the appropriate simplified forms of eq 4 are listed in Table II. The fit of the k_{1app} values predicted by eq 4 to the experimental data using any of the four sets of constants in Table II is shown in Figure 4 where the solid line gives the predicted values of k_{1app}.

The existence of four simplified versions of reaction II giving equivalent fits to the data is due to the inability of the kinetic method to distinguish between reaction paths such as

$$H_2P + CN^- \xrightarrow{k_5}$$

and

$$HP + HCN \xrightarrow{k_8}$$

in which the total number of ionizable protons on the two reactants is the same but the positions of their attachment are different. Although some of the simplified forms of reaction II may appear more reasonable than others there is no reason on purely kinetic grounds to favor any one over another. As well as the four simplified forms of reaction II any appropriate linear

[4] IBM SHARE library (1964), PROGRAM SDA 3094, modified for use on a University of Alberta IBM 360 computer.

TABLE I: Rate and Equilibrium Constants for the Binding of Cyanide by Lactoperoxidase at 25°.

pH	Buffer	Total Ionic Strength	Exptl Apparent Rate Constants[a] k_{1app} (M^{-1} sec^{-1})	Exptl Apparent Rate Constants[a] k_{-1app} (sec^{-1})	$K_{diss} = \frac{[\overline{LP}][\overline{CN}]}{[\overline{LPCN}]}$ From Kinetic Data	K_{diss} Spectrophotometric Determinations
4.55	Acetate	0.11	1.9×10^4	7	3.7×10^{-4}	
5.15	Acetate	0.11	1.2×10^5	11	9.2×10^{-5}	
5.80	Cacodylate	0.11	3.2×10^5	22	6.8×10^{-5}	6.9×10^{-5}
6.23	Phosphate	0.11	6.3×10^5	22	3.5×10^{-5}	
6.60	Phosphate	0.11	8.0×10^5	28	3.5×10^{-5}	2.9×10^{-5}
7.00	Phosphate	0.11	10.5×10^5	20	1.9×10^{-5}	4.2×10^{-5}
7.20	Phosphate	0.11	9.8×10^5	34	3.5×10^{-5}	
7.40	Phosphate	0.11	9.7×10^5	28	2.9×10^{-5}	
7.55	Phosphate	0.11	10.5×10^5	29	2.8×10^{-5}	3.3×10^{-5}
7.80	Phosphate	0.11	8.8×10^5	36	4.1×10^{-5}	
7.90	Phosphate	0.11	8.2×10^5	36	4.4×10^{-5}	5.0×10^{-5}
8.00	Phosphate	0.15	8.4×10^5	29	3.5×10^{-5}	
8.00	Phosphate	0.11	8.4×10^5	31	3.7×10^{-5}	
8.00	Borate	0.11	7.5×10^5	29	3.9×10^{-5}	
8.40	Borate	0.15	5.7×10^5	31	5.4×10^{-5}	5.5×10^{-5}
8.97	Borate	0.15	3.3×10^5	33	1.0×10^{-4}	
9.50	Borate	0.11	2.0×10^5	37	1.9×10^{-4}	
9.50	Glycinate	0.11	2.0×10^5	28	1.4×10^{-4}	
9.80	Glycinate	0.15	9.5×10^4	32	3.4×10^{-4}	2.5×10^{-4}
10.20	Glycinate	0.15	5.8×10^4	30	5.2×10^{-4}	3.0×10^{-4}
10.60	Glycinate	0.15	3.2×10^4	37	1.2×10^{-3}	

[a] The k_{1app} and k_{-1app} values are probably accurate to within 10 and 20%.

TABLE II: Rate and Equilibrium Constants of Simplified Forms of Mechanism II.[a]

Constant	a	b	c	d
k_5	$(6.0 \pm 1.1) \times 10^8$ M^{-1} sec^{-1}	$(6.0 \pm 1.1) \times 10^8$ M^{-1} sec^{-1}	*b*	*b*
k_6	*b*	*b*	*b*	*b*
k_7	$(1.6 \pm 0.3) \times 10^7$ M^{-1} sec^{-1}	*b*	$(1.6 \pm 0.3) \times 10^7$ M^{-1} sec^{-1}	*b*
k_8	*b*	*b*	$(1.3 \pm 0.2) \times 10^6$ M^{-1} sec^{-1}	$(1.3 \pm 0.2) \times 10^6$ M^{-1} sec^{-1}
k_9	$(1.7 \pm 1.4) \times 10^4$ M^{-1} sec^{-1}	$(1.7 \pm 1.4) \times 10^4$ M^{-1} sec^{-1}	$(1.7 \pm 1.4) \times 10^4$ M^{-1} sec^{-1}	$(1.7 \pm 1.4) \times 10^4$ M^{-1} sec^{-1}
k_{10}	*b*	$(6.9 \pm 1.4) \times 10^5$ M^{-1} sec^{-1}	*b*	$(6.9 \pm 1.4) \times 10^5$ M^{-1} sec^{-1}
K_2	$(4.9 \pm 1.8) \times 10^{-7}$ M	$(4.9 \pm 1.8) \times 10^{-7}$ M	$(4.9 \pm 1.8) \times 10^{-7}$ M	$(4.9 \pm 1.8) \times 10^{-7}$ M
K_3	$(2.4 \pm 0.6) \times 10^{-8}$ M	$(2.4 \pm 0.6) \times 10^{-8}$ M	$(2.4 \pm 0.6) \times 10^{-8}$ M	$(2.4 \pm 0.6) \times 10^{-8}$ M

[a] The error limits given are approximately 95% confidence limits from the nonlinear least-squares analysis. [b] Signifies a rate constant effectively equal to zero.

combination of them is capable of giving the same fit to the experimental data.

It should be emphasized that although there is uncertainty about which enzyme and ligand species are reacting there is none regarding the existence of the ionizable groups on the enzyme involved in the binding reaction. All four versions of the reaction give the same values $(4.9 \pm 1.8) \times 10^{-7}$ and $(2.4 \pm 0.6) \times$

10^{-8} M, for K_2 and K_3, respectively, from the analysis of the forward rate data. The corresponding pK values for these ionizable groups are 6.3 and 7.6. Neglecting possible differences between molecular and group ionization constants, the former value falls within the range of pK values usually attributed to the imidazole group of histidine residues and the latter to the α-amino group of amino acid residues (Dixon and Webb, 1964). Structural studies have already established that LP contains only a single free α-amino group, the end terminal leucine (Rombauts *et al.*, 1967). Since there are no spectral shifts for LP in the pH range of the present study, it is unlikely that these groups are ligands coordinated to the heme iron. Further studies will be required to establish clearly the nature of the ionizable groups and their role in enzymatic catalysis.

No attempt was made to analyze the reverse-rate data because of the large uncertainty in the rate constants relative to the over-all changes in k_{-1app} with pH.

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