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A Study of Ligand Binding to Spleen Myeloperoxidase†

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Received December 1, 1986; Revised Manuscript Received March 11, 1987

ABSTRACT: The ligand binding properties of spleen myeloperoxidase, a peroxidase formerly called "the spleen green hemeprotein", were studied as functions of temperature and pH, using chloride and cyanide as exogenous ligands. Ligand binding is influenced by a proton dissociable group with a pK_a of 4. The protonated, uncharged form of cyanide binds to the unprotonated form of the enzyme, while chloride ion binds to the enzyme when this group is protonated. In both cyanide and chloride binding, the pH-dependent change in the apparent ligand affinity is due to a change in the apparent association rate with pH. The proton dissociable group on the enzyme involved in ligand binding has a ΔH value of about 8 kcal·mol^{-1}. The present results suggest that this ionizable group is the imidazole group of a histidine residue located near the ligand binding site.

The presence of a green peroxidase in bovine spleen, with optical absorption properties somewhat similar to those of myeloperoxidase isolated from granulocytes, was first reported by Davis and Averill (1981), who named the enzyme "green heme peroxidase". The enzyme was called "the spleen green hemeprotein" in the paper of Babcock et al. (1984). Resonance Raman data indicate that the structure of the chromophore of the spleen green hemeprotein is identical with that of myeloperoxidase, an iron chlorin (Babcock et al., 1984; Sibbett & Hurst, 1984; Ikeda-Saito et al., 1985). The optical absorption, electron paramagnetic resonance, and ligand binding properties of the spleen enzyme are very similar to those of myeloperoxidase (Ikeda-Saito, 1985). Magnetic and natural circular dichroism studies (Sono et al., 1986b) have further indicated that the spleen green hemeprotein and myeloperoxidase have common prosthetic group environments and endogenous ligands to the central iron. These data have established that the spleen enzyme can be used as a more readily obtainable model to investigate the active center and its environment in myeloperoxidase (Ikeda-Saito, 1985). In their initial paper, Davis and Averill (1981) reported the spleen green hemeprotein as a monomeric peroxidase having a molecular weight of 57 000 with substrate specificity different from that of myeloperoxidase. Recent studies, however, have revealed that the spleen enzyme consists of two heavy subunits (M_r 6×10^4 with a single prosthetic group per subunit) and two light subunits (M_r 1.5×10^4) forming a tetramer of M_r 1.5×10^5, indistinguishable from granulocyte myeloperoxidase (Ikeda-Saito, 1986), and that the spleen enzyme also exhibits catalytic activities identical with those of myeloperoxidase (Ikeda-Saito, 1985). Together, these findings have led to the

† Supported by Research Grant GM-39492 from the National Institutes of Health.

0006-2960/87/0426-4344$01.50/0

proposal that the spleen green hemeprotein and myeloperoxidase are in fact the same (Ikeda-Saito, 1986).[1] Thus, in this paper, the spleen enzyme will be called "spleen myeloperoxidase" rather than "the spleen green hemeprotein". Previous studies on spleen and granulocyte myeloperoxidase suggested the presence of an ionizable group with a pK_a value of 4 on the enzyme (Bolscher & Wever, 1984a; Ikeda-Saito, 1985). When chloride and cyanide were used as exogenous ligands, equilibrium data were analyzed in terms Scheme I where E, K_a, K_{Cl}, and K_{CN} are enzyme, dissociation constant of an ionizable group on the enzyme, dissociation constant for the enzyme–chloride complex, and dissociation constant of the enzyme–cyanide complex (Ikeda-Saito, 1985), respectively.

Scheme I

$$E{\cdot}HCN \underset{HCN}{\overset{K_{CN}}{\leftrightarrow}} E \underset{H^+}{\overset{K_a}{\leftrightarrow}} E{\cdot}H^+ \underset{Cl^-}{\overset{K_{Cl}}{\leftrightarrow}} E{\cdot}H^+{\cdot}Cl^-$$

Although measurement of ligand binding is a classical technique, it has provided useful information about the structure of active sites of peroxidases (Sono et al., 1986a; Campbell et al., 1982; Araiso & Dunford, 1981; Dunford & Stillman, 1976). In order to further characterize the nature of the ionizable group with a pK_a value of 4 on the enzyme, the initial ligand binding study has been extended by including the determination of the rate constants involved in cyanide and chloride binding, and the ΔH of the ionization of this functional group on the enzyme. Cyanide and chloride were chosen primarily because they had been used in the initial study of the spleen enzyme (Ikeda-Saito, 1985) and that of granulocyte myeloperoxidase (Bolscher & Wever, 1984a). Cyanide is a well-established low-spin heme ligand, and cyanide binding mimics a part of the mechanism of compound I formation (Bolscher & Wever, 1984a). There are a number of observations which support the notion that chloride binds directly to the iron center in spleen and granulocyte myeloperoxidase to form a six-coordinate high-spin complex (Bolscher & Wever, 1984a; Ikeda-Saito et al., 1985, Ikeda-Saito, 1985; Ikeda-Saito & Inubushi, 1987). The present results indicate that the ionizable group with a pK_a of 4 is probably the distal histidine residue.

Experimental Procedures

Myeloperoxidase was extracted from bovine spleens obtained from the local abattoir by the method of Davis and Averill (1981) and purified as described previously (Ikeda-Saito, 1985). The preparation had a ratio of absorbance at 428 nm to that at 280 nm of 0.83.

The chloride affinity of the enzyme was measured by a stepwise titration method using a HITACHI 557 spectrophotometer and is expressed in terms of the apparent dissociation constant, K_{app}, which was estimated by an iterative least-squares fitting as described previously (Ikeda-Saito, 1985). The rate of cyanide binding was determined by a stopped-flow apparatus, which consisted of a 50-W halogen lamp light source, a Bausch & Lomb monochromator (33-86-78), an EMI photomultiplier (Model RSI/F), an absorbance detector amplifier (Biomedical Instrumentation Group, University of Pennsylvania), and a Union Giken rapid mixing device (RA-441). The time course of the absorbance change at 458 nm due to the formation of cyanide complex was digitized by a Nicolet digital oscilloscope (Model 3091) and then stored in a computer for analysis. All the kinetics were of single-exponential character, and the rate constants were estimated by iterative least-squares fitting. The enzyme (1.5 μM iron) was mixed with cyanide (between 25 and 250 μM). Under these conditions, the observed rate constant was linearly proportional to the cyanide concentration, and rate saturation was not observed. Three or four measurements were performed at each set of experimental conditions. Similar methods were used to estimate the dissociation rate of cyanide from the enzyme by mixing the enzyme–cyanide complex with buffer solution containing chloride.

Because the rates of chloride binding were found too rapidly to follow with the stopped-flow apparatus, the temperature jump method was used to estimate the chloride association and dissociation rates. The instrument used was based on a Messanalagen Studiengeselschaft type SBA 7 temperature jump unit. A 100-W halogen lamp operated with a Hewlett-Packard Model 6267B direct-current power supply was used as a light source. A transmittance change at 445 nm after a temperature jump of about 5 °C obtained by a discharge of 18 kV was detected by a photodiode detector, and the relaxation spectrum was digitized by the Nicolet digital oscilloscope and stored in the computer. The dead time of the instrument was about 10 μs. All the relaxation spectra were expressed by single-exponential character, and the relaxation time was estimated by a least-squares fitting. The association and dissociation rates were calculated by using the following equations, since the dissociation equilibrium constants for the enzyme–chloride complex were already determined (Ikeda-Saito, 1985):

$$1/\tau = k_{off} + k_{on}([\text{enzyme}] + [\text{chloride}])$$

$$k_{on} = k_{off}/K$$

where τ, k_{off}, k_{on}, K, [enzyme], and [chloride] are relaxation time, dissociation rate constant, association rate constant, dissociation equilibrium constant, equilibrium concentration of the enzyme, and equilibrium concentration of chloride, respectively (Eigen & DeMayer, 1963). The enzyme concentration was 5.3 μM iron chlorin, and the chloride concentration was between 12.5 μM and 2.5 mM. The temperature after a jump was 20 °C. The average of three jumps was used to estimated the relaxation time under a given set of experimental conditions. Because of errors inherent in the temperature jump technique, the values of the rate constants probably are accurate to within about 20%.

All the calculations were done on an AT&T 6300 personal computer. Buffers used were 0.1 M phosphate (pH 5.7–8) and 0.1 M citrate–phosphate (pH 2.5–5.7), and experiments, other than the temperature jump series, were performed at 5, 20, and 35 °C.

Results

Figure 1 shows the pH dependence of the apparent equilibrium dissociation constants for enzyme–chloride complex formation at 5, 20, and 35 °C. The data set at 20 °C was taken from Ikeda-Saito (1985). Since chloride ion binds to the protonated form of spleen myeloperoxidase (Scheme I), the apparent dissociation constant for the enzyme–chloride complex, K_{app}, is expressed as

$$K_{app} = K_{Cl}(1 + K_a/[H^+]) \quad (1)$$

where K_{Cl} and K_a are, respectively, the equilibrium dissociation constant of the enzyme–chloride complex and the dissociation constant of the ionizable group of the enzyme. The curves are

[1] The identity of the spleen green heme protein and granulocyte myeloperoxidase has been further supported by the antibody cross-reactivities and by the electron microscopic examination of the size and shape of these two enzymes (T. G. Frey, S. Kimura, and M. Ikeda-Saito, unpublished results).

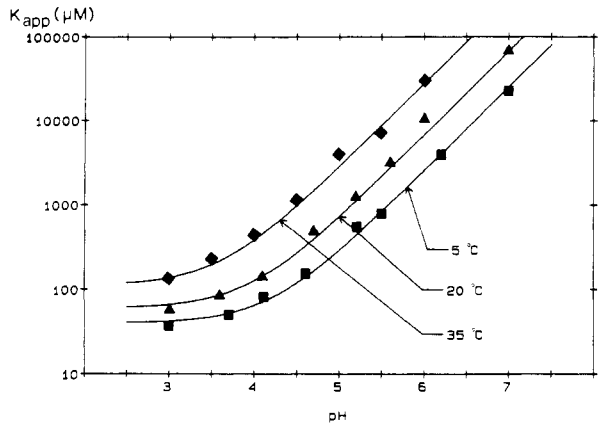

FIGURE 1: pH dependence of the apparent dissociation constant of the chloride complex at 5, 20, and 35 °C. Symbols are experimental data points, and the lines were drawn by use of eq 1 with the pK_a and K_{Cl} values listed in Table I.

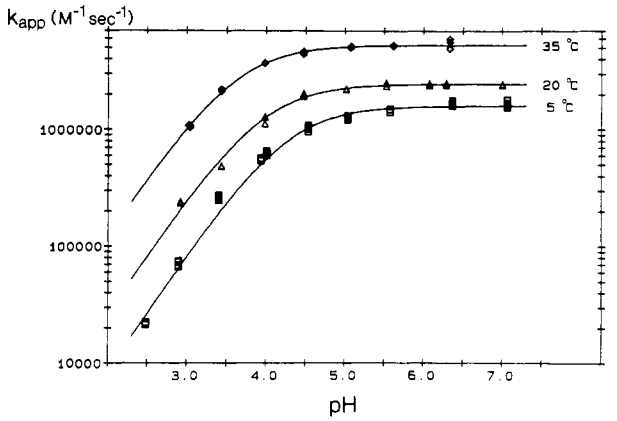

FIGURE 2: pH dependence of the apparent association rate of cyanide complex formation at 5, 20, and 35 °C. Symbols are experimental data points, and the lines were drawn by use of eq 2 with the pK_a and K_{Cl} values listed in Table I.

Table I: Dissociation Constant for Chloride Complex Formation and Association Rate for Cyanide Complex Formation

	chloride complex formation		cyanide complex formation	
temp (°C)	$K_{Cl} \times 10^5$ (M)	pK_a	$k_{on}^{CN} \times 10^{-6}$ (M^{-1}·s^{-1})	pK_a
5	4 ± 0.4	4.2 ± 0.05	1.60 ± 0.02	4.29 ± 0.03
20	6 ± 0.5	3.9 ± 0.05	2.46 ± 0.03	3.96 ± 0.03
35	11 ± 1	3.6 ± 0.06	5.29 ± 0.08	3.63 ± 0.04

drawn with the best-fitted values of pK_a and K_{Cl} which are listed in Table I.

The temperature dependence of the ionization constant of the functional group on the enzyme involved in cyanide binding was studied by measuring the pH dependence of the cyanide association rate at 5, 20, and 35 °C. Figure 2 illustrates the pH dependence of the apparent association rate constants, k_{app}, for cyanide complex formation. The association rate is essentially independent of pH between pH 5 and 7 but declines below pH 5, analogous to the cyanide affinity of the enzyme reported previously (Ikeda-Saito, 1985). Below pH 4, the slope of the pH dependence of the apparent association rate approached unity, showing involvement of a proton in the reaction of enzyme with cyanide. From Scheme I, in which the protonated form of cyanide, HCN, reacts with the unprotonated form of the enzyme, the apparent association rate, k_{app}, is written as

$$k_{app} = k_{on}^{CN}/(1 + [H^+]/K_a) \quad (2)$$

Table II: Rate of Cyanide Complex Formation and That of Compound I Formation by Hydrogen Peroxide

	cyanide complex formation		compound I formation	
peroxidase	$k \times 10^{-6}$ (M^{-1}·s^{-1})	pK_a	$k \times 10^{-7}$ (M^{-1}·s^{-1})	pK_a
myeloperoxidase[a]	2.5	3.96	2	4.15
myeloperoxidase[b]	1.3	4.0	2.3	4.3
horseradish peroxidase[c]	0.12	3.9	1.8	3.9
chloroperoxidase[d]	0.052	<3	1	<3
cytochrome *c* peroxidase[e]	0.0044	5.4	4.5	5.5
bromoperoxidase[f]			2.1	3.94

[a] This paper and unpublished results on the spleen enzyme. [b] Bolscher and Wever on the granulocyte enzyme (1984a). [c] Dunford et al. (1978). [d] Hager et al. (1975) and Lambeir et al. (1983). [e] Loo & Erman (1975). [f] Manthey & Hager (1985).

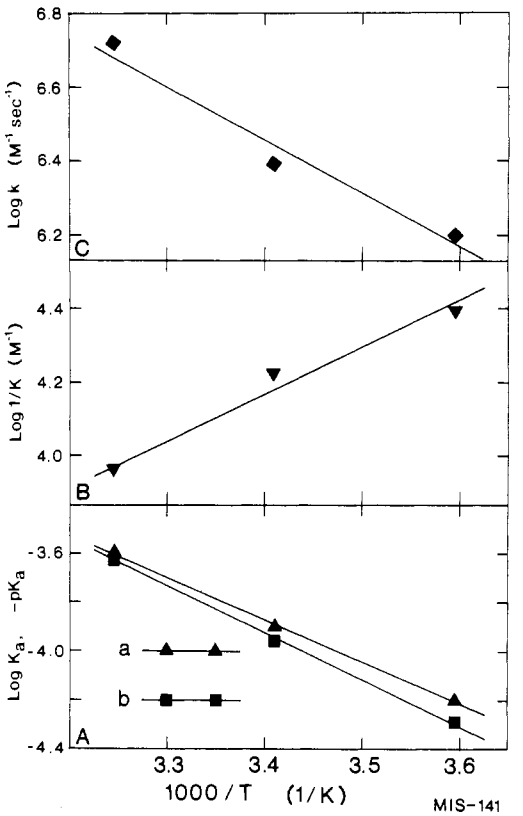

FIGURE 3: (A) Temperature dependence of pK_a obtained from chloride equilibrium [data set a (▲)] and from the cyanide association rate [data set b (■)]. (B) Effect of temperature on the association equilibrium constant of chloride complex formation. (C) Arrhenius plot for the formation of the cyanide complex. Symbols are experimental data points, and lines are drawn by least-squares fitting.

where k_{on}^{CN} is an association rate constant for cyanide complex formation. The curves are drawn by using the best-fitted parameters which are listed in Table I. The alternative but formally equivalent reaction scheme in which cyanide ion reacts with the protonated enzyme would lead to an association rate of the order of 10^{10} M^{-1}·s^{-1}, which appears to be too rapid for ligand binding to heme proteins (Dunford & Stillman, 1976). This indicates that Scheme I adequately expresses the pH-dependent cyanide binding property of spleen myeloperoxidase. Combined with the previously reported pH dependence of the apparent cyanide affinity of the enzyme (Ikeda-Saito, 1985), the cyanide dissociation rate is expected to be independent of pH in the pH range between 2.5 and 7. The rate constants and pK_a values of the rate of formation of cyanide complex for various peroxidases are listed in Table

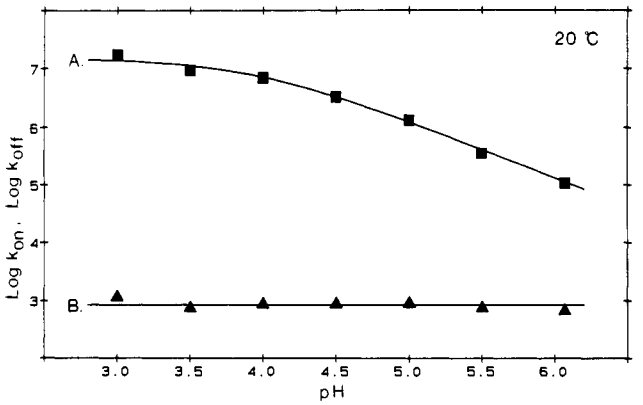

FIGURE 4: pH dependence of the logarithm of the apparent association rate [data set A (■)] and the logarithm of the dissociation rate [data set B (▲)] for reactions of the enzyme with chloride at 20 °C. Symbols are experimental data, and the line for data set A was drawn with the use of eq 3 using $pK_a = 3.95$ and $k_{on}^{Cl} = 1.5 \times 10^7$ $M^{-1}\cdot s^{-1}$, and that for data set B was drawn with $k_{off}^{Cl} = 8 \times 10^2$ s^{-1}.

II together with those of compound I formation by reaction with hydrogen peroxide.

Figure 3A plots the $-pK_a$ values of the ionizable group on the enzyme estimated from the chloride equilibrium experiments (data set a) and from the cyanide reaction kinetics (data set b) against the inverse absolute temperature ($1/T$). From these plots, ΔH for the ionization of the functional group in the enzyme involved in cyanide binding and chloride binding was determined as about 8.7 ± 0.3 and 7.9 ± 0.2 kcal·mol^{-1}, respectively. In Figure 3B, the logarithms of the association equilibrium constants for chloride binding to spleen myeloperoxidase [log ($1/K_{Cl}$)] are plotted against $1/T$. The ΔH and ΔS values for chloride binding to the enzyme were calculated as −5.8 ± 0.8 kcal·mol^{-1} and −0.4 ± 2.8 cal·mol^{-1}·deg^{-1}, respectively.[2] Similarly, an Arrhenius plot of log k_{CN} vs. $1/T$ (Figure 3C) suggests an energy of activation of 6.8 ± 0.5 kcal·mol^{-1} and an Arrhenius frequency factor of (3.9 ± 0.9) × 10^{11} $M^{-1}\cdot s^{-1}$. From this plot, the cyanide association rate is calculated for 10 °C as 1.9×10^6 $M^{-1}\cdot s^{-1}$, which is close to the value of 1.3×10^6 $M^{-1}\cdot s^{-1}$ reported for granulocyte myeloperoxidase (Bolscher & Wever, 1984a).

Figure 4 plots the logarithm of the apparent association and dissociation rates of the reaction of spleen myeloperoxidase with chloride at 20 °C obtained by the temperature jump method. The dissociation rate does not exhibit marked pH dependence. The apparent association rate varies considerably with pH, analogous to the apparent chloride affinity of the enzyme (Figure 1). From Scheme I, where the protonated form of the enzyme reacts with chloride ion, the apparent chloride association rate, k_{app}, is expressed as

$$k_{app} = k_{on}^{Cl}/(1 + K_a/[H^+]) \quad (3)$$

where k_{on}^{Cl} is an association rate constant for enzyme–chloride complex formation. The experimental data were best fitted with the values of $k_{on}^{Cl} = 1.5 \times 10^7$ $M^{-1}\cdot s^{-1}$ and $pK_a = 3.95$ as seen in Figure 4. The dissociation rate was estimated as 8×10^2 s^{-1} over the pH range of Figure 4. If the alternative but kinetically equivalent scheme in which the unprotonated

[2] In my previous paper (Ikeda-Saito, 1985), the ΔH value of chloride binding was reported as −7.8 kcal·mol^{-1} at pH 4.1, which differs by −2 kcal·mol^{-1} from the intrinsic ΔH of chloride binding determined in the present study. The apparent difference in the ΔH value is due to the inclusion of the heat of partial ionization of the functional group with a pK_a value of 4 in the previous data.

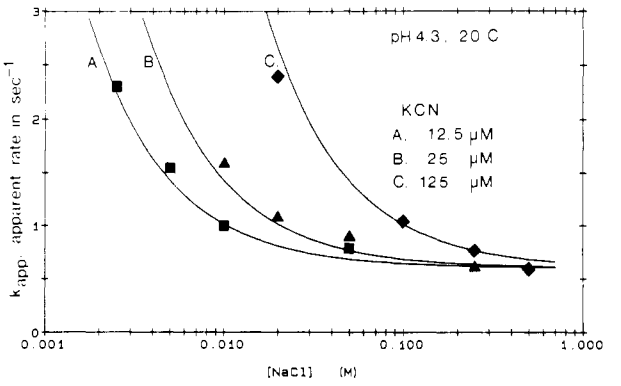

FIGURE 5: Chloride concentration dependence of the rate of the ligand replacement reaction of the cyanide complex at pH 4.3, 20 °C. The symbols are experimental data points, and the lines were drawn by using eq 4 with the following parameters: $k_{off}^{CN} = 0.65$ s^{-1}, $k_{off}^{Cl} = 8 \times 10^2$ s^{-1}, $A = 4.6 \times 10^6$ $M^{-1}\cdot s^{-1}$, and $B = 1.7 \times 10^6$ $M^{-1}\cdot s^{-1}$.

form of the enzyme binds with protonated chloride is used, the association rate would be of the order of 10^{12} $M^{-1}\cdot s^{-1}$ using a pK_a value of −2.2 for HCl. This rate appears to be too rapid for ligand binding to heme proteins (Dunford & Stillman, 1976). This confirms that Scheme I is adequate to describe the pH-dependent change in chloride binding by the enzyme.

In a previous paper (Ikeda-Saito, 1985), I reported that the addition of a large excess of chloride to the enzyme–cyanide complex at low pH displaced cyanide from the enzyme. This showed the possibility of determining the cyanide dissociation rate by measuring the rate of approach to equilibrium after mixing the enzyme–cyanide complex with buffer containing chloride. Figure 5 plots the rate of the conversion from the cyanide complex to the chloride complex determined at various concentrations of cyanide and chloride at pH 4.3, 20 °C. By modification of the expression given by Antonini and Brunori (1971), the rate of the conversion, k, is written as

$$k = (k_{off}^{CN}A[Cl] + k_{off}^{Cl}B[CN])/(B[CN] + A[Cl]) \quad (4)$$

where $A = k_{on}^{Cl}/(1 + K_a/[H^+])$ and $B = k_{on}^{CN}/(1 + [H^+]/K_a)$. At pH 4.3, where the experiments were carried out, the apparent association rates for cyanide and chloride binding are similar ($A \approx B$; see Figures 2 and 3). If the relationship [NaCl] ≫ [KCN] holds, then eq 4 can be reduced to $k = k_{off}^{CN} + k_{off}^{Cl}[CN]/[Cl]$. From this equation, lim $k = k_{off}^{CN}$ as [Cl] → ∞. Therefore, the rate of the conversion approaches the value of k_{off}^{CN} at higher NaCl concentration. From the data in Figure 5, k_{off}^{CN} is estimated as 0.65 s^{-1}, which is in a good agreement with the dissociation rate of 0.56 s^{-1} reported for the granulocyte myeloperoxidase–cyanide complex by Bolscher and Wever (1984a). Using the values of A and B calculated for pH 4.3 from the data in Table I with eq 1 and 2, together with the cyanide and chloride dissociation rates, the NaCl concentration dependence of the conversion rate was calculated for KCN concentrations of 12.5, 25, and 125 μM by eq 4 and is plotted in Figure 5. The three sets of data are well fitted by the calculated curves, indicating the proper estimation of the rates by either the stopped-flow or the temperature jump technique. The dissociation and association rates yield an equilibrium dissociation constant for the enzyme–cyanide complex of 0.26 μM, which is close to the 0.6 μM determined by equilibrium measurement (Ikeda-Saito, 1985) and 0.43 μM reported for granulocyte myeloperoxidase (Bolscher & Wever, 1984a). Minor discrepancies in these values seem to come from the uncertainties involved in determinations of the dissociation constant by titration methods, due to the high cyanide affinity of the enzyme.

Discussion

The present investigation determines both the on and off rates of cyanide binding to spleen myeloperoxidase. Both rates and the pH profile are in good agreement with data reported for granulocyte myeloperoxidase (Bolscher & Wever, 1984a), further supporting our proposal that the ligand binding active site of spleen myeloperoxidase is identical with that of granulocyte myeloperoxidase. The present determination of the pH dependence of the apparent rates involved in cyanide and chloride binding to spleen myeloperoxidase concludes that a proton dissociable group with a pK_a value of 4 regulates the association reaction of the enzyme with ligands. Not only the pK_a value but also the ΔH values for the ionization of the functional group on the enzyme involved in binding of chloride and cyanide are very close. This establishes the adequacy of Scheme I in which the same ionizable group regulates both cyanide and chloride binding to the enzyme (Bolscher & Wever, 1984a; Ikeda-Saito, 1985). This ionizable group must be protonated for the binding of chloride ion but unprotonated for cyanide binding to the enzyme. This is a characteristic commonly seen in ligand binding to peroxidases and infers that spleen myeloperoxidase has an active-site environment typical to those of common peroxidases. It is appropriate to point out that Sono et al. (1981a) also concluded that HCl, a strong acid, binds as a chloride anion to the acid form of chloroperoxidase whereas HCN, a weak acid, reacts as the unprotonated molecule to the neutral form of chloroperoxidase, in accordance with the present findings.

The salient feature of the ligand binding properties of spleen myeloperoxidase is that the enzyme has a relatively fast cyanide association rate in comparison with other peroxidases. The rates of compound I formation of various peroxidases by hydrogen peroxide are all about 10^7 $M^{-1}\cdot s^{-1}$, while the cyanide association rates are slower, but with marked variation, as listed in Table II. The slower rate of cyanide association than that of compound I formation has been considered to be due to steric constraints around the binding site of peroxidases (Dunford et al., 1978): the spatial arrangement of the amino acids around the binding site facilitates compound I formation from the hydrogen peroxide molecule to form a bent Fe–O–O structure (Poulos & Kraut, 1980) but retards cyanide binding, which energetically prefers linear iron ligand bonding (Fe–C–N), by mandating a tilted or bent Fe–C–N structure (Yoshikawa et al., 1985). The relatively faster cyanide association rate for myeloperoxidase may indicate that the structure of the distal site of the iron center in myeloperoxidase is composed in such a way that cyanide experiences less steric pressure, and/or smaller structural alteration is required for cyanide binding in comparison with other peroxidases.

The intimate relationship between ligand binding properties and the state of ionization of the functional group with a pK_a value of 4 on the enzyme is indicative of close proximity between this functional group and the ligand binding site, the iron center of the enzyme. One may suggest one of the carboxyl groups of the propionic acid side chains of the iron chlorin prosthetic group as a candidate of this ionizable group. The participation of these chlorin peripheral groups in the covalent linkage between the prosthetic group and the apoprotein in myeloperoxidase (Schultz et al., 1983) renders this possibility unlikely. Then, either the distal group or the proximal axial iron ligand is the probable candidate for this functional group. Proton nuclear magnetic resonance, nitric oxide electron paramagnetic resonance, and resonance Raman data are indicative of the presence of an imidazole residue as the proximal axial ligand of the iron center in granulocyte and spleen myeloperoxidase (Ikeda-Saito & Inubishi, 1987; Bolscher & Wever, 1984b; Babcock et al., 1984). The possibility of the involvement of the proximal group seems to be remote. The light absorption and magnetic circular dichroism spectra of spleen myeloperoxidase are independent of pH between pH 4 and 8 (Sono et al., 1986b). If the protonation of the axial imidazole group of a histidine residue is involved, we would expect to detect spectral differences between the protonated and unprotonated forms of the enzyme. The distal group is the most likely functional group which controls the ligand binding property of spleen myeloperoxidase, as proposed for hydroperoxidases in general by Davies et al. (1976) and in particular for chloroperoxidase (Sono et al., 1986a) and granulocyte myeloperoxidase (Bolscher & Wever, 1984a).

The distal group has been suggested to be a histidine residue (Ikeda-Saito & Prince, 1985). Although the value of 4 is relatively low for a pK_a value of the imidazole group of a histidine residue, such a pK_a has been reported for the imidazole group of the distal histidine residue in leghemoglobin *a* (Johnson et al., 1978; Fuchsman & Appleby, 1979). The nature of the putative distal group in myeloperoxidase may be further explored by the aid of the thermodynamic parameters of its ionization. While the pK_a values of the imidazole group of a histidine in proteins may vary considerably according to their environment, the ΔH values for the ionization are in the range of 7–8 kcal·mol^{-1} (Cohn & Edsall, 1943). The ΔH value of about 8 kcal·mol^{-1} obtained in this study agrees well with this value. An alternative possibility for the ionizable group would be a carboxylic acid group of an amino acid residue, the pK_a values of which are usually between 1 and 6. At first glance, this seems a more likely assignment than histidine, but the ΔH values of amino acid carboxylic groups in proteins are in the range of ± 1.5 kcal·mol^{-1} (Christensen & Izatt, 1976), which is considerably smaller than the ΔH value of 8 kcal·mol^{-1} measured here. On the basis of these considerations, the ionizable group with a pK_a value of 4 on the enzyme is assignable to an imidazole group of the distal histidine residue. The distal histidine residue seems to control access of exogenous ligands to the binding site in the enzyme.

The ^{15}N nuclear magnetic resonance and infrared studies on cyanide complexes of hemes and hemeproteins have provided evidence for cyanide binding as a metal cyanide (Fe–C≡N) and against HCN being the ligand in nitrile-like bonding (Fe–N≡C–H) (Morishima & Inubushi, 1978; Yoshikawa et al., 1985; Behere et al., 1986). This implies that a proton must be released from HCN upon its binding to the enzyme. The pH dependence of the cyanide binding data indicates that cyanide binding to the enzyme is not associated with proton release. An ionizable group on the enzyme must be protonated upon cyanide binding. Although Araiso and Dunford (1981) proposed that the proximal histidine is protonated upon cyanide binding to horseradish peroxidase, in light of recent NMR results, such a possibility seems to be remote (de Ropp et al., 1985; Behere et al., 1986). Sono et al. (1986a) suggested the distal histidine residue as a possible candidate for this ionizable group in ligand binding to chloroperoxidase. As discussed above, the distal histidine residue is not protonated in the resting neutral form of myeloperoxidase which reacts with HCN. The distal histidine, which is located close to the cyanide binding site, most likely traps the proton released from HCN upon binding to spleen myeloperoxidase. This idea infers that the distal histidine residue not only controls access to the iron site but also directly participates in the cyanide binding reaction. The rate vs. pH profile of compound I formation of spleen myeloperoxidase

is similar to that of cyanide complex formation: hydrogen peroxide reacts with the enzyme when the ionizable group with a pK_a of 4 is unprotonated (unpublished result), as expected from the results on the granulocyte enzyme (Bolscher & Wever, 1984a). Trends similar to this have also been reported for horseradish peroxidase, cytochrome *c* peroxidase, and chloroperoxidase (Dunford & Stillman, 1976; Loo & Erman, 1975; Lambeir & Dunford, 1983). The distal histidine group in spleen myeloperoxidase is considered to play a role in the heterolytic hydroperoxide cleavage as proposed in the model based on the X-ray structure of cytochrome *c* peroxidase (Poulos & Kraut, 1980). Because of identical active-site structures of the spleen and granulocyte enzyme, the present proposal should also be applicable for granulocyte myeloperoxidase.

ACKNOWLEDGMENTS

I thank T. Yonetani for his facilities, M. Kiwaki for her assistance, R. C. Prince for his comments, and an anonymous reviewer of this paper for recommending the change in the name of the enzyme from the spleen green hemeprotein to spleen myeloperoxidase.

Registry No. Myeloperoxidase, 9003-99-0; Cl^-, 16887-00-6; CN^-, 57-12-5; histidine, 71-00-1.

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