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Kinetic Analysis of the Acid–Alkaline Conversion of Horseradish Peroxidases†

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ABSTRACT: The nature of the acid–alkaline conversion of horseradish peroxidases was studied by measuring four rate constants in reactions, $E + H^+$ (k_1) $\rightleftharpoons$ (k_2) EH^+ and $E + H_2O$ (k_3) $\rightleftharpoons$ (k_4) $EH^+ + OH^-$, where EH^+ and E denote the acid and alkaline forms of the enzymes. The values of k_1, ($k_2 + k_3$), and k_4 were obtained by measuring the relaxation rates of the acid → alkaline and alkaline → acid conversions by means of the pH jump method with a stopped-flow apparatus. The value of k_3 could also be obtained by measuring the rate of reactions between hydrogen peroxide and peroxidases at alkaline pH. The measurements were conducted with four peroxidases having different pK_a values: peroxidase A (pK_a = 9.3), peroxidase C (pK_a = 11.1), diacetyldeuteroperoxidase A (pK_a = 7.7), and diacetyldeuteroperoxidase C (pK_a = 9.1). The value of k_1 was about 10^{10} M^{-1} s^{-1} in the reaction of the four enzymes while k_4 was quite different between the enzymes. The pK_a was determined by k_3 and k_4 for the natural peroxidases and by k_1 and k_2 for the diacetyldeuteroperoxidases. The mechanism of the acid–alkaline conversion was discussed in comparison with that of metmyoglobin.

High-spin hemoproteins, such as methemoglobin, metmyoglobin, and plant peroxidases, change their electronic and magnetic properties at alkaline pH (Theorell, 1947; Keilin & Hartree, 1951). The change has been attributed to the proton dissociation of an iron-bound water molecule. This idea seems to conform to the fact that the rates of the acid–alkaline conversion are very fast in methemoglobin and metmyoglobin (Ilgenfritz & Schuster, 1971; Iizuka et al., 1976). On the other hand, the slow rate of the conversion in the case of horseradish peroxidase C has recently been interpreted in terms of the involvement of a conformation change of the protein (Epstein & Schejter, 1972; Iizuka et al., 1976). Evidently, this mechanism contradicts the classical one. The evidence that supports the new mechanism, however, seems insufficient.

This paper reports a detailed investigation of the kinetics of the acid–alkaline conversion of horseradish peroxidases A and C containing a proto- or diacetyldeuterohemin; the pK_a values are reported to be much different between these peroxidases (Makino & Yamazaki, 1972). The present paper also deals with a general rule in the relation between the pK_a and four rate constants involved in the acid–alkaline conversion.

Materials and Methods

Horseradish peroxidases were prepared from wild horseradish root by the method of Shannon et al. (1966) with slight modification. Peroxidase isoenzymes used in this experiment were peroxidase A and peroxidase C, according to nomenclature by Paul (1958) and Shannon et al. (1966). Peroxidase A was a mixture of A_1 and A_2; the difference of catalytic and chemical properties between A_1 and A_2 has not been determined, although they possess different charge properties (Kay et al., 1967; Shin et al., 1971). Artificial peroxidases, containing 2,4-diacetyldeuterohemin in the place of protohemin IX of natural peroxidases, were prepared by the method of Makino & Yamazaki (1972, 1973).

All measurements were performed at 20 ± 0.2 °C and the

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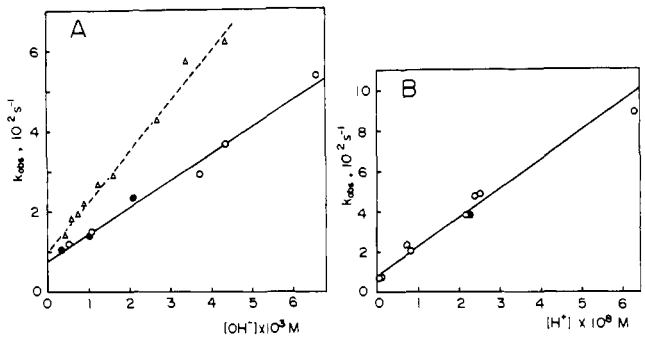

FIGURE 1: The plots of k_{obsd} vs. $[OH^-]$ (A) and $[H^+]$ (B) in the acid-alkaline conversion of peroxidase C. The rate was measured by the stopped-flow technique at 403 or 416 nm. The two solutions mixed were: (A) (○) 4–8 μM peroxidase C in 0.1 M KCl and NaOH (varied) in 0.1 M KCl; (●) 4–8 μM peroxidase C in distilled water and glycine–NaOH in an NaCl solution. In A, the data of Epstein & Schejter (1972) at 23 °C are replotted with △. In B, 4–8 μM peroxidase C in an NaOH solution (pH ≈ 12) and (○) potassium phosphate (μ = 0.2) or (●) potassium phosphate (μ = 0.05) + KCl (μ = 0.15).

ionic strength in reaction solutions was adjusted to 0.1 by the addition of KCl. Buffer systems used in this experiment were potassium phosphate for pH 7.0–8.2, sodium bicarbonate for pH 8.8–10.3, and glycine–NaOH for pH 10.3–11.5. The ionic strength contributed from the buffers was about 0.01. Tris-HCl and sodium borate were found to be not suitable for this pH-jump experiment. Spectrophotometric measurements were performed with a Shimazu recording spectrophotometer, Model MPS-5000 for ordinary time-scale experiments and with a Union Giken rapid reaction analyzer, RA-1300 for rapid reactions. The details of the latter instrument were described previously (Araiso et al., 1976). The reactions were started by mixing two solutions in equal volume. The pH of the reaction solutions (μ = 0.1) was measured after the two solutions were mixed, with an assembly of Radiometer (Copenhagen) equipped with a combined electrode, type GK 2320 C.

Results and Kinetics

Natural Horseradish Peroxidases. The rate of conversion of peroxidase C from acid to alkaline form has been measured at varied pH values by Epstein & Schejter (1972). By rapid-scan spectrophotometric measurement we found that one set of isosbestic points was observed during the conversion. The apparent first-order rate constant could be measured graphically by plotting logarithm of the absorbance difference at a fixed wavelength against the time. It is known that when the following system (reaction 1) is approaching its equilibrium the first-order rate constant (k_{obsd}) of the relaxation contains the contributions from the forward and backward reactions additively as shown in eq 2 (Eigen & De Maeyer, 1963).

$$A \underset{k_b}{\overset{k_f}{\rightleftharpoons}} B \quad (1)$$

$$k_{obsd} = k_f + k_b \quad (2)$$

This simple example can be applied to the conversion between the acid and alkaline forms of peroxidases when the conversion occurs at a constant pH. In this case, however, one has to consider two reaction paths in each direction of the conversion. If the acid and alkaline forms of peroxidases are denoted as EH^+ and E the conversion is formulated by eq 3 and 4.

$$E + H^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EH^+ \quad (3)$$

$$E + H_2O \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EH^+ + OH^- \quad (4)$$

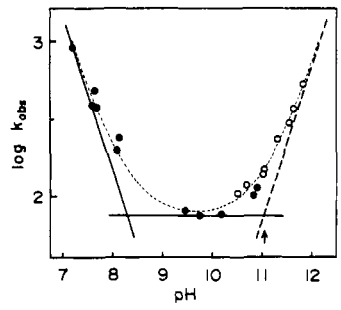

FIGURE 2: The log k_{obsd} vs. pH plot in the acid-alkaline conversion of peroxidase C. Data were replotted from Figure 1, except for data between pH 10 and 11 in the alkaline → acid conversion. The solid and the broken lines indicate the alkaline → acid and the acid → alkaline conversions, respectively. The dotted line was calculated from eq 5 with values in Table II. The arrow indicates the pK_a value. The solid lines denote the pH dependence of log $k_1[H^+]$ and log k_3, and the broken line that of log $k_4[OH^-]$.

When pH is kept constant during the conversion, the first-order rate constant of the relaxation can be expressed as[1]

$$k_{obsd} = k_1[H^+] + k_2 + k_3 + k_4[OH^-] \quad (5)$$

The rate constant was measured at varied pH values. The plot of k_{obsd} vs. $[OH^-]$ exhibited a linear relationship in the acid → alkaline conversion of peroxidase C (Figure 1A). From the slope and intercept, k_4 and $(k_2 + k_3)$ were estimated at 6.7×10^4 M^{-1} s^{-1} and 75 s^{-1}, respectively. The contribution from $k_1[H^+]$ is negligibly small at these pH values even if k_1 is a diffusion-limiting rate constant.

Likewise, the rate of conversion of the enzyme from the alkaline to acid form could be measured (Figure 1B). The values of 1.5×10^{10} M^{-1} s^{-1} and 75 s^{-1} were obtained for k_1 and $(k_2 + k_3)$, respectively. It is evident that under these conditions the term $k_4[OH^-]$ is negligible. Figure 2 demonstrates overall pH dependence of the logarithm of k_{obsd} in the acid-alkaline conversion of peroxidase C.

It seemed reasonable that nearly identical intercepts were obtained by the two experiments shown in Figures 1A and 1B. The next problem to be solved is to determine which rate constant, k_2 or k_3, is responsible for the pH-independent term. It was concluded from the following two independent processes that k_3 was about 75 s^{-1}.

The value of pK_a for the acid-alkaline conversion of peroxidase C has been reported by many workers to be about 11.1 (Theorell, 1942; Harbury, 1957; Ellis & Dunford, 1969; Makino & Yamazaki, 1972). At pH = pK_a the following equation should hold

$$k_1[H^+] + k_3 = k_2 + k_4[OH^-] \quad (6)$$

At pH 11.1, eq 6 becomes 0.13 s^{-1} + k_3 = k_2 + 75 s^{-1}. Since $(k_2 + k_3)$ = 75 s^{-1}, it was concluded that k_3 = 75 s^{-1} and $k_2 \ll k_3$.

Secondly, k_3 could be measured by a direct method. As suggested by Chance (1949) and Marklund et al. (1974) peroxidase compound I cannot be formed from a direct reaction between H_2O_2 and the alkaline form of the enzyme. If compound I is formed only via the acid form, the rate constant of the conversion from the alkaline form to compound I will reach $k_1[H^+] + k_3$ as the concentration of H_2O_2 is increased. A typical experiment of measuring the rate of compound I formation is demonstrated in Figures 3A and 3B. The rates

[1] After we submitted this paper we noticed the paper of Job, D., Ricard, J., & Dunford, H. B. (1977) *Arch. Biochem. Biophys. 179*, 95, who reported kinetic analysis of the alkaline conversion of turnip peroxidases on the basis of eq 5.

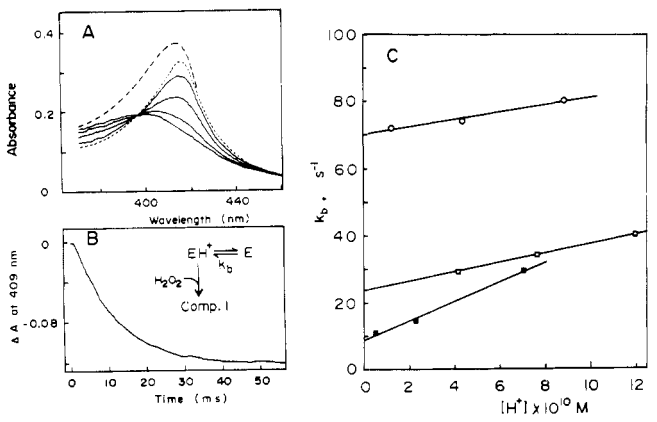

FIGURE 3: Formation of compound I from the alkaline form (E) of peroxidases. (A) Rapid-scan spectrophotometric measurement of peroxidase C at pH 11.05 (glycine-NaOH). The reaction was started by mixing 7.8 μM enzyme with 8 mM H_2O_2 in equal volume. Solid lines from the top: 2, 8, 20, and 40 ms after mixing (scanning speed = 75 nm/ms). The broken line shows an absorption spectrum of 3.9 μM peroxidase C at pH 11.05. The dotted line shows an absorption spectrum of a mixture of 1.95 μM E and 1.95 μM compound I, which is supposed to be obtained within dead time (about 1 ms). (B) A time course of the reaction of peroxidase C monitored at 409 nm, which is isosbestic between the acid (EH^+) and the alkaline forms of the enzyme. (C) The rates of the compound I formation at varied pH values. The apparent first-order rate constant (k_b) = $k_1[H^+] + k_3$. Higher concentrations of H_2O_2 are needed for the reaction of peroxidases A because the reactions between the enzymes and H_2O_2 are slower (Marklund et al., 1974). (○) Peroxidase A, mixed with 50 mM H_2O_2, measured at 416 nm; (□) diacetyldeuteroperoxidase A, mixed with 50 mM H_2O_2, measured at 428 nm; (●) diacetyldeuteroperoxidase C, mixed with 4 mM H_2O_2, measured at 428 nm. Each wavelength was a peak of absorption spectrum of the alkaline form of the enzymes.

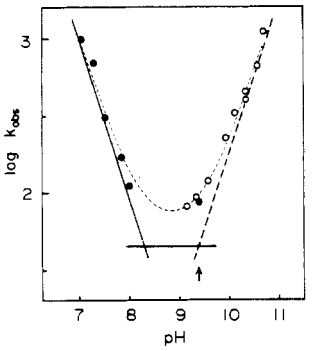

FIGURE 4: The log k_{obsd} vs. pH plot in the acid-alkaline conversion of peroxidase A. The experimental procedures and the plot were as described in Figures 1 and 2, respectively.

TABLE I: Apparent Rate Constant of the Alkaline → Acid Conversion of Peroxidase C Measured from the Reaction of the Enzyme with H_2O_2 at Alkaline pH.[a]

pH	Buffer	$[H_2O_2]$ (mM)	Rate constant (s^{-1})
10.30	Glycine-NaOH	4	87
10.62	Glycine-NaOH	25	95
10.38	NaOH-KCl	50	86
10.48	NaOH-KCl	25	95
11.20	NaOH-KCl	4	73

[a] Experimental procedures were as described in Figure 3.

measured at varied experimental conditions are listed in Table I. Since $k_1[H^+] \ll 75$ at alkaline pH described in Table I, the rate should be independent of pH and equal k_3. This method appears to be suitable for the measurement of k_3 because the reaction of H_2O_2 with the acid form of peroxidase is very fast and practically irreversible. It was thus concluded that in the acid-alkaline conversion of peroxidase C the pH-independent terms of eq 5 are governed by k_3.

Similar experiments were performed with peroxidase A, the pK_a value of which has been reported to be 9.3 by Makino & Yamazaki (1972), 9.5 by Tamura & Hori (1972) and 9.2 by Ohlsson & Paul (1973). The pH dependence of k_b (see Figure 3B) and log k_{obsd} is shown in Figures 3C and 4, respectively. From a comparison of Figure 4 with Figure 2, it may be seen that the k_1 value was nearly identical between the two isoenzymes and the difference in the pK_a value was mainly ascribable to the k_4 values. Figure 4 also indicates that both $k_1[H^+]$ and $k_4[OH^-]$ contributed to k_{obsd} around pH 9 in the reaction of peroxidase A. Unlike the case of peroxidase C, it was difficult to measure ($k_2 + k_3$) directly from the plot of k_{obsd} vs. $[H^+]$ or $[OH^-]$. The value of k_3, however, could be obtained by measuring the rate of compound I formation at alkaline pH. The rate depended upon pH. Figure 3C shows the plot of k_b vs. H^+ concentration. Since $k_b = k_1[H^+] + k_3$, k_3 was estimated at 70 s^{-1} from the intercept. Based on the fact that $pK_a = 9.3$ it was concluded that the k_{obsd} at pH = pK_a depended largely upon k_3 and $k_4[OH^-]$. The simulation of experimental data in Figure 4 was achieved by using the value of 9.3 for pK_a and with an assumption that $k_2k_3 = k_1k_4K_w$, where K_w denotes the ion product of water. The rate constants determined by a least-squares analysis are listed in Table II. The k_3 value slightly differed from that obtained from Figure 3C.

Diacetyldeutero Derivatives of Horseradish Peroxidases. The values of pK_a for the acid-alkaline conversion of diacetyldeutero derivatives of peroxidases A and C were reported to be 7.7 and 9.1, respectively (Makino & Yamazaki, 1972). In spite of the similarity in pK_a values of peroxidase A and diacetyldeuteroperoxidase C, the plots of log k_{obsd} vs. pH were different between the two enzymes (compare Figures 4 and 5A). It should be noticed that the values of k_1 were similar between the two enzymes. A striking difference appeared in the value of k_3, which could be obtained from the rate of compound I formation at alkaline pH. Figure 3C demonstrates that the k_3 value of diacetyldeuteroperoxidase C was about one-tenth of that of peroxidase A. The result accords with the fact that in this case the pK_a was identical with pH at the left corner of the log k_{obsd} vs. pH curve. The pH was indicated with an arrow in Figure 5A. The four rate constants determined as described in the case of peroxidase A are listed in Table II. Using these values, the pH dependence of the logarithm of each term in eq 5 is demonstrated in Figure 5A. The insertion shows the pH dependence of the apparent first-order rate constant of compound I formation which equals k_b, the sum of $k_1[H^+]$ and k_3. Obviously, this method could be applied to the reactions above pH near pK_a and only a lower part of the log k_b vs. pH plot was measured. On the other hand this part of the plot could not be measured directly by the pH-jump method and was calculated on the assumption that $k_2k_3 = k_1k_4K_w$. We concluded that the assumption was correct since the plot in the insertion of Figure 5A was nearly coincident with the calculated one.

Similar experiments were also carried out with diacetyldeuteroperoxidase A. As shown in Figure 5B, the k_{obsd} was relatively high in this case. The values contained considerable experimental errors because the relaxation time was close to the limit of resolution by our instrument. At any rate the rate constants could be determined by a least-squares analysis and the approximate values are listed in Table II. The value of k_3 could be also obtained by measuring the rate of compound I formation at alkaline pH. The result is shown in Figure 3C and in the insertion of Figure 5B.

TABLE II: Rate Constants.[a]

Enzyme	pK_a[b]	k_1 (M^{-1} s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (M^{-1} s^{-1})
Peroxidase A[c]	9.3	1.0×10^{10}	5.0	45	2.2×10^6
		(1.1×10^{10})		(75)	
Peroxidase C[d]	11.1	1.5×10^{10}	0.13	75	6.7×10^4
				(87)	
Diacetyldeuteroperoxidase A[c]	7.7	0.8×10^{10}	150	15	3×10^7
		(1.3×10^{10})		(23)	
Diacetyldeuteroperoxidase C[c]	9.1	2.4×10^{10}	20	6.5	4.8×10^5
		(2.9×10^{10})		(8.5)	

[a] These values were used to simulate the log k_{obsd}–pH relation (dotted lines) in Figures 2, 4, and 5. The values in parentheses were obtained from the rates of compound I formation at alkaline pH (Table I and Figure 3C). [b] Cited from Makino & Yamazaki (1972). [c] Rate constants were determined by a least-square analysis from experimental data with the use of the pK_a values listed and of eq 5 and 8. [d] Rate constants were obtained from Figure 1 with eq 8. From these values, pK_a was calculated at 11.05.

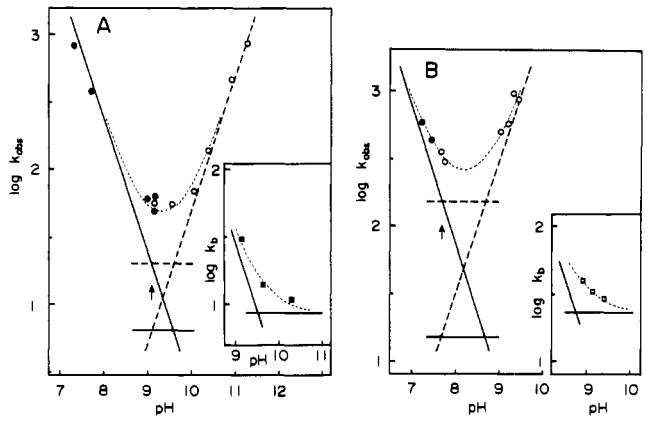

FIGURE 5: The log k_{obsd} vs. pH plot in the acid–alkaline conversion of diacetyldeuteroperoxidases. The experimental procedures and the plot were as described in Figures 1 and 2, respectively. The rate constant, k_b, measured from the compound I formation was replotted from Figure 3C in the insertions for ease of comparison between the two independent experiments. (A) Diacetyldeuteroperoxidase C. (B) Diacetyldeuteroperoxidase A.

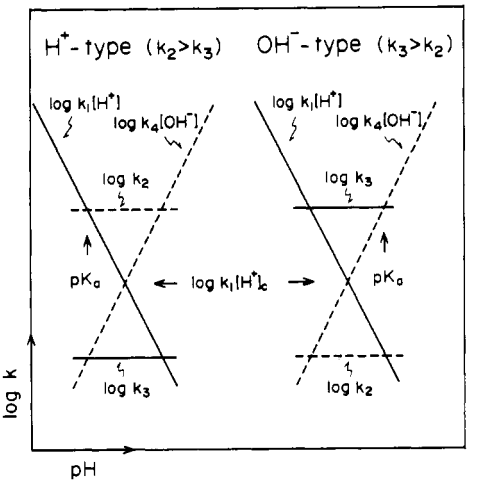

FIGURE 6: Schematic demonstration of two types of the acid–alkaline conversion. Each line denotes the logarithm of the respective term in the right-hand side of eq 5. It is evident from eq 8 that log k_2 − log $k_1[H^+]_c$ = log $k_1[H^+]_c$ − log k_3. Aplysia metmyoglobin and diacetyldeuteroperoxidases A and C belong to the H^+ type and peroxidases A and C, and probably mammalian metmyoglobin belongs to the OH^- type. The pH dependence of log k_{obsd}, log k_f, and log k_b can be simulated in each figure when the ordinate and abscissa scales are determined.

Discussion

Epstein & Schejter (1972) have formulated the alkaline ionization of horseradish peroxidase C as eq 7:

$$\underset{\text{(high spin)}}{\text{peroxidase-H}^+} \overset{K_i}{\underset{\text{fast}}{\rightleftharpoons}} \underset{\text{(high spin)}}{\text{peroxidase}} + \text{H}^+ \overset{k_f}{\underset{k_b}{\rightleftharpoons}} \underset{\text{(low spin)}}{\text{peroxidase}} \quad (7)$$

the parameters, K_i, k_f, and k_b, being measured as 5.5×10^{-13} M, 2.98×10^3 s^{-1} and 80 s^{-1}, respectively. However, their experimental data are not much different from ours as indicated in Figure 1A. It is obvious that the slow rate of the alkaline ionization does not necessarily mean an involvement of a slow conformation change of the protein. The pH dependence of k_{obsd} in the alkaline → acid conversion shown in Figure 1B cannot be explained by the mechanism of eq 7.

The present results clearly demonstrate the existence of two mechanisms in the acid–alkaline conversion, H^+ and OH^- types. The rule is schematically explained in Figure 6. At equilibria of reactions 3 and 4, one obtains eq 8

$$k_2k_3 = k_1k_4K_w = k_1^2[H^+]_c^2 \quad (8)$$

where $[H^+]_c$ denotes the concentration of H^+ at the crossing point of $k_1[H^+]$ and $k_4[OH^-]$ lines. The equilibrium at pH = pK_a is governed by reaction 3 in the H^+ type and by reaction 4 in the OH^- type. Evidently, the H^+-type conversion occurs when $k_2 > k_3$ and the OH^- type when $k_3 > k_2$. If $k_2 = k_3$, pK_a = p$[H^+]_c$. The values of k_2 and k_3 are obtained independently in the reactions of diacetyldeuteroperoxidases A and C; the relation of eq 8 can be verified within experimental error (Table II).

According to Giacometti et al. (1975), the acid and alkaline conversion of Aplysia metmyoglobin belongs to the H^+ type. Although the pH dependence of k_{obsd} has not been reported in the reaction of mammalian metmyoglobin, it may be concluded that its acid–alkaline conversion is grouped into the OH^- type. The reason is as follows: If it belonged to the H^+ type, k_1 was calculated to be 10^{13} M^{-1} s^{-1} on the basis that pK_a = 9 and $k_{obsd} \approx 10^4$ s^{-1} (assumed from the result of Iizuka et al., 1976). This is unlikely because the calculated k_1 is much higher than the diffusion-controlled limit (Eigen, 1963). It is predicted from the OH^--type mechanism that $k_3 \approx 10^4$ s^{-1} and $k_4 \approx 10^9$ M^{-1} s^{-1}. Table II demonstrates that nearly diffusion-controlled values are given to k_1 independently of the kind of hemoproteins and also that k_4 varies drastically from hemoprotein to hemoprotein.

We do not have sufficient structural evidence to explain such variation in k_4 values. In general, however, there are two factors that control k_4. First, the dissociable proton of the water molecule at the 6th position is hydrogen bonding. Second, the reaction of the proton with an OH^- ion is inhibited by a steric hindrance and (or) an electrostatic repulsion. It appears that the difference in k_4 between the peroxidase and metmyoglobin is caused by the two factors. The difference in k_4 between the

two peroxidase isoenzymes is probably due to the difference in the hydrogen bonding of the water molecule at the 6th position. The characteristic difference between peroxidases A and C has been explained in terms of the basic strength of the distal amino acid residue (Yamada & Yamazaki, 1974, 1975; Yamada et al., 1975). Crooks (1975) has described that the rate of proton dissociation by OH^- ion is much less than the diffusion-controlled limit when the proton forms a strong intramolecular hydrogen bond; the rate constant is lowered even to about 10^4 M^{-1} s^{-1}. When protoheme of natural peroxidases is replaced with diacetyldeuteroheme, the dissociation of proton from the water molecule becomes easier and the release of OH^- from the alkaline form becomes more difficult because of the electron-withdrawing effect of the acetyl groups (Table II).

Since Keilin & Hartree (1951) it has been generally accepted that a water molecule coordinates to the iron atom in high-spin ferric hemoproteins. However, George & Lyster (1958) asserted that, though the model is applicable to metmyoglobin and methemoglobin, peroxidase and catalase have a crevice structure without the coordination of water. In order to elucidate this problem relaxation experiments by magnetic resonance have been carried out, but the conclusion is conflicting in the case of peroxidase (Lanir & Schejter, 1975; Vuk-Pavlovič & Benko, 1975). Morrison & Schonbaum (1976) have described that a water molecule coordinates to the iron atom, giving a labile complex. The crevice structure in peroxidases cannot be precluded by the present study, but the model of Keilin & Hartree (1951) conforms well to the present data.

It is certainly true that the respective rate constant in the acid–alkaline conversion is much different between peroxidase C and mammalian metmyoglobin. But, it should be emphasized here that large differences likewise appear among the four peroxidases which will possess similar structure and function.

Acknowledgments

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