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# OPTICAL AND MAGNETIC MEASUREMENTS OF HORSERADISH PEROXIDASE

## III. ELECTRON PARAMAGNETIC RESONANCE STUDIES AT LIQUID-HYDROGEN AND -HELIUM TEMPERATURES

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SUMMARY

Electron paramagnetic resonance (EPR) spectra of horseradish peroxidase (donor: $H_2O_2$ oxidoreductase, EC 1.11.1.7) isozymes, acid and neutral enzymes, have been measured at both liquid-hydrogen and -helium temperatures. Both acid and neutral enzymes give similar EPR spectra exhibiting the prominent rhombic splitting at $g = 6$ region in acid solution. At alkaline pH, the acid enzyme forms two different kinds of low-spin compounds during the transition from "acid form" to "alkaline form"; one with the $g$ values at 3.18, 2.06 and 1.23 and the other at 2.94, 2.08 and 1.63. The former is observed at neutral pH and the latter at alkaline pH. On the other hand, the neutral enzyme shows one low-spin compound, known as an "alkaline form", at liquid-hydrogen temperature. At liquid-helium temperature, however, the neutral enzyme exhibits new EPR absorptions having $g$ values at 3.20, 2.05 and 1.23. From the pH dependence of EPR absorptions, the p$K$ values of the transitions between these three components are determined for both peroxidase isozymes; $pK_1 \approx 9$, $pK_2 \approx 11$ for the neutral enzyme and $pK_1 \approx 6.5$, $pK_2 \approx 9$ for the acid enzyme, respectively. The results obtained by the EPR measurements are in good agreement with those obtained by low-temperature spectrophotometry.

## INTRODUCTION

It is accepted that horseradish peroxidase (donor: $H_2O_2$ oxidoreductase, EC 1.11.1.7) forms two components during the acid–base transitions, the "acid form" and the "alkaline form." This transition has been explained simply by the ionization of water molecule coordinated in the sixth position of the heme iron to the hydroxyl ion, as shown in Eqn 1.

$$Fe^{3+}(H_2O) \longleftrightarrows Fe^{3+}(OH^-) + H^+ \qquad (1)$$

The equilibrium expressed by Eqn 1 has been well studied by optical[1,2], magnetic susceptibility[3], and electron paramagnetic resonance[4,5] measurements. The p$K$ value of the transition has been determined to be approximately p$K$ = 11 at room temperature[2,3], which is a much higher value than that of either methemoglobin or metmyoglobin.

As shown previously[6], the temperature dependence of the paramagnetic susceptibility and optical absorption spectra at cryogenic temperatures suggested the presence of the two different kinds of alkaline forms; one is in thermal equilibrium between high- and low-spin states, and the other one is purely low-spin for the whole temperature range. It is still unknown whether the temperature dependent transition between high- and low-spin states is due to the change of "effective pH" or the change in the ice crystals in the frozen state. In order to clarify the above, we measured the EPR absorption spectra of horseradish peroxidase isozymes, the acid and neutral enzymes, at liquid-hydrogen and helium temperatures over a wide pH range. The results obtained are complicated and cannot be explained by Eqn 1. The details are presented in this report.

## MATERIALS

Horseradish peroxidase was purified from wild horseradish roots by the method of Kenten and Mann[7]. Chromatography on DEAE- and CM-cellulose columns were carried out according to the method of Shannon *et al.*[8]. The neutral enzyme used was the fraction eluted first from the CM-cellulose column, and the acid enzyme was the main fraction adsorbed on the DEAE-cellulose column. The R.Z. values of the neutral and the acid enzymes are 3.1 and 3.4, respectively. The concentration of peroxidase was calculated by the use of the millimolar extinction coefficient of 107.7 $mM^{-1} \cdot cm^{-1}$ at 403 nm[9].

## METHODS

EPR absorption was measured using an EPR spectrometer of Japan-Electroptics, JES-3BX equipped with 100 KHz field modulation. Quartz sample tubes with 3 mm inner diameter were used. The sample tube was placed in the dewar containing liquid hydrogen, and the dewar was inserted into the sample cavity. The frequency of the klystron was determined by the use of a frequency meter. The measurement of the magnetic field strength was made by using the nuclear magnetic resonance of protons in water. Diphenylpicrylhydrazyl was used for the internal standard at $g$ = 2.00. The measurements at liquid-helium temperature were made using a Varian EPR spectrometer, Model V-4502, with 100 KHz field modulation. A Varian variable temperature accessory was used with liquid helium as coolant. Both EPR spectrometers were operated at X-band. All the EPR spectra were recorded in an approximately first derivative display at a microwave power level well below saturation (approx. 20 mW).

The optical absorption spectra were measured with a split beam spectrophotometer, Shimadzu 40 DFS. The measurements at cryogenic temperatures were carried out using the double dewar cell compartment designed by Hagihara and Iizuka[10]. Measurements of pH were done at room temperature.

RESULTS

*EPR absorption spectra at liquid-hydrogen temperature*

Fig. 1A shows the EPR spectra of the neutral enzyme in the frozen solution at different pH values at 20 °K. The spectrum at pH 7 exhibits a prominent rhombic splitting at $g = 6$, showing that the enzyme is mainly in the high-spin state at this temperature. At pH 11, on the other hand, the resonance amplitude at around $g = 6$ decreases, and the low-spin type absorptions with the principal values of $g$-tensors at $g_x = 1.63$, $g_y = 2.08$, $g_z = 2.94$ appear. This change is known as a transition from "acid form" to "alkaline form".

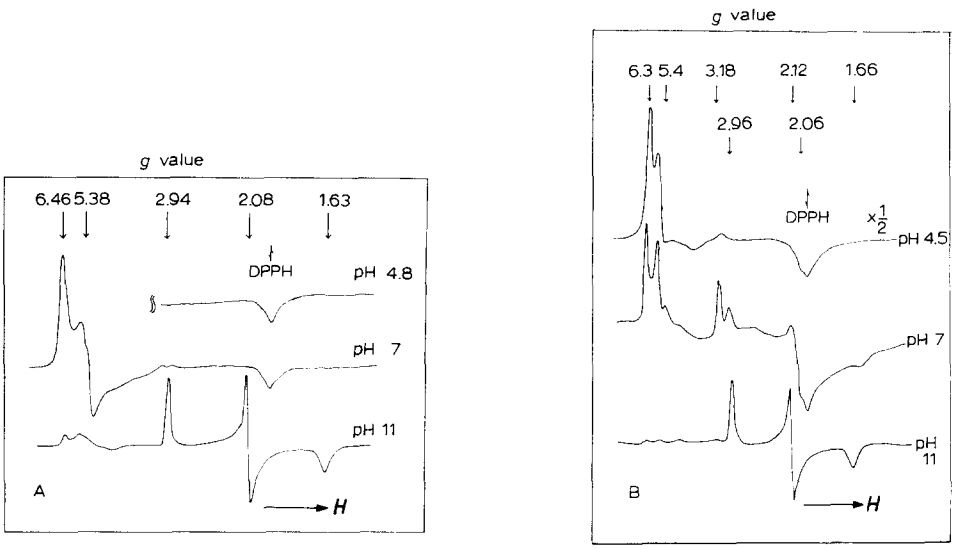


Fig. 1. EPR absorption spectra of peroxidase isozymes at different pH at 20 °K. Buffer concentration, 0.1 M. Phosphate buffer (pH 4.5, 4.8, 7). Glycine–NaOH (pH 11). (A) Neutral enzyme, 150 $\mu$M. (B) Acid enzyme, 150 $\mu$M. Microwave frequency: (A) 9116 MHz; (B) 9097 MHz. Modulation amplitude: 10 G.

Fig. 1B shows the EPR spectra of the acid enzyme at 20 °K at various pH values. A well-resolved large anisotropy is observed at $g = 6$. The spectrum at pH 4.5 is similar to that of the neutral enzyme at pH 7 (*cf.* Fig. 1A). The absorption at $g = 6$ markedly decreases at pH 7, while a new anisotropic absorption appears at $g = 2$, attributable to the low-spin compound, indicating the coexistence of the high-spin and low-spin compounds under these conditions. Furthermore, there exists two different kinds of the low-spin compounds in the sample as judged from the distinct signals at $g = 3.18$ and 2.96. However, the other $g$ values of these two low-spin compounds cannot be determined because they are broad and overlap. At pH 11, the acid enzyme shows the typical low-spin spectrum which is similar to that of the neutral enzyme measured at the same pH.

The pH dependence of the EPR spectra of the low-spin compounds is shown in more detail in Fig. 2. Fig. 2A shows that the signal of the neutral enzyme at $g = 3.2$, which has an amplitude almost comparable to that of $g = 2.94$ signal at pH 7, has

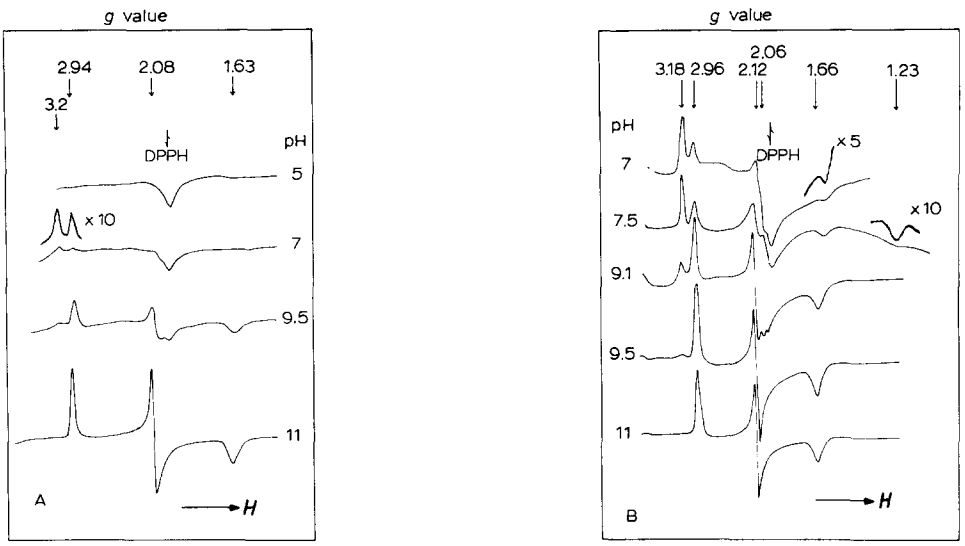


Fig. 2. pH dependence of the EPR absorption spectra of peroxidase isozymes in the low-spin region at 20 °K. (A) Neutral enzyme, 150 $\mu$M. Buffer concentration, 0.1 M. Phosphate (pH 5 and 7). Glycine–NaOH (pH 9.5 and 11). (B) Acid enzyme, 150 $\mu$M. Buffer concentration, 0.1 M. Phosphate (pH 7 and 9.1). Sodium acetate (pH 7.5). Glycine–NaOH (pH 9.5 and 11). Microwave frequency: (A) 9116 MHz; (B) 9097 MHz. Modulation amplitude: 10 G.

diminished at pH 11. On the other hand, the signals at $g$ values at $g_x = 1.63$, $g_y = 2.08$, $g_z = 2.94$ increase with increasing pH. These results suggest the presence of two different kinds of low-spin compounds involved in this transition. The pH-dependent transition between the two kinds of low-spin compounds can be observed more clearly in the acid enzyme (Fig. 2B). At pH 7.5, the spectrum clearly shows two sets of low spin compounds at $g_x = 1.23$, $g_y = 2.06$, $g_z = 3.18$, and $g_x = 1.66$, $g_y = 2.12$, $g_z = 2.96$. The ratio of the signal amplitudes at $g_z = 3.18$ and 2.96 inverts between pH 7.5 and 9.1. The spectra thus suggest that the ratio of these two low-spin compounds is inverted between pH 7.5 and 9.1.

*EPR spectra at liquid-helium temperature*

Fig. 3 illustrates the EPR spectra of the two peroxidase isozymes at liquid-helium temperature (4.2 °K). The spectrum of the acid enzyme at pH 6 (top), is similar to that measured at pH 7 and 20 °K (Fig. 1B). On the other hand, the spectra of the neutral enzyme at pH 8.4 and 9.4 are similar to those of the acid enzyme at pH 6 and 7. The signals at $g_x = 1.23$, $g_y = 2.05$ and $g_z = 3.20$ are scarcely observed at 20 °K, but are clearly observed at 4.2 °K. The ratio of the signal amplitudes at $g_z = 3.20$ and 2.94 is inverted between pH 9.4 and 10.8 as it is for the acid enzyme. The results suggest that the absorption at $g_z = 3.20$ of the neutral enzyme is highly temperature sensitive, while that of the acid enzyme is not, since this latter absorption can be observed at 20 °K (*cf.* Fig. 2B). Table I summarizes the $g$ values for both peroxidase isozymes. The results obtained here are in agreement with those reported previously[4,5].

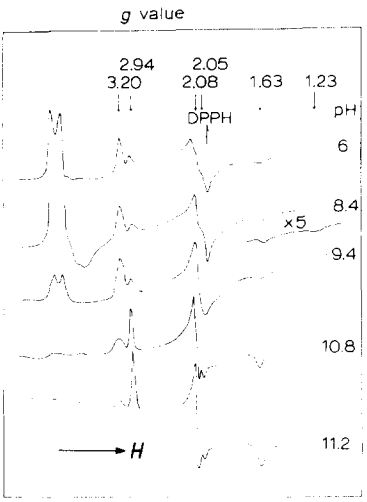


Fig. 3. EPR absorption spectra of peroxidase isozymes at 4.2 °K. Acid enzyme (pH 6). Neutral enzyme (pH 8.4–11.2). Acid enzyme, 70 $\mu$M. Neutral enzyme, 100 $\mu$M. Buffer concentration, 0.1 M. Phosphate (pH 6). Tris–HCl (pH 8.4). Glycine–NaOH (pH 9.4, 10.8, 11.2). Microwave frequency: 9120 MHz. Modulation amplitude: 12 G.

TABLE I

THE $g$ VALUES OF THE VARIOUS COMPOUNDS OF BOTH PEROXIDASE ISOZYMES UNDER DIFFERENT CONDITIONS

| *Horseradish peroxidase* | *Condition* | *Compound* | *g values* |
|---|---|---|---|
| Acid | Low pH | Acid form | 6.3, 5.4, 2.0 |
| | Neutral pH | (I)* | 3.18, 2.06, 1.23 |
| | High pH | Alkaline form | 2.96, 2.12, 1.66 |
| Neutral | Low pH | Acid form | 6.46, 5.38, 2.00 |
| | Neutral pH | (I)* | 3.20, 2.05, 1.23 |
| | High pH | Alkaline form | 2.94, 2.08, 1.63 |

* See discussion.

*pH dependence of EPR signal amplitude*

The pH dependence of the EPR signal amplitude of the high-spin compound ("acid form") and the two low-spin compounds is summarized in Fig. 4. The pH dependence of the high-spin EPR signal amplitude of the neutal enzyme is unchanged between 20 and 4.2 °K. In the case of the neutral enzyme (Fig. 4A), the amplitude at $g = 6.46$ ("acid form") decreases above pH 7, the amplitude at $g_z = 3.20$ increases with pH to a maximum at around pH 10 and then decreases, while the amplitude at $g_z = 2.94$ increases markedly above pH 9. These results show that these two low-spin absorptions originate from the pH-dependent interconvertible compounds of peroxidase itself, and not from the co-existence of the different peroxidase isozymes in the sample. Similar curves are obtained for the acid enzyme except for the difference of pH at which these transitions occur, as shown in Fig. 4B. From the figure, we can

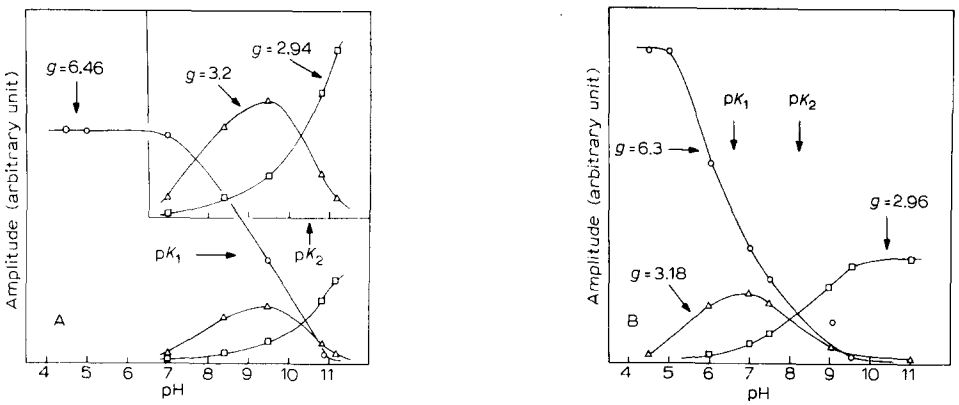


Fig. 4. pH dependence of EPR signal amplitude of peroxidase isozymes. (A) Neutral enzyme. The data at $g = 6.46$ is that obtained at 20 °K and others are at 4.2 °K. The higher pH region is shown expanded in the insert. (B) Acid enzyme. All the data are at 20 °K.

TABLE II

THE p$K$ VALUES OF THE ACID–BASE TRANSITION OBTAINED BY EPR, OPTICAL AND MAGNETIC SUSCEPTIBILITY MEASUREMENTS

p$K_1$ and p$K_2$ are defined by Eqn 2.

| *Horseradish peroxidase* | *Cryogenic temp.* | | | | | | *Room temp.* |
|---|---|---|---|---|---|---|---|
| | *EPR* | | *Optical* | | *Magnetic susceptibility* | | $pK_{OH^-}$ |
| | $pK_1$ | $pK_2$ | $pK_1$ | $pK_2$ | $pK_1$ | $pK_2$ | |
| Neutral | 9 | ≈10.8 | ≈9 | — | ≈9* | — | 10.8–11 |
| Acid | ≈6.5 | ≈ 9 | ≈7 | — | <8** | — | 9.5 |

* M. Tamura, unpublished.

** Recalculated from the results reported previously[6] by assuming that $n^2_{eff}$ values at 77 °K are constant at 25 below pH 5.

roughly estimate the p$K$ values of the transitions between these compounds. The p$K_1$ is determined from the decrement of the high-spin EPR absorption ($g = 6$ region), and p$K_2$ is the p$K$ value of the transition between two low-spin compounds. The values of p$K_1$ and p$K_2$ for both isozymes are listed in Table II.

*Optical absorption spectra at room and liquid-nitrogen temperatures*

The optical absorption at different pH values has been measured at both room and liquid-nitrogen temperatures, as shown in Fig. 5. At 293 °K, the optical absorption of the neutral enzyme (Fig. 5A) is almost unchanged between pH 5 and 9.5, characteristic of the high-spin state. The absorption at pH 11.5 is characteristic of the low-spin state known as "alkaline form." The spectrum at pH 11 is intermediate between that at pH 5 and 11.5. The p$K$ value of this transition, p$K_{OH^-}$, is almost 11 in this case. However, when measured at 77 °K, the pH dependence of the optical absorption is markedly different from that at 293 °K. The 77 °K spectra at pH 5 and 7 are similar to those at 293 °K of the high-spin state. However, the spectrum at pH

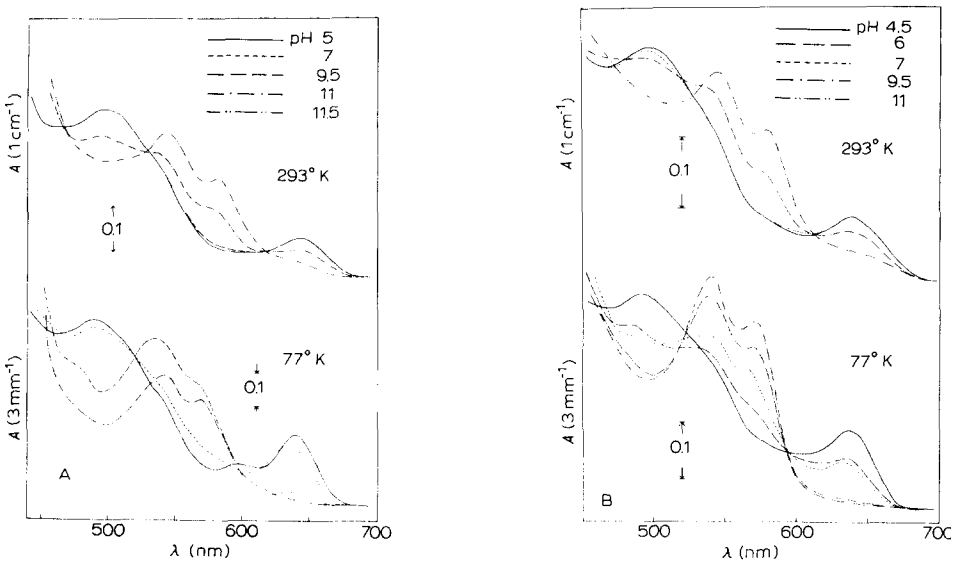


Fig. 5. Optical absorption spectra measured at 293 °K and 77 °K at different pH. (A) Neutral enzyme, 30 $\mu$M. Buffer concentration, 0.1 M. Acetate (pH 5). Phosphate (pH 7). Glycine–NaOH (pH 9.5, 11, 11.5). (B) Acid enzyme, 25 $\mu$M. Buffer concentration, 0.1 M Phosphate (pH 4.5, 6 7). Glycine–NaOH (pH 9.5 and 11).

9.5, which is identical to that at pH 7 and 5 at 293 °K, is changed into that of the low-spin state on cooling to 77 °K. The 77 °K spectra at pH 11 and 11.5 are typical of low-spin states, which are similar to that at pH 11.5 at 293 °K. The apparent p$K$ value at 77 °K is almost 9 which is lower than that at 293 °K, (approx. 11), since the spectrum at pH 9.5 is indicative of almost purely low-spin state at this temperature. Similar temperature induced shifts of the p$K$ values are seen in the case of the acid enzyme, as shown in Fig. 5B. The spectra at pH 6 and 7 at 77 °K, are a mixture of high- and low-spin states, while the spectrum at pH 11 which is similar to that at pH 11.5 of the neutral enzymes (*cf.* Fig. 5A), is unchanged between 293 °K and 77 °K. The p$K$ values are estimated roughly to be 9.5 and 6.5 at 293 °K and 77 °K, respectively. The pH dependence of the optical absorption at 77 °K is in agreement with the results obtained from the EPR measurements, showing that the p$K$ values determined optically at 77 °K might correspond to the $pK_1$ obtained from the EPR measurements (Fig. 4). Thus the values determined optically at 77 °K are listed in Table II as $pK_1$.

Fig. 6 shows the temperature dependence of the optical absorption of the acid enzyme at pH 7 below 273 °K. The transition from low-spin state to high-spin state is clearly seen with increasing temperature, showing that the sample is in thermal equilibrium between high- and low-spin states. On the other hand, at 293 °K, the "alkaline form" appears at pH 11 and is almost a low-spin state (*cf.* Fig. 5B). Therefore the compound appearing at neutral pH with $g_x = 1.23$, $g_y = 2.06$ and $g_z = 3.18$, is in thermal equilibrium between two spin states, while the "alkaline form" with $g_x = 1.66$, $g_y = 2.12$, $g_z = 2.96$ is purely low-spin state for the whole temperature range. A similar result is also obtained in the case of the neutral enzyme; the optical absorption of the low-spin compound with $g_x = 1.23$, $g_y = 2.05$, $g_z = 3.20$ is tem-

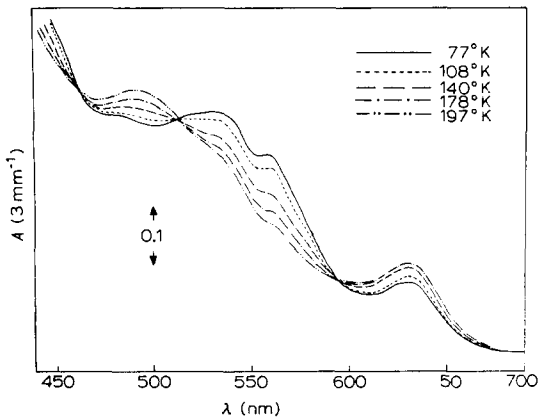


Fig. 6. The temperature dependence of the optical absorption spectrum of the acid enzyme at pH 7. Acid enzyme, 35 $\mu$M. 0.1 M phosphate (pH 7).

perature dependent (M. Tamura, unpublished), but that of the "alkaline form" with $g_x = 1.63$, $g_y = 2.08$, $g_z = 2.94$ is not (*cf.* Fig. 5A).

## DISCUSSION

Present EPR studies clearly demonstrate that there exist three components involved in the acid–base transition, *i.e.*

$$\text{Horseradish peroxidase (acid)} \xrightarrow{pK_1} \text{horseradish peroxidase (I)} \xrightarrow{pK_2} \text{horseradish peroxidase (alkaline)} \quad (2)$$

where horseradish peroxidase (acid) and horseradish peroxidase (alkaline) represent the "acid" and "alkaline form", which corresponds to $Fe^{3+}$ ($H_2O$) and $Fe^{3+}$ ($OH^-$) of Eqn 1, respectively. Horseradish peroxidase (I) is a new component whose EPR spectrum is more anisotropic than that of the "alkaline form" (*cf.* Table I). This EPR absorption of horseradish peroxidase (I) was hardly detected at liquid-nitrogen temperature coming from its broadening of the rapid relaxation rate, while that of "alkaline form" was detected easily at the same temperature. It should be noted here that the measurements of EPR and optical absorption spectra were made at quite different temperatures; the former at 20 °K and 4.2 °K and the latter at above 77 °K. However, as shown by the magnetic susceptibility measurements[6], the temperature dependence of magnetic susceptibility of those samples obeys the Curie law in the temperature range below 170 °K, showing that the relative amounts of the high-spin and low-spin components in the sample are unchanged in the temperature range from 4.2 to 77 °K. Therefore, the results obtained by EPR and optical measurements can be correlated in each other as $pK_1$. In Table II, the values of $pK_1$ and $pK_2$ are compared with the $pK_{OH-}$ of the "acid" and "alkaline" transition measured optically at room temperature. The results obtained from the paramagnetic susceptibilities at 77 °K are also included. These values are in agreement with each other. It should be noted that $pK_1$ is not observed optically at room temperature, and $pK_2$ is almost the same as $pK_{OH-}$. As shown in Fig. 6, the absence of $pK_1$ at room temperature can be

explained by the fact that horseradish peroxidase (I) is in thermal equilibrium between high- and low-spin states, which is high-spin state at room temperature, since "acid form" is also high-spin state at this temperature. As "alkaline form" is low-spin at room temperature, $pK_2$ can be observed as the transition between "acid" and "alkaline form". The presence of the thermally equilibrated compound, horseradish peroxidase (I), strongly supports the magnetic susceptibility measurements which show the presence of the crossing point in the $n^2_{eff} - 1/T$ curves near room temperature between pH 5 and 9[6], where $n_{eff}$ is the effective Bohr magneton number and $T$ is absolute temperature. It will be pointed out that "alkaline forms" of catalase (EC 1.11.1.6)[11] and cytochrome *c* peroxidase (EC 1.11.1.5)[12,13] behave quite similarly to horseradish peroxidase (I), and those of methemoglobin and metmyoglobin are in thermal equilibrium between the two spin states, which are more than 50% of the high-spin state at room temperature[14,15]. Therefore, it would seem likely to conclude that there exists the compound which is in thermal equilibrium between two spin states at alkaline solution for these various hemoproteins. It is interesting that the $pK$ value of the transition between "acid form" and the thermal equilibrium compound, horseradish peroxidase (I), ($pK_1$) is lower than pH 10 in both peroxidase isozymes. These results lead us to speculate that the histidine residue is concerned with this transition as in hemoglobin and myoglobin.

From the measurements of the differential pH titrations between holo- and apo-horseradish peroxidase, Theorell and Paul[16] discussed the presence of the three different kinds of compounds involved over the pH range from 4 to 11. They proposed that "alkaline form" of the horseradish peroxidase was the structure attached in both the 5th and 6th positions of heme iron to the two $OH^-$, and there existed another compound which was coordinated at the 6th position of the iron to one $OH^-$ such as "alkaline form" of methemoglobin and metmyoglobin. The scheme of Eqn 2 is not incompatible with the results obtained by these authors, however, if horseradish peroxidase (I) has the same structure as alkaline methemoglobin and alkaline metmyoglobin, the electronic state of horseradish peroxidase (I) is quite different from that of hemoglobin and myoglobin, since the EPR characteristics are different; (*cf.* $g_x = 1.82$, $g_y = 2.19$, $g_z = 2.56$ for alkali metmyoglobin[17] and $g_x = 1.85$, $g_y = 2.17$, $g_z = 2.55$ for alkali methemoglobin)[18].

Among the various enzymic properties, $pK_{OH-}$ ($pK_2$) of the acid enzyme and the neutral enzyme are very different from each other (see Figs 5A, 5B). The $pK_{OH-}$ of the acid enzyme is almost 2 pH units shifted to lower pH than that of the neutral enzyme, and similar phenomenon is observed in the case of $pK_1$. However, the EPR spectra of these three components, "acid form", horseradish peroxidase (I) and "alkaline form" are quite similar between both peroxidase isozymes except for the difference of the values of $pK_1$ and $pK_2$. Another horseradish peroxidase isozyme, paraperoxidase[19], also gives the two different kinds of low-spin compounds during the acid–base transition (M. Tamura, R. Nakajima and I. Yamazaki, unpublished). So, it would be concluded that all the horseradish peroxidase isozymes behave similarly in the acid–base transition, which follows the scheme of Eqn 2. The pH dependence of the paramagnetic susceptibility of the neutral enzyme gives similar results, and these will appear in the following paper.

Recently, we have prepared the various synthetic horseradish peroxidases which contain the modified hemins. It is shown that the modifications of side groups

of the porphyrin ring strongly affect the p$K$ values of the acid–base transition (p$K_{OH-}$ or p$K_2$)[20]. The measurements of EPR and paramagnetic susceptibilities of these synthetic enzymes are very interesting and will be reported soon.

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