Presence of Endogenous Calcium Ion and Its Functional and Structural Regulation in Horseradish Peroxidase*

(Received for publication, January 3, 1986)

Yoshitsugu Shiro, Masuo Kurono, and Isao Morishima†

From the Division of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 606, Japan

The endogenous calcium ion (Ca²⁺) in horseradish peroxidase (HRP) was removed to cause substantial changes in the proton NMR spectra of the enzyme in various oxidation/spin states. The spectral changes were interpreted as arising from the substantial alterations in the heme environments, most likely the heme proximal and distal sides. The comparative kinetic and redox studies revealed that these conformational changes affect the reduction process of compound II, resulting in the decrease of the enzymatic activity of HRP. It is also revealed from the ESR spectrum and the temperature dependences of the NMR and optical absorption spectra of the Ca²⁺-free enzyme that the heme iron atom of the Ca²⁺-free enzyme is in a thermal spin mixing between ferric high and low spin states, in contrast to that of the native enzyme. These results show that Ca²⁺ functions in maintaining the protein structure in the heme environments as well as the spin state of the heme iron, in favor of the enzymatic activity of HRP.

Horseradish peroxidase (HRP) is a heme enzyme catalyzing the oxidation of a wide variety of aromatic molecules by hydrogen or alkyl peroxides. The enzymatic reaction cycle has been established and normally proceeds by the following mechanism (1, 2),

\[
\begin{align*}
\text{HRP} + \text{H}_2\text{O}_2 & \xrightarrow{k_1} \text{compound I} + \text{H}_2\text{O} \\
\text{compound I} + \text{AH}_2 & \xrightarrow{k_2} \text{compound II} + \text{AH} \\
\text{compound II} + \text{AH}_2 & \xrightarrow{k_4} \text{HRP} + \text{AH} \\
2\text{AH}^- & \xrightarrow{k_5} \text{A}_2\text{H}_2 \text{ or A} + \text{AH}_2
\end{align*}
\]

where compound I and compound II represent the enzymatic reaction intermediates, and AH₂ the reducing substrate. This enzyme has been established to contain iron(III)-protoporphyrin IX as a noncovalently bound prosthetic group. The fifth ligand of the heme iron is now confirmed to be a histidyl imidazole (3, 4), and the sixth coordinated position has been suggested to be occupied by a water molecule (5). The distal histidyl imidazole sits within the hydrogen-bonding dis-

* This research was supported by Grants-in-Aid 60540285 and 6079022 for Scientific Research from the Ministry of Education, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.
† To whom correspondence should be addressed.
1 The abbreviations used are: HRP, horseradish peroxidase; metMb, metmyoglobin; metHb, methemoglobin; PABA, \(p\)-aminobenzoic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

However, a recent x-ray crystallographic study on cytochrome-c peroxidase (5, 6), an analogous protein to HRP, revealed that the geometry of the distal histidine in peroxidase is not proper for the hydrogen bonding to the water coordinated to the heme iron, while the distal imidazole in metMb is normally hydrogen bonded to the bound water molecule. Indeed, there is reason to believe that the water ligand at the sixth position in HRP is less strongly bound and in a somewhat different environment than it is in metMb. For example, results from absorption (7), ESR (6, 9), resonance Raman (10), and nuclear magnetic relaxation (11) measurements have indicated that in HRP the axial water ligand is either absent or loosely bound. However, as was displayed on the Evans and Sutherland Picture System, the distal histidine of peroxidase is oriented to readily hydrogen bond with the iron-coordinated peroxides, resulting in a heterolytic cleavage of the peroxide O-O bond by its acid-alkaline catalysis. Furthermore, the x-ray analysis also showed that in peroxidase the proximal histidine contacts and probably hydrogen bonds with the side chain of glutamine, which in turn interacts with the buried carboxylyte group of glutamic acid, while in metMb the residue is hydrogen bonded to a backbone carbonyl oxygen atom. This former set of interactions in peroxidase may impart sufficient anionic character to the proximal histidine to stabilize higher oxidation states of the heme iron during the peroxidase catalytic cycle (6, 12). These arguments seem to indicate that the specific structures in the heme environments may regulate the activities of the heme prosthetic group in peroxidases.

Recently, Haschke and Friedhoff (13) and present authors (14) reported that HRP contains 2 mol of Ca²⁺/mol of enzyme and that removal of the bound Ca²⁺ from the enzyme causes a 2-fold decrease in its enzymatic activity. They also studied the effect of Ca²⁺ binding on the thermal stability of the protein. These results allowed us to expect that Ca²⁺ binding by HRP is essential in maintaining the protein structure which is suitable for the peroxidase activity. However, more detailed studies including kinetic and spectroscopic measurements have not yet been carried out to see the calcium binding to HRP in relation to its catalytic mechanism. Furthermore, the effect of the calcium binding to the enzyme on the spin state of the heme iron and heme microenvironmental structure have remained open to further studies.

We have attempted here to study kinetics and redox potential measurements of Ca²⁺-free HRP and to delineate the Ca²⁺-binding effect on the catalytic process of the enzyme in more detail by comparing these results with those previously reported for the native enzyme. The relevance of the present results to the structural effect of calcium binding revealed by ¹H NMR, ESR, and absorption spectral measurements is also

9382
discussed to shed light on the relationship of HRP between the specific structure in the heme environment and its characteristic function.

**MATERIALS AND METHODS**

**Enzyme Preparation**—Horseradish peroxidase type VI (BZ = 3.2) was purchased as a lyophilized salt-free powder from Sigma. Calcium removal from the enzyme was achieved by the method of Haschke and Friedhoff (13). The Ca**2+-reconstituted enzyme was prepared by adding CaCl₂ to the Ca**²⁺-free enzyme solution or incubation of the Ca**²⁺-free enzyme against CaCl₂ solution, and purified by chromatography on CM-52. The concentration of the enzyme was determined spectrophotometrically by using molar absorptivity of 102 cm**⁻¹**mm**⁻¹** at 403 am and pH 7 (15). The cyanide or benzohydroxamic acid complex of the enzyme was prepared by adding a 5-fold excess amount of the ligand or the substrate to the enzyme solution, respectively. The ferrous enzyme was generated by addition of sodium dithionite under N₂.

The specific activities of the enzymes were measured spectrophotometrically by following the 480-nm absorption accompanied by the oxidation of o-aminophenol in the presence of hydrogen peroxide at 20°C.

**Kinetics and Redox Potential Measurements**—The reaction rate constants of the enzymes, k₆, k₇, and k₈, were measured with a Union Giken rapid reaction analyzer RA-1300 at 50°C. The measurements of the k₆ and k₇ values were carried out within 5 min after compounds I and II were generated. The spectrophotometric wavelength used to monitor the reaction was an isosbestic point between the other two. PABA, which was obtained from Nakarai Chemical Co. as its potassium salt, was used as a substrate for compound II formation. Its concentration was monitored spectrophotometrically in phosphate buffer with the molar absorptivity of 14.5 mM⁻¹ cm⁻¹ at 265 nm and pH 7.6 (16). Deionized water obtained through Organo G-10 with extremely small electric conductivity was distilled twice for kinetic measurements.

The oxidation-reduction potentials for the ferric-ferrous couple of HRP were measured by the methods described previously (18). Sodium dithionite and ferricyanide were employed as a reductant and an oxidant, respectively.

**Spectral Measurements**—Proton NMR spectra were recorded at 300 MHz on a Nicolet NTC-300 Fourier transform NMR spectrometer equipped with a NMC-1280 computer system. For recording the typical proton spectra, 5,000–15,000 transients were accumulated to obtain the Fourier transformed spectra with 8,000 data points and a 5.7-μs 90° pulse. Large proton peaks of the solvent water were suppressed by a 500-μs low power 180° pulse prior to the high power observe pulse. Proton chemical shift is referenced with respect to the proton signal of H₂O in the protein solution, assigning a positive value for the low-field resonance.

In all the sample solutions for NMR spectral measurements were prepared in appropriate buffer solutions between pH 5 and 10 such as PIPES except phosphate buffer, which contaminates calcium ion. Protein concentration was about 1.0–2.0 mM in 90% H₂O and 10% ²H₂O. The pH titration was performed by direct addition of 0.1 N NaOH or HCl to the enzyme solution in the NMR sample tube. The pH values were measured with a radiometer model PHM-100 pH meter, equipped with an Ingold microcombination glass electrode.

The visible absorption spectra were recorded with a Union-Giken SM-401 spectrometer by using a cell with a 1-cm path length.

**RESULTS**

**Kinetic and Redox Measurements**—The bound Ca**²⁺ in HRP can be readily removed from the enzyme by incubation of the enzyme in the presence of guanidine hydrochloride and EDTA as confirmed by atomic absorption spectroscopic measurements. Before and after the removal of the bound Ca**²⁺, we measured the enzymatic activity of HRP at pH 7. The activity of the enzyme was reduced to approximately 40% after calcium removal and returned to 80% of the initial activity upon reconstitution with Ca**²⁺. When the enzyme was purified by the use of CM-cellulose after addition of Ca**²⁺ (Ca**²⁺-reconstituted enzyme), the activity was almost restored. This result is the same as that reported by Haschke and Friedhoff (13).

In order to clarify the effect of Ca**²⁺ removal on the enzymatic reaction of HRP, the kinetic measurements were made at pH 7 for the native (Ca**²⁺-bound), Ca**²⁺-free, and Ca**²⁺-reconstituted enzymes of HRP and analyzed under pseudo-first order approximation (19–21). The rate constants, k₁, k₆, and k₈, determined by dividing the pseudo-first order constants by substrate concentrations are listed in Table I. Inspection of the table shows that significant differences are formed for all the constants between the native and the Ca**²⁺-free enzymes and were almost recovered upon Ca**²⁺ reconstitution. With these rate constants, we calculated the specific activities of these enzymes on the basis of the expression relating the specific activity and the rate constants under the steady-state condition (22, 23).

\[ k₀ = \frac{2k₆k₇[HRP][PABA][H₂O₂]}{(k₆ + k₆ + k₈)[H₂O₂] + k₈[PABA]} \]

The calculated value for the Ca**²⁺-free enzyme is 47% of the native one, which is quite consistent with those obtained from the static activity measurement. This result indicates that the differences in the rate constants arise from Ca**²⁺ removal.

Upon Ca**²⁺ removal, substantial change of k₃, which was reduced to 44% of the initial value, is more remarkable, compared with k₁ and k₇, keeping in mind that the k₅ process is a rate-determining step in the enzymatic cycle of HRP and thus governs the overall activity, it seems reasonable to conclude that the decrease in the k₃ may be predominantly responsible for a loss of the enzymatic activity upon Ca**²⁺ removal. Then, in order to see the effect of Ca**²⁺ removal on k₃ value in more detail, the oxidation of PABA by compound II was examined for the native, Ca**²⁺-free, and Ca**²⁺-reconstituted enzymes over a wide range of substrate concentration (up to 9 mM) at pH values of 4.4 and 7.0. At both pH values, nonlinear responses were observed in k₇ versus [PABA] plots and analyzed according to the following equations (16).

\[ \text{Compound II} + \text{PABA} \xrightarrow{k_{11}} \text{HRP} + \text{P} \\
\text{K}_n \uparrow \text{PABA} \\
\text{Compound II} + \text{PABA} \xrightarrow{k_{12}} \text{HRP} + \text{P} \]

\[ k_{obs} = \frac{k_{11}[\text{PABA}] + k_{12}[\text{PABA}]/K_n}{1 + [\text{PABA}]/K_n} \]

The obtained kinetic parameters, k₇₁, k₇₂, and 1/Kₚ (Kₚ:

---

*Irreversibility of the enzymatic activity upon recombination of Ca**²⁺ may be due to a partial denaturation of the protein by treatment of strong denaturant, guanidine hydrochloride. However, the NMR spectral features such as resonance positions and their temperature dependences were all reversible with respect to the Ca**²⁺ reconstitution.
Functional and Structural Regulation by Ca²⁺ in HRP

TABLE II

| Enzymes                | pH 7.0 | pH 4.4 |
|------------------------|--------|--------|
|                        | \( k_{41} \times 10^{-2} \) | \( k_{42} \times 10^{-2} \) | \( 1/K_{m} \times 10^{2} \) | \( k_{41} \times 10^{-2} \) | \( k_{42} \times 10^{-2} \) | \( 1/K_{m} \times 10^{2} \) |
| Native HRP             | 4.2    | 2.2    | 1.1   | 5.7   | 3.1   | 0.2   |
| Ca²⁺-free HRP          | 2.3    | 6.1    | 1.4   | 6.3   | 4.0   | 0.3   |
| Reconstituted HRP      | 4.4    | 2.4    | 1.5   | 6.5   | 4.0   | 0.4   |

\( ^{a} \) In 1 mM PIPES-NaOH.
\( ^{b} \) In 3 mM citric acid-sodium citrate.

In order to gain further insight into Ca²⁺ binding by HRP, we obtained the proton NMR spectra in various spin and oxidation states. Fig. 5 shows the hyperfine-shifted proton NMR spectra of the cyanide complexes of the Ca²⁺-free and the native enzymes at pH 7 and 22 °C. The heme methyl...
Functional and Structural Regulation by Ca\textsuperscript{2+} in HRP

proton peaks of the cyanide complex of the Ca\textsuperscript{2+}-free enzyme are observed at 21.0 and 26.8 ppm downfield from the H\textsubscript{2}O resonance, whereas those of the cyanide complex of the native enzyme are located at 21.5 and 26.6 ppm. Some minor spectral differences are also noticeable between these spectra in the 5–20 ppm range. Upon addition of Ca\textsuperscript{2+} to the cyanide complex of the Ca\textsuperscript{2+}-free enzyme, the signals of the Ca\textsuperscript{2+}-free and the native enzymes were concomitantly observed when a half-saturated amount of Ca\textsuperscript{2+} was added.

In Fig. 6A are compared the proton NMR spectrum of ferrous Ca\textsuperscript{2+}-free HRP with that of the native enzyme. The heme peripheral proton signals of the enzyme are observed at 22.6, 17.9, 14.2, and 8.5 ppm for the native enzyme, while at 20.0, 15.8, 9.2, and 6.3 ppm for the Ca\textsuperscript{2+}-free one. The broad and single-proton peak for the ferrous native enzyme, assigned to proximal histidyl NH signal, is observed at 74.0 ppm (26, 27), whereas the one for ferrous Ca\textsuperscript{2+}-free enzyme appears at 79.7 ppm. Fig. 6B shows the corresponding absorption spectral change in which the Soret peak shifted from 437 to 428 nm upon calcium removal. The NMR and visible spectral changes were reversible with respect to removal or recombination of Ca\textsuperscript{2+}.

In order to reveal the heme microenvironmental structural changes induced by calcium removal, we have also examined pH dependence of the spectra for Ca\textsuperscript{2+}-free HRP with recourse to proton NMR measurements. In Fig. 7A is shown the downfield portions of proton NMR spectra of reduced HRP in H\textsubscript{2}O as a function of pH from 5.2 to 9.0. The pH-dependent spectral changes for Ca\textsuperscript{2+}-free HRP are similar to those for the native HRP reported by La Mar and de Ropp (27). There are two pH-induced interconvertible protein conformers as is manifested by pH-induced spectral alterations. However, integration of individual methyl resonances or the proximal histidyl NH signal as a function of pH yields a pH of 5.5, which is associated most probably with the ionization of the distal histidine. With further raising of the pH, all hyperfine shifted NMR signals decreased in intensities and finally disappeared at pH 9.0. These spectral changes were reversible with respect to raising or lowering pH. Disappearance of the hyperfine shifted resonances above pH 9 may suggest that the heme iron in ferrous Ca\textsuperscript{2+}-free HRP becomes diamagnetic in the alkaline region, which is in sharp contrast to ferrous native HRP of which proton NMR spectrum was insensitive to the pH variation in this pH region. Corresponding pH-dependent alteration in absorption spectrum is shown in Fig. 7B. With raising pH, there is a steep increase in intensity of low spin band at 425, 530, and 560 nm. The resultant spectrum at pH 9.7 bears a strong resemblance to that of diamagnetic pyridine hemochromogen, which is in good agreement with the proton NMR result stated above (28).

Fig. 8 illustrates the effects of Ca\textsuperscript{2+} removal from the enzyme on the proton NMR spectrum of the HRP-substrate complex.

![Figure 2: Proton NMR spectra at 24°C and pH 6.7 in H\textsubscript{2}O of isoenzyme c of horseradish peroxidase before (native) and after (Ca\textsuperscript{2+} free) and the treatment of the enzyme with guanidine hydrochloride and EDTA. The downfield region of the hyperfine shifted spectra is illustrated. Proton chemical shift is referenced with respect to the signal of H\textsubscript{2}O in the sample solution. HDO, residual H\textsubscript{2}O in D\textsubscript{2}O.](image-url)
Functional and Structural Regulation by Ca"+ in HRP

The present spectroscopic data for Ca"+-free HRP such as proton NMR, ESR, and optical spectra revealed that the heme environmental structure of Ca"+-free HRP is substantially different from that of the native enzyme in various iron spin/oxidation states (Figs. 2-8), and kinetic results showed that Ca"+ removal from HRP affects all the rate constants in the catalytic cycle of the enzyme (Tables I and II). These results allow us to expect that Ca"+ binding is essential for the structural and functional characteristics of HRP. Bearing in mind that the Ca"+ removal from HRP reduces its enzymatic activity, thermal stability (13), and compound I stability (14), all the present results and discussion may provide us with a clue to unveil the relationship between the heme microenvironmental structures and the catalytic function of HRP. The spectral and kinetic observations will be discussed in structural terms relevant to the peroxidase activity.

The structural changes of HRP in the heme vicinity upon Ca"+ removal are sensitively manifested in the proton NMR spectra as the spectral shift of the hyperfine-shifted proximal histidyl NH resonance for a ferrous high spin form (27, 29). As shown in Fig. 6A, the downfield shift by 6 ppm of this signal induced by Ca"+ removal from the enzyme significantly reflects the subtle perturbation at the heme proximity. Since the heme iron of ferrous form enzyme has no sixth coordination ligand and eventually has no direct interaction with the distal histidine, the spectral change in the NH signal may result from the changes in the binding profiles of the proximal imidazole such as bond compression or tilting of the ironimidazole bond or modulation of the NH hydrogen bonding. The structural change in the proximal side was also supported by the NMR spectral change of the proximal histidyl C6H signal by 2 ppm for the ferric low spin cyanide complexes (Fig. 5), which has been suggested to be a probe for the extent of the proximal histidine NH hydrogen bonding (30, 31). Furthermore, the NMR spectral shift for the proximal NH, H of the HRP-benzohydroxaminic acid complex in a pure ferric high spin state appears to be also consistent with this suggestion. This implies that the conformational changes in the heme proximal side associated with the Ca"+ removal from the enzyme could occur irrespective of spin/oxidation states of the heme iron.

These results remind us of the previous studies by Kastner and co-workers (32-34) which have emphasized the influence of protein structure on the spin equilibrium through a control of axial ligation. More recently, Neya and Morishima (35) suggested on the basis of paramagnetic NMR shifts for heme-N3-(1- or 2-methylimidazole) model systems that the
FIG. 5. Hyperfine shifted proton NMR spectra of the cyanide complexes of the Ca²⁺-free and the native enzymes of horseradish peroxidase at pH 7.6 and 21°C. Asterisks show the heme methyl signals of the residual cyanide complex of the native enzyme.

FIG. 6. Proton NMR (A) and absorption (B) spectra of the ferrous Ca²⁺-free and the ferrous native enzymes of horseradish peroxidase at pH 6.3 and 22°C.

binding nature of proximal ligand to the heme iron could modulate the spin equilibrium of the iron. Therefore, the structural change in the heme proximal side, most probably the proximal imidazole, may be responsible for the spin equilibrium of the Ca²⁺-free enzyme in a ferric spin state, in which some change of the binding nature of proximal histidine is induced by removal of Ca²⁺ from the enzyme. Other structural factors such as van der Waals interaction between the heme peripheral and peptide chains and effects from the distal side may account for the iron spin state change of the ferric
**A**

**Fig. 7.** A, downfield protons of the proton NMR spectra ferrous Ca$^{2+}$-free HRP at various pH values: 5.2, 5.8, 7.2, and 9.0. All chemical shifts were referenced with respect to H$_2$O. B, optical absorption spectra of ferrous Ca$^{2+}$-free HRP at pH values 7.0 and 9.7. All spectra were taken at 22°C.

**B**

enzyme, and these possibilities could not be necessarily ruled out at the present stage. In any event, it is evident that Ca$^{2+}$ removal elicits the structural perturbation in the heme proximal side.

Further insight into the conformational change in the heme vicinity was gained on the basis of the pH dependence of the NMR spectra of the reduced enzyme. It has been well known that the spectral change of native HRP in a ferrous high spin is attributable to the ionisation of the distal histidine (27). The pK value of the distal histidine is lowered by 2 pH units although the NMR spectral feature of ferrous Ca$^{2+}$-free HRP associated with its ionization is similar to that of the native enzyme. Furthermore, it is to be noted that the drastic spectral change is encountered in the alkaline region as is manifested by proton NMR and absorption spectra (Fig. 7). This result indicates that some internal nitrogenous ligand from the distal amino acid residue, most likely the imidazole group of the distal histidine, occupies the sixth coordination position of the heme iron, thereby producing the diamagnetic species like pyridine hemochrome. The above spectral change was not observed for the native enzyme in the same pH region, and the Ca$^{2+}$ reconstitution converted the above spectral changes to those for native HRP. It is thus tempting to suggest that Ca$^{2+}$ removal causes the conformational changes in the heme distal side, which appears to be more drastic than that in the proximal side. These conformational changes in the heme vicinity induced by Ca$^{2+}$ removal may substantially affect the enzymatic activity of HRP.

We then discuss these structural changes upon Ca$^{2+}$ removal in the heme environment with relevance to the unique changes in the reaction rates of HRP; $k_4$ experiences a substantial change as compared with $k_1$ and $k_2$. The $k_4$ process involves two reaction steps, the binding of a substrate to the protein moiety and the subsequent reduction of the heme iron from ferryl to ferric states. It is reasonable to assume that the
Functional and Structural Regulation by Ca\textsuperscript{2+} in HRP

former and/or the latter steps are disturbed by the structural change induced by calcium removal. In other words, the Ca\textsuperscript{2+} removal may alter the structures relevant to these processes. Indeed, the redox property of HRP was significantly altered upon Ca\textsuperscript{2+} removal, as manifested as a cathodic shift by 30 mV of the redox potential for the Fe(III)/Fe(II) couple. However, a little change in the binding constant of PABA by compound II at pH 7 (1/K\textsubscript{a}) allows us to expect that the protein structural change around the substrate-binding site may be insignificant (Table II). This is also supported by the measurement of the binding constant of indolepropionic acid to HRP on the basis of the proton NMR spectral shifts of the heme methyl of the enzyme (36), which was not affected by calcium removal. These findings thus suggest that the change of the reaction rate k\textsubscript{4} induced by calcium removal, which is directly related to the loss of the specific activity, is essentially caused at the reduction step of the heme iron rather than at the step of substrate binding.

The redox potential of HRP for the Fe(III)/Fe(II) couple is characteristically low (-273 mV), as compared with metMb (+50 mV) (37). This feature is invariant for peroxidases (38). Mechanistically this requirement for a lowered redox potential in peroxidases may be understandable, since the Fe(IV) and/or porphyrin π-cation radical in compound I must be stabilized (39). These unique redox properties of peroxidases have been primarily explained as due to the anionic character of the proximal imidazole. The validity of this view could be assessed by Doeff and his co-workers (40) in the study of the model complex heme-imidazolyl NH. Thereby, the increase of redox potential of HRP upon Ca\textsuperscript{2+} removal from -273 to -244 mV (Fig. 1) may be interpreted in terms of the structural change in the proximal side such as a decrease of an anionic character of proximal imidazole. This conformational change, if any, appears to be reasonably responsible for instability of compound I of Ca\textsuperscript{2+}-free HRP relative to native compound I (14).

However, one may ask why there is no significant difference in k\textsubscript{a} at pH 4.4 between native and Ca\textsuperscript{2+}-free HRPs (Table II) in spite of the different nature of the heme fifth ligand as inferred from the different proton NMR spectra. Thus, we must take into account another possible cause which is more predominantly affected upon Ca\textsuperscript{2+} removal and governs the redox property of HRP rather than the proximal histidine. Yamazaki and his co-workers (39, 41, 43) have postulated that the protonation of the distal His is significantly related to the redox property of HRP rather than the proximal histidine. This structural change may be responsible for the change of the redox potential of the enzyme and eventually for a decrease of the rate constant k\textsubscript{4}.

A variety of studies including the x-ray analysis of cytochrome-c peroxidase (5, 6) suggested that the distal histidine of peroxidase plays a crucial role in its enzymatic reaction. The proposed mechanism for peroxidase-catalyzed heterolytic cleavage of the peroxide O=O bond to generate compound I suggested that the distal histidine serves as a scid-alkaline catalyst to facilitate the transfer of a proton from one oxygen atom of peroxide to the other one (6, 12). One might expect the histidine residue to form a hydrogen bond with the oxygen atom of peroxide. In fact, disruption of hydrogen-bonded lattice caused by selective and reversible modification of the imidazole group with p-chlorobenzoylchloride decreases or abolishes the enzymatic activity of HRP (9, 44), although the modification induces no significant structural changes in the heme environment (45). Thus, the structural changes in the heme distal side as well as the change of the imidazole basicity of the distal histidine possibly affect k\textsubscript{a} and k\textsubscript{4}. Moreover, it is also likely that these structural alterations are accompanied by the change in the distance between the heme and the substrate as is manifested as a different ratio of k\textsubscript{a1} to k\textsubscript{a2} between Ca\textsuperscript{2+}-free and native HRP (Table II).

As mentioned so far, the endogenous Ca\textsuperscript{2+} binding maintains the heme environmental structures of HRP, the heme proximal and distal side, which is relevant to its activity. The Ca\textsuperscript{2+} titration to the Ca\textsuperscript{2+}-free enzyme followed by proton NMR spectra indicates that one Ca\textsuperscript{2+} is essential for the protein structure of HRP, although the enzyme contains 2 mol of Ca\textsuperscript{2+}/mol of the enzyme. It thus follows that one Ca\textsuperscript{2+} is a structural factor to favor the peroxidase activity of HRP. However, there is no evidence for identification of the Ca\textsuperscript{2+} binding sites. To gain insights into the different roles of two Ca\textsuperscript{2+} ions and the structures at the Ca\textsuperscript{2+} site, the metal substitutions for Ca\textsuperscript{2+} in HRP were carried out and will be shown in the subsequent paper.

Acknowledgments—We are grateful to Professor K. Hiromi and Dr. H. Nakata for making the stopped flow spectrometers available to us and for helpful discussions. We also thank Dr. R. Makino for measuring the redox potential of the enzymes.

REFERENCES

1. Chance, B. (1952) Arch. Biochem. Biophys. 41, 416-424
2. George, P. (1953) Biochem. J. 54, 267-276
3. La Mar, G. N., and de Ropp, J. S. (1979) Biochim. Biophys. Acta 587, 406-421
4. La Mar, G. N., de Ropp, J. S., Smith, K. M., and Langry, K. C. (1980) J. Biol. Chem. 255, 6646-6652
5. Poulo, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Erikkson, B., Xuong, N., Yonetani, T., and Kraut, J. (1980) J. Biol. Chem. 255, 575-580
6. Poulo, T. L., and Kraut, J. (1980) J. Biol. Chem. 255, 8199-8205
7. Kobayashi, K., Tamura, M., Hayashi, K., Hori, H., and Morimoto, H. (1978) J. Biol. Chem. 253, 2239-2242
8. Gupta, R. K., Mlvdvan, A. S., and Schonbaum, G. R. (1979) Biochem. Biophys. Res. Commun. 89, 1334-1340
9. Gupta, R. K., Mlvdvan, A. S., and Schonbaum, G. R. (1979) in Biochemical and Clinical Aspects of Oxygen (Caughey, W. S., ed) pp. 177-193, Academic Press, Orlando, FL
10. Teraoka, J., and Kitagawa, T. (1981) J. Biol. Chem. 256, 3969-3977
11. Gupta, R. K., Mlvdvan, A. S., and Schonbaum, G. R. (1980) Arch. Biochem. Biophys. 202, 1-7
12. Morrison, M., and Schonbaum, G. R. (1976) Annu. Rev. Biochem. 45, 861-888
13. Haschke, R. H., and Friedhoff, J. M. (1979) Biochem. Biophys. Res. Commun. 86, 1039-1042
14. Ogawa, S., Shiro, Y., and Morishima, I. (1979) Biochem. Biophys. Res. Commun. 90, 674-678
15. Schonbaum, G. R., and Lo, S. (1972) J. Biol. Chem. 247, 3353-3360
16. Dunford, B. H., and Cotton, M. L. (1975) J. Biol. Chem. 250, 2932-2942
17. Cotton, M. L., and Dunford, H. B. (1973) Can. J. Chem. 51, 583-587
18. Makino, R., Chiang, R., and Hager, L. P. (1976) Biochemistry 15, 4748-4754
19. Dolmaa, D., Newell, G. A., Thulrow, M. D., and Dunford, H. B. (1975) Can. J. Biochem. 53, 495-501
20. Job, D., and Dunford, H. B. (1978) Can. J. Chem. 56, 1327-1334
Functional and Structural Regulation by Ca\textsuperscript{2+} in HRP

21. Hubbard, C. D., Dunford, H. B., and Hewson, W. D. (1975) *Can. J. Chem.* 53, 1563-1569
22. Cormier, M. J., and Frichard, P. M. (1968) *J. Biol. Chem.* 243, 4706-4714
23. Hasinoff, B. B., and Dunford, H. B. (1970) *Biochemistry* 9, 4930-4939
24. Makino, R., Sakaguchi, K., Iizuka, T., and Ishimura, Y. (1980) *J. Biol. Chem.* 255, 11883-11891
25. Beetlesone, J., and George, P. (1964) *Biochemistry*, 3, 707-714
26. La Mar, G. N. (1981) in *NATO Advanced Study Institute on Coordination Chemistry Environments in Iron-Coordinating Proteins and Enzymes*, pp. 357-373, D. Reidel Publishing Co., Dordrecht, Holland
27. La Mar, G. N., and de Ropp, J. S. (1982) *J. Am. Chem. Soc.* 104, 5205-5206
28. Paul, K. G., Theorell, H., and Akeson, A. (1953) *Acta Chem. Scand.* 7, 1284-1287
29. Nagai, K., La Mar, G. N., Jue, T., and Bunn, H. F. (1982) *Biochemistry* 21, 842-847
30. Chacko, V. P., and La Mar, G. N. (1982) *J. Am. Chem. Soc.* 104, 7002-7007
31. La Mar, G. N., de Ropp, J. S., Chacko, V. P., Satterlee, J. D., and Erman, J. E. (1982) *Biochim. Biophys. Acta* 708, 317-325
32. Makino, T., Kastner, M. E., Spartalian, K., Sceidt, W. R., and Reed, C. A. (1978) *J. Am. Chem. Soc.* 100, 6354-6362
33. Perutz, M. F., Sanders, J. K. M., Chenerv, D. H., Noble, R. W., Pennelly, R. R., Fund, I. M.-M., Ho, C., Giannini, L., Porschke, D., and Winkler, H. (1978) *Biochemistry* 17, 3640-3652
34. Messana, C., Cerdonio, M., Shenkin, P., Noble, R. W., Fermi, R., Perutz, R., and Perutz, M. F. (1978) *Biochemistry* 17, 3652-3662
35. Neya, S., and Morishima, I. (1982) *J. Am. Chem. Soc.* 104, 5658-5661
36. Morishima, I., and Ogawa, S. (1979) *J. Biol. Chem.* 254, 2814-2820
37. Cassatt, J. C., Marini, C. P., and Bender, J. (1971) *Biochemistry* 14, 5470-5475
38. Conroy, C. W., Tyma, P., Daum, P. H., and Erman, J. E. (1978) *Biochim. Biophys. Acta* 537, 62-69
39. Yamada, H., Makino, R., and Yamazaki, I. (1975) *Arch. Biochem. Biophys.* 169, 344-353
40. Doeff, M. M., Sweigart, D. A., and O'Brien, P. (1983) *Inorg. Chem.* 22, 851-852
41. Butunori, M., Saggese, U., Rotilio, G. C., Antonini, E., and Wyman, J. (1971) *Biochemistry* 10, 1604-1609
42. Deleted in proof
43. Hayashi, Y., and Yamazaki, I. (1979) *J. Biol. Chem.* 254, 9101-9106
44. Schonbaum, G. R., Houtchens, R. A., and Caughey, W. S. (1979) *Biochemical and Clinical Aspects of Oxygen*, pp. 195-211, Academic Press, Orlando, FL
45. Shiro, Y., and Morishima, I. (1984) *Biochemistry* 23, 4879