Articles

Structural Characterization of Lactoperoxidase in the Heme Environment by Proton NMR Spectroscopy†

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ABSTRACT: The heme environmental structures of lactoperoxidase (LP) have been studied by the use of hyperfine-shifted proton NMR and optical absorption spectra. The NMR spectra of the enzyme in native and cyanide forms in H_2O indicated that the fifth ligand of the heme iron is the histidyl imidazole with an anionic character and that the sixth coordination site is possibly vacant. These structural characteristics are quite similar to those of horseradish peroxidase (HRP), suggesting that these may be prerequisite to peroxidase activity. The pH dependences of the spectra of LP in cyanide and azide forms showed the presence of two ionizable groups with pK values of 6 and 7.4 in the heme vicinity, which is consistent with the kinetic results. The group with pK = 7.4 is associated with azide binding to LP in a slow NMR exchange limit, which is in contrast to the fast entry of azide to HRP.

Lactoperoxidase (LP) is a constituent of mammalian milk, saliva, and tears. In common with other peroxidases, the enzyme catalyzes the oxidation of a large number of substrates by hydrogen peroxide and is therefore a component of the biological defense system of mammalians. It also relates to the biosynthesis of the hormon thyroxine through an iodination reaction (Morrison & Schonbaum, 1976). The early works have shown that the prosthetic group of LP is not protoheme IX but is a mesoheme IX in conjunction with the porphyrin skeleton and hydroxyl groups attached to the side chain (Hultquist & Morrison, 1963; Morrison et al. 1970), or the heme is covalently bound to the protein through an alkaline-labile chemical bond such as an ester or amide linkage (Morell & Clezy, 1963). The spectral properties of LP suggested that a group ionizing with pK = 11.2, which may be either the guanidine of Arg or the ϵ-amino of Lys, is probably the coordinating group to the heme iron (Morrison et al., 1966, 1970). These proposed structures of the active site of LP are much different from those of plant peroxidases such as horseradish peroxidase (HRP), although both enzymes essentially have the same catalytic function.

Recently, however, Sievers (1979) showed the heme of LP to be protoheme IX by isolating the prosthetic group from a pronase hydrolysate of the enzyme and indicated that it is buried in a crevice of the protein molecule. On the basis of his comprehensive studies of LP, he also suggested that the proximal ligand of the heme iron might be the histidyl imidazole (Sievers, 1980; Sievers et al., 1983, 1984). Although some information on LP has been accumulated in the past decade (Kimura et al., 1981), a knowledge of the structure of LP in the heme vicinity has not yet been sufficient for understanding its characteristic function and the heme environmental structure.

Proton NMR studies of hemoproteins in paramagnetic forms can provide us with some structural details in the heme vicinity relevant to their functions. Recently, La Mar and his co-workers (La Mar et al., 1977, 1979, 1980; Cutnell et al., 1981) showed the proton NMR spectra of some hemoprotein derivatives in H_2O and assigned an exchangeable proton resonance located in the paramagnetically shifted region as the histidyl imidazole N_1H. We have studied here proton NMR spectra of LP in H_2O and ^2H_2O solutions in comparison with those for HRP and myoglobin (Mb) and assigned the proximal histidyl N_1H proton NMR resonance of LP. The pH dependences of the NMR spectra of LP derivatives show the presence of the ionizable groups in the heme vicinity responsible for regulating the external ligand binding to the heme iron. These spectral data are also discussed in relation to the structural specificities of peroxidases for their function and their ligand-binding properties (Morishima et al., 1977, 1978).

Materials and Methods

Lactoperoxidase purchased from Sigma (type L–2005) as a salt-free lyophilized powder was further purified by dialysis at pH 7 and following application to CM-52. The purity index, RZ value, of the enzyme was 0.7. The concentration of the enzyme was determined spectrophotometrically at 412 nm by using an absorptivity of 114 cm^{-1} mM^{-1} at pH 7. Sperm whale myoglobin (type II) was purchased from Sigma, and horseradish peroxidase (type G-I-C, RZ = 3.3) was obtained from Toyobo Co. (Osaka). The cyanide or azide complexes of the proteins were prepared by adding a 5- to 10-fold excess amount of the ligand to the enzyme solution. The pH titrations were performed by directly adding 0.1 N NaOH or HCl to the enzyme solution. The pH values were measured with a Radiometer Model PHM-100 pH meter, equipped with an Ingold microcombination glass electrode.

Proton NMR spectra were recorded at 300 MHz on a Nicolet NTC-300 spectrometer equipped with a 1280 computer system. Typical spectra of the enzyme consisted of 40000–100000 transients using 8K data points and 5.7-μs 90° pulse after the strong solvent resonance in H_2O solution was suppressed by a 500-μs low-power 180° pulse. Proton chemical shift is referenced with respect to the proton signal of the water in the enzyme solution, assigning a positive value for low-field resonance.

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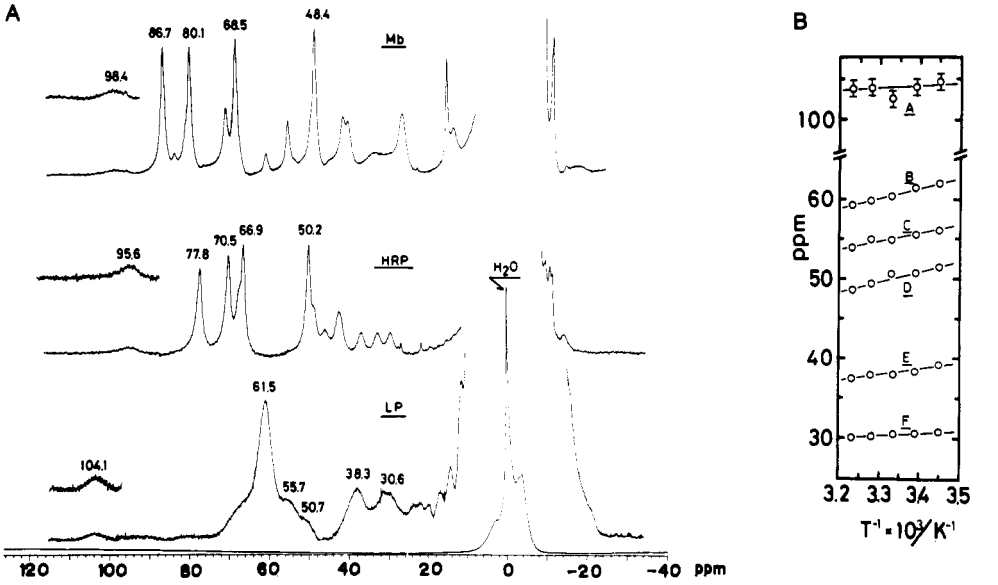

FIGURE 1: (A) Proton NMR spectra of aquometmyoglobin, horseradish peroxidase, and lactoperoxidase in H_2O at pH 7 and 23 °C. The downfield region of the hyperfine-shifted spectra is illustrated. Chemical shift is referenced with H_2O signal. (B) Temperature dependence of the heme peripheral proton signals of LP at pH 7.

The spin–lattice relaxation time, T_1, of the water proton was measured at 100 MHz with a JEOL FX-100 pulsed spectrometer by using a 180°–τ–90° pulse sequence. The frequency dependence of the T_1 between 0.01 and 20 MHz was measured with an originally designed NMR spectrometer at IBM, Thomas J. Watson Research Center, Yorktown Heights, NY (Fabry & Koenig, 1966; Gupta & Koenig, 1971; Fabry et al., 1971). The paramagnetic relaxivity of the enzyme was analyzed with Solomon and Bloembergen, Luz and Meiboom, and Swift and Connik equations (Dewk, 1973; Mildvan & Cohn, 1970).

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

Results

The hyperfine-shifted portions of the 300-MHz proton NMR spectrum of ferric lactoperoxidase (LP) in H_2O are compared with those of aquometmyoglobin (aquometMb) and ferric horseradish peroxidase (HRP) at 22 °C and neutral pH in Figure 1A. In the spectrum of LP, the poorly resolved proton peaks, probably arising from the heme peripheral groups, are observed at 61.5, 55.7, 50.7, 38.3, and 30.6 ppm. The spectral characteristics are quite different from those for the two other hemoproteins, where four well-resolved heme methyl signals are observed in the 50–90 ppm region. However, a broad single-proton resonance that was not detected in ^2H_2O solution is observed at 104.1 ppm. The resonance is close in position and width to the proximal histidyl N_1H signals for aquometMb and HRP (La Mar & de Ropp, 1979; La Mar et al., 1980). The temperature dependence of the paramagnetic shifts strictly follows the Curie law over a temperature range of 17–37 °C as is shown in Figure 1B.

In order to obtain the structural implication of the heme sixth site of LP, we have studied the temperature dependence of the water proton magnetic relaxivity of LP (Mildvan & Cohn, 1970; Dewk, 1973). The spin–lattice relaxivities (T_1^{-1}) measured at pH 7 and 100 MHz are plotted in Figure 2A against inverse absolute temperature between 9 and 40 °C (Gupta & Mildvan, 1975; Lanir & Schejter, 1975). T_1^{-1} of LP monotonically increased with decreasing temperature (Morishima & Ogawa, 1982), showing the presence of a rapidly exchanging water molecule in and out of the paramagnetic coordination sphere of the enzyme (Gupta et al., 1980). From the spin–lattice relaxivity due solely to the fast-exchanging mechanism, the interspin distance (r) between the heme iron and the water protons bound to the enzyme can be calculated when the correlation time (τ_c) is known. To obtain an accurate correlation time, we have measured the frequency dependence of T_1^{-1} between 0.01 and 20 MHz at 25 °C for native LP. The relaxation rates are plotted against the Lamor frequency in Figure 2B, from which τ_c of LP is estimated as 5.5×10^{-10} s at 25 °C. Then, on the basis of the values of τ_c and T_1^{-1} at 25 °C, the interspin distance between the heme iron and the water protons located at a site in the heme vicinity of LP was calculated by the usual method (Dewk, 1973; Mildvan & Cohn, 1970; Gupta & Mildvan, 1975) to yield r = 3.6 Å at 25 °C and pH 7.

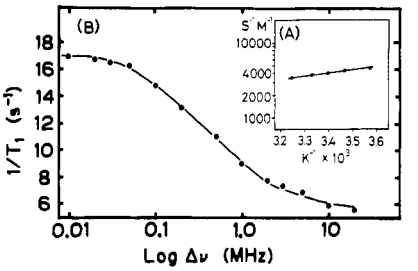

FIGURE 2: (A) Longitudinal paramagnetic solvent water proton relaxivity of LP at pH 7 and 100 MHz as a function of temperature. The relaxivity is normalized to the heme unit and is plotted against inverse absolute temperature. (B) Frequency dependence of the spin–lattice relaxation rates between 0.01 and 20 MHz for LP solution at pH 7 and 25 °C.

The NMR spectrum of the ferric low-spin cyanide complex of LP (LP·CN^-) in H_2O is compared with those of cyanide complexes of Mb (Mb·CN^-) and HRP (HRP·CN^-) in Figure 3. The spectral pattern of LP·CN^- is rather unusual. With a heme methyl proton signal at 20 ppm, some minor peaks are located in the downfield region. Goff et al. described in a recent paper (1985) that these signals arise from some minor components of LP. In the ^2H_2O solution, the peaks at 64.4, 44.7, 32.3, 25.4, and 11.8 ppm are observed, while the peak at 21.5 ppm disappeared. After dialysis of the ^2H_2O enzyme solution against H_2O for several days, this peak came out at 21.5 ppm, showing that this proton is slowly exchanging with the solvent water proton. In the spectra of Mb·CN^- and

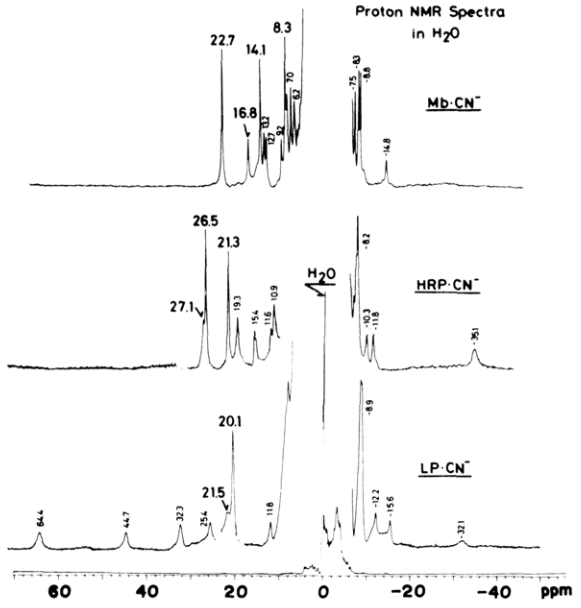

FIGURE 3: Proton NMR spectra of cyanide complexes of Mb (Mb·CN^-), HRP (HRP·CN^-), and LP (LP·CN^-) at pH 7.0 and 22 °C.

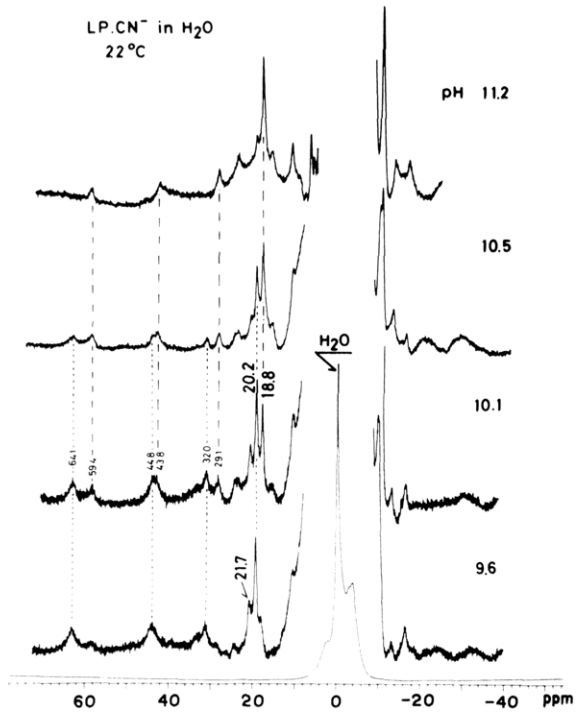

FIGURE 4: pH dependence of the proton NMR spectra of LP·CN^- between 9 and 11 at 23 °C.

HRP·CN^-, the H–D exchangeable signals assigned to the proximal histidyl N_1H's were observed at 16.8 and 27.1 ppm, respectively (Cutnell et al., 1981). In contrast to the downfield region, the spectral pattern of LP·CN^- in the upfield region is similar to that of HRP·CN^-. A signal at −32.1 ppm for LP·CN^- appears to correspond to that at −35.1 ppm for HRP·CN^-, which has been tentatively assigned to the C_2H signal of the proximal imidazole of HRP (Chacko & La Mar, 1982; La Mar et al., 1982).

The ^1H NMR spectrum of LP·CN^- changed in two steps depending on pH. A striking and reversible spectral alteration was encountered at pH 10–11 as illustrated in Figure 4. With raising pH above 10, the heme methyl peak at 20.2 ppm decreased in intensity and concurrently a new peak appeared at 18.8 ppm. In parallel with the spectral change for the

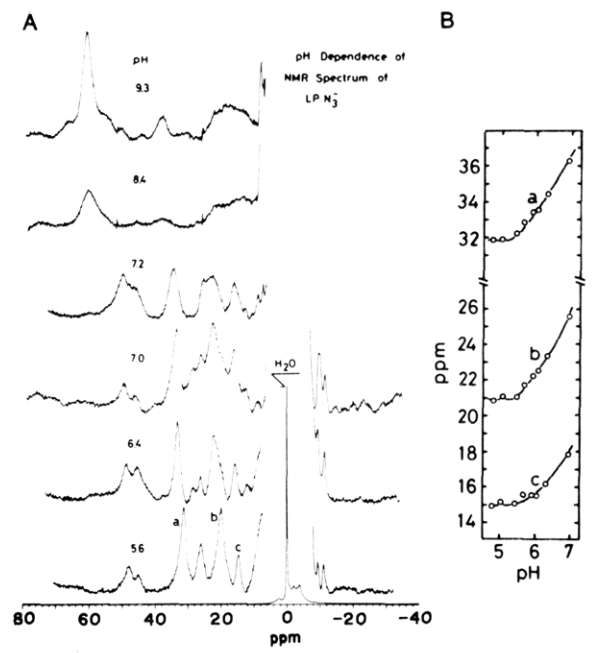

FIGURE 5: (A) pH dependence of the proton NMR spectra of azide complex of LP (LP·N_3^-) between pH 9 and 5 at 23 °C. (B) Plot of the signal positions of heme peripheral proton signals of LP·N_3^- against pH. The region between pH 7 and 5 is depicted in detail. The titration curves with reflection point at about pH 6 are noted.

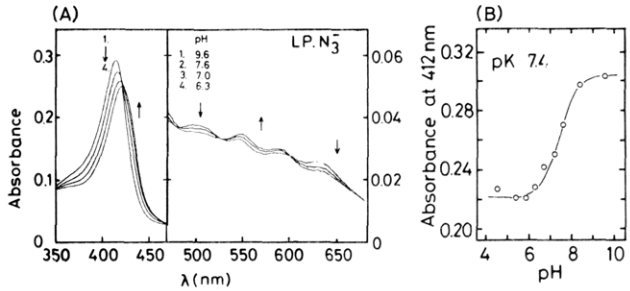

FIGURE 6: (A) Absorption spectral change of LP in the presence of azide ion between pH 6 and 10 at 23 °C. (B) pH dependence of the absorbance change at 412 nm. The titration curve with the reflection point at pH 7.4 is noted.

methyl resonance, anomalously shifted peaks in the downfield region are also replaced by a new set of peaks. On the other hand, the spectral change around pH 6 of LP·CN^- was not so drastic as that observed around pH 10. Around pH 6, the peak at 20 ppm showed a small but significant upfield shift by 1 ppm depending on pH. The plot of its chemical shift against pH gave a reflection point with pK = 6.

We also examined the azide binding to LP. Figure 5A shows the pH dependence of the NMR spectra of LP in the presence of azide ion. Above pH 9, the spectrum is indistinguishable from that of the native enzyme in a ferric high-spin state, indicating that the enzyme forms no complex with azide ion in this pH region. However, with lowering pH, the signals of the resting enzyme decreased in their intensities, and concomitantly signals between 50 and 10 ppm appeared and increased in their intensities. Below pH 7 the enzyme was completely combined with azide (LP·N_3^-). This pH-dependent azide binding by LP was also followed by the absorption spectral measurements (Figure 6A). The absorption changes at 412 nm were plotted against pH (Figure 6B) to yield pK = 7.4 for this change. These spectral changes show that LP forms no complex with azide above pH 8, but protonation of the ionizable group with pK = 7.4 facilitates azide binding to the heme iron of LP. In addition to these spectral changes,

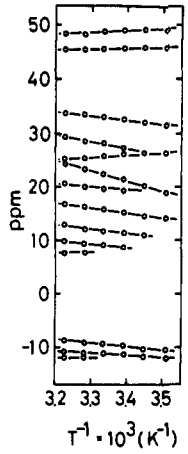

FIGURE 7: Temperature dependences of the heme peripheral proton signals of LP·N_3^- at pH 6.4. In this pH region, the enzyme is completely combined with the azide ion.

when the pH was lowered, the NMR signals of LP·N_3^- shifted to upfield by 3 ppm with pK = 6.0 as shown in Figure 5B.

In Figure 7 is shown the temperature dependence (12–37 °C) of the NMR spectrum of LP·N_3^- at pH 5. All the peaks do not follow the normal Curie law behavior, suggesting that the heme iron of LP·N_3^- is not in a single spin state but may exhibit a thermal spin equilibrium between ferric high- and low-spin states like azide complexes of other hemoproteins and model hemes (Morishima & Iizuka, 1974; Iizuka & Morishima, 1974; Neya & Morishima, 1982).

Figure 8 illustrates the difference spectra of LP in the Soret region upon a successive addition of azide ion to the enzyme solution at pH 5. Four isosbestic points are clearly observed at 349, 418, 482, and 524 nm. From the double-reciprocal plots of the absorbance increments at 430 nm, the dissociation constant (K_d) of azide ion from LP is found to be 0.27 mM, which is smaller than that for HRP (33 mM) (Morishima et al., 1978; Tamura, 1971), but larger than that for metMb (0.026 mM) (Bailey et al., 1969).

Discussion

Identification and Characterization of the Heme Iron Ligands of Lactoperoxidase. Lactoperoxidase (LP) is a true peroxidase that reacts with H_2O_2 to form compound I similarly to HRP, but its spectral properties have aspects somewhat different from those of HRP. For example, the absorption spectrum of native ferric LP (412, 500, 550, 600, 635 nm) is much different from that of native HRP (403, 498, 640 nm), and the absorption maxima of its pyridine ferrohemochrome (423, 525, 563 nm) do not match that of protoheme IX (418, 524, 557 nm). The present ^{1}H NMR spectrum of LP is also very unique in comparison to those of Mb and HRP (Figure 1A). However, the recent study by Sievers (1979) demonstrated that the heme isolated from pronase-degradated LP is protoheme IX. A resonance Raman study of intestimal peroxidase seems to be also in agreement with this suggestion (Kimura et al., 1981). If this is so, however, the structure at the heme proximity such as fifth or sixth heme ligands may be so unique as to make the spectra distinctly different from those of other protoheme-containing hemoproteins.

In spite of extensive studies by Sievers and his co-workers (Sievers et al., 1979, 1980, 1983, 1984), identification of the fifth ligand of LP has not yet been established. They could only preclude the possibility of carboxylate, phenolate, amino groups, and thiolate and equivocally assign the histidyl imidazole as its fifth ligand. In our NMR spectrum of native LP (Figure 1), a broad and exchangeable single-proton peak is

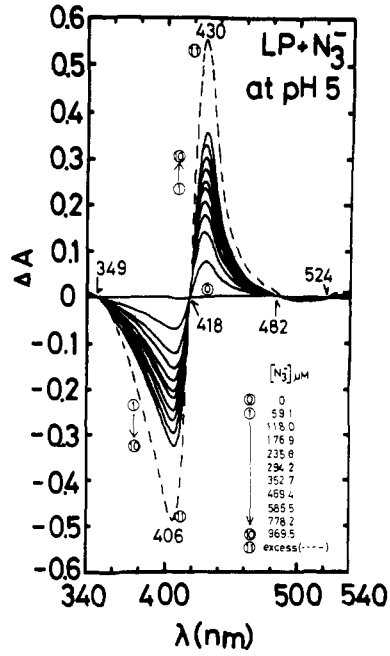

FIGURE 8: Soret absorption changes of LP upon successive addition of NaN_3 by means of difference absorption spectroscopy at pH 5.

observed at 104.1 ppm, in the same region as the proximal histidyl N_1H signals of aquometMb (98.4 ppm) and native HRP (95.6 ppm). In the spectrum of LP·CN^- (Figure 3), an exchangeable proton peak is also observed at 21.5 ppm, consistent with the proximal N_1H signals for Mb·CN^- (16.8 ppm) and HRP·CN^- (27.1 ppm). These results suggest that these exchangeable proton signals should arise from the proximal histidyl imidazole N_1H of LP. Additional support for this view was provided from the upfield-shifted proton NMR spectrum of LP·CN^-, where a proton resonance is observed at −32.1 ppm. Judging from the resemblance of the spectral pattern in the upfield region between cyanide complexes of LP and HRP, the −32.1 ppm peak is most likely assignable to the C_2H proton of the imidazole coordinated to the heme iron. Recently, La Mar and his co-workers suggested that the far-upfield bias of this signal in HRP·CN^- relative to Mb·CN^- is taken as evidence for appreciable imidazolate character for the axial ligand and that the degree of its upfield shift is indicative of the extent of the histidyl N_1H hydrogen bonding (Chacko & La Mar, 1982; La Mar et al., 1982). Hence, these observations may allow us to conclude that the fifth ligand of the heme iron of LP is the histidyl imidazole with an anionic character resulting from the strong hydrogen-bonded N_1H. This suggestion appears to be consistent with that from recent ^{13}C and ^{15}N NMR studies of LP derivatives (Behere et al., 1985).

It has been well recognized that the proximal imidazole in peroxidase such as HRP and CCP is more anionic, compared with oxygen-carrying proteins, which results from the strong interaction of its NH with the nearby proton-acceptor residue, probably glutamate (Poulos et al., 1980). The anionic proximal imidazole has something to do with the absence of the iron-bound water at the heme sixth site in ferric resting HRP and CCP (Kobayashi et al., 1980; Gupta et al., 1979; Teraoka & Kitagawa, 1981; Gupta et al., 1980), while aquometMb and Hb have a water molecule as an iron sixth ligand (Morishima et al., 1985). This leads us to the discussion on the nature of the heme sixth ligand in native LP. The temperature dependence of the nuclear magnetic longitudinal relaxation rate (T_1^{-1}) of the water proton in LP (Figure 2A) is essentially the same as that of HRP. The average distance of 3.6 Å of the water protons is also close to that of CCP as visualized by

X-ray analysis (Poulos et al., 1980). From the analogy to other peroxidase, it would be concluded that the iron sixth site of LP is also vacant or occupied by a water molecule loosely interacting with the heme iron. Unusually low affinity of N_3^- to LP and its pH dependence (Figures 5 and 6; vide infra) could be also interpreted in terms of the trans ligand effect from the anionic proximal imidazole, as was found for HRP (Morishima et a., 1977).

Our proposed structure of LP with respect to the iron ligands is essentially the same as those of other peroxidases such as HRP and CCP. The common structural features at the heme sixth site of various peroxidases may be related to their fundamental functions, in which the compound I formation requires the formation of an "inner-sphere" complex between peroxide and the heme iron. It seems reasonable that the vacant structure at the sixth position in peroxidases is favorable for binding of peroxides to the heme iron. If the carboxylate of the peptide binds to the iron in native LP as suggested by Sievers et al. (1983), the compound I formation must accompany the ligand exchange between the internal and the external ligands. Evidently, this mechanism is unlikely to interpret a very fast reaction rate of LP with H_2O_2, 10^7 M^{-1} s^{-1} (Kimura & Yamazaki, 1979), which is the same order as that for HRP.

Acidic–Alkaline Transition and Heme-Linked Ionizable Groups of Lactoperoxidase. The pH variation studies of hemoproteins provide useful information regarding structural details in the heme vicinity (Morishima et al., 1977). One of the prevalent arguments on this point is the acidic–alkaline transition, in which the spin state of the heme iron is converted from ferric high-spin to low-spin states. The acidic–alkaline transition of the plant peroxidases such as HRP has been attributable to the ligand exchange at the heme sixth site, in which a distal ligand, probably the histidyl imidazole, occupies the sixth coordination position to form a ferric low-spin form in the alkaline region (Morishima et al., 1977; Teraoka & Kitagawa, 1981). This characterizes the structural specificity in the heme environment of HRP, compared with metMb and metHb, in which the water molecule coordinated to the heme iron is ionizied into hydroxide ion (Antonini & Brunori, 1971).

For mammalian peroxidases, the acidic–alkaline transition has not yet been established. Yamazaki et al. (1982) described that a lack of alkaline transition of the ferric enzyme is one of the specific properties of mammalian peroxidases. In fact, the present NMR and absorption spectra of native LP did not change between pH 7 and 12. However, the NMR spectral alteration of LP·CN^- around pH 11 (Figure 5) with a NMR slow-exchange feature, where two sets of signals are concomitantly observed, is analogous to that associated with the acidic–alkaline transition for HRP·CN^- (Morishima et al., 1977) but is inconsistent with the NMR fast-exchange feature in the acidic–alkaline transition of metMb and its cyanide complex. The NMR spectral change of HRP·CN^- has been rationalized in terms of a conformational change in the heme vicinity that is attributable to the acidic–alkaline transition for a low-spin cyanide complex (Morishima et al., 1977). By analogy to the spectral feature of HRP·CN^-, the NMR change of LP·CN^- allows one to expect that a rearrangement of protein structure in the heme distal side occurs around pH 11. This leads us to the suggestion that this structural change results from "acidic–alkaline transition" of LP. From these results, it may be deduced that the acidic–alkaline transition should be present above pH 12 for native LP.

The pH titration for the heme peripheral proton signals of the cyanide and the azide complexes of LP also afforded two pK's, 6 and 7.4. These pK's are virtually identical with those suggested from the kinetic study of cyanide complex formation (Dolman et al., 1968), in which Dunford and his co-workers attributed these pK's to arise from the histidyl imidazole and amino group of N-terminal Leu, respectively. As manifested in the pH-dependent spectral change for LP·CN^- and LP·N_3^- with slight but significant shifts of the heme proton signals, the ionizable group with pK = 6 links with the heme iron in a way that the protonation of this group affects the electronic structure of the heme iron through its direct or indirect interaction with the central heme iron. On the other hand, the group with pK = 7.4 does not link with the heme iron as revealed by no significant change in the NMR spectrum of LP·CN^- in association with protonation of this group. However, it is noteworthy that this group is significantly responsible for the azide binding to the heme iron (Figures 5 and 6).

It has been shown that binding of external azide ion to HRP is quite specific (Morishima et al., 1977, 1978) compared with metMb, which binds azide ion at the heme iron at any pH between 5 and 10 to produce a stable complex and consequently exhibits no pH-dependent spectral change. We have described in the previous kinetic and proton and ^{15}N NMR spectral studies that binding of azide to HRP occurs only at acidic pH, while above pH 7 the ligand is not accessible to the heme iron of the enzyme (Morishima et al., 1977). This characteristic feature is now attributable to a strong linkage of the azide binding to HRP with a proton uptake of an ionizable group, which has been possibly assigned to the histidyl imidazole located in the heme distal side on the basis of comprehensive studies including proton balance, redox, and kinetic measurements (Yamada & Yamazaki, 1974; Yamada et al., 1975). This may be also the case for LP, in which the group with pK = 7.4, most probably distal histidine, modulates the binding of azide to the enzyme. Although the assignment is in disagreement with the previous one by Dolman et al. (1968), the pK value falls into the possible range for that of imidazole. The protonation of this group has been also found to affect the binding of benzhydroxamic acid to LP (Kimura & Yamazaki, 1979).

The present study shows that the structure in the heme environment with respect to the iron axial ligand, acidic–alkaline transition, and heme-linked ionizable groups is quite similar to those for other peroxidases such as CCP and HRP. In particular, it must be noted that an anionic proximal imidazole and the ionizable group that regulates the external ligand binding are present in LP, as the case for HRP. In the hypothetical reaction mechanism of peroxidases that involves redox rearrangement resulting in the formation of compound I subsequent to a formation of an inner-sphere complex between ferric enzyme and peroxide, acid–base catalysis by the distal base and anionic proximal ligand act synergistically to facilitate the heterolytic scission of the O–O bond of peroxide (Morrison & Schonbaum, 1976). The structural similarities of LP to HRP may allow one to expect that these structural specificities are prerequisite of peroxidase reactivity. However, the slow entry of azide ion to the heme iron in LP on the NMR time scale is in sharp contrast to the fast one for HRP (Morishima et al., 1977), which is noticeably responsive to a structural difference between LP and HRP. This is supposed to be due to the heme being buried more deeply in a crevice of the protein molecule than HRP, as suggested by Sievers (1979). This remains open to further study.

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Registry No. LP, 9003-99-0; heme, 14875-96-8.

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