Nuclear Magnetic Resonance Studies of Hemoproteins. Acid–Alkaline Transition, Ligand Binding Characteristics, and Structure of the Heme Environments in Horseradish Peroxidase†

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ABSTRACT: The structure of the heme environment of ferric horseradish peroxidase was studied by high resolution proton nuclear magnetic resonance spectroscopy at 220 MHz covering the large paramagnetically shifted resonances of protons on and near the heme. The pH-dependent features of the NMR spectra of the ferric enzyme and its complexes with cyanide and azide were discussed in terms of the heme environmental structures, comparing these with the case of metmyoglobin. The results were interpreted as follows. (1) There exists an ionizable amino group near the heme responsible for the ligand binding reactions of the enzyme, which modulates the entry of external azide to the heme iron through protolytic equilibrium of this group. The pK value of this group was determined to be 5.9 by monitoring the pH-dependent shift of the heme peripheral methyl signals of the native enzyme, indicating that the group is likely to be histidyl residue. (2) Acid–alkaline transition of metmyoglobin was confirmed to associate with the proton dissociation of an iron-bound water molecule, whereas, in horseradish peroxidase, pH-dependent spin-state change characterized with pK = 11 is attributed not to the simple protolytic reaction of the iron bound water but to the direct coordination of an amino acid residue of the polypeptide chain to the ferric heme iron. Histidyl imidazole is a possible candidate for the new sixth iron ligand in alkaline peroxidase above pH 11.

The heme environment of hemoprotein, together with the heme prosthetic group, plays a crucial role in modulating the redox and oxygen binding properties of the heme iron. Toward some understanding of the structural feature of the heme proximity, a number of studies have been conducted with hemoproteins by the use of x-ray, UV,[1] NMR, IR, Mössbauer, and more recently resonance Raman spectroscopies. Although horseradish peroxidase (HRP) is one of the ferric enzymes well studied by these methods (Yamazaki, 1974; Saunders, 1973; Leigh et al., 1975; Peisach et al., 1971; Rakskit and Spiro, 1974; Felton et al., 1976), most of the studies were concentrated on the reaction mechanism since the analysis of the kinetic studies had been reported on the enzyme by Chance (Chance, 1952) and George and Hanania (1955). For HRP the knowledge of the structures of the heme environment has not been accumulated in sufficient detail to understand its unique catalytic reaction. It contains iron-protoporphyrin IX as a noncovalently bound prosthetic group like the oxygen-carrying hemoproteins such as myoglobin and hemoglobin. The fifth axial iron ligand is probably histidyl imidazole as evidenced by the ESR spectrum of its nitrosyl complex (Yonetani et al., 1972; Henry and Mazza, 1974) and by ultraviolet spectra (Brill and Sandberg, 1968; Critchlow and Dunford, 1972; Mauk and Girotti, 1974), while the sixth ligand has been speculated to be a water molecule (Lanir and Schejter, 1975; Vuk-Pavlovic and Benko, 1975). In spite of these basic structural similarities to myoglobin and hemoglobin, HRP shows quite different behavior from these oxygen-carrying hemoproteins in ligand binding reactions and pH-dependent behaviors (George and Lyster, 1958; Hayashi et al., 1976). For example, ferric HRP has been known to react with cyanide, azide, fluoride, and hydroxylamine forming derivatives that can be detected spectroscopically (Saunders, 1973). Although these ligands cause the same sort of alternation in the spin state of HRP as in metmyoglobin and methemoglobin, the ligand binding constant and its pH dependence of HRP are appreciably different from those of metmyoglobin and methemoglobin (George and Lyster, 1958). The particular case of this has been known in the azide complexes of these hemoproteins since the azide complex of HRP is only formed below pH 4.5, whereas in metmyoglobin and methemoglobin the complex formation is almost pH independent (Theorell and Ehrenberg, 1951; Keillin and Hartree, 1951). Therefore, the influence of structural factors on reactivity of the heme, notably the heme crevice structure of HRP, has thus remained open to further studies.

Since the external ligand occupies the sixth coordination position of the heme iron, the studies on pH-dependent aspects of hemoprotein and its complex with the external ligand are expected to provide information on the structural details of the heme vicinity. One of the prevalent arguments on this point in hemoprotein research is to attribute the alkaline ionization of ferric high spin hemoprotein to the proton dissociation of an iron-bound water molecule (Antonini and Brunori, 1971; Ilugenfriz and Schuster, 1971; Yamada and Yamazaki, 1974). Although kinetic and NMR studies of such ionization with pK = 8.9 of metmyoglobin have indicated that it can be reasonably interpreted as the simple protolytic reaction of the water ligand (Ilugenfriz and Schuster, 1971; Iizuka and Morishima, 1975), the results of alkaline ionization of peroxidase could not be interpreted along these lines (Ellis and Dunford, 1969; Epstein and Schejter, 1972; Job et al., 1977). Since it has been noted that the replacement of the iron axial ligand of ferric hemoproteins drastically alters the pattern and symmetry of the unpaired spin distribution over the heme ring (Wüthrich,

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[1] Abbreviations used are: HRP, horseradish peroxidase; NMR, nuclear magnetic resonance; ESR, electron spin resonance; UV, ultraviolet; IR, infrared; DSS, sodium 2,2-dimethyl-2-silopentane-5-sulfonate.

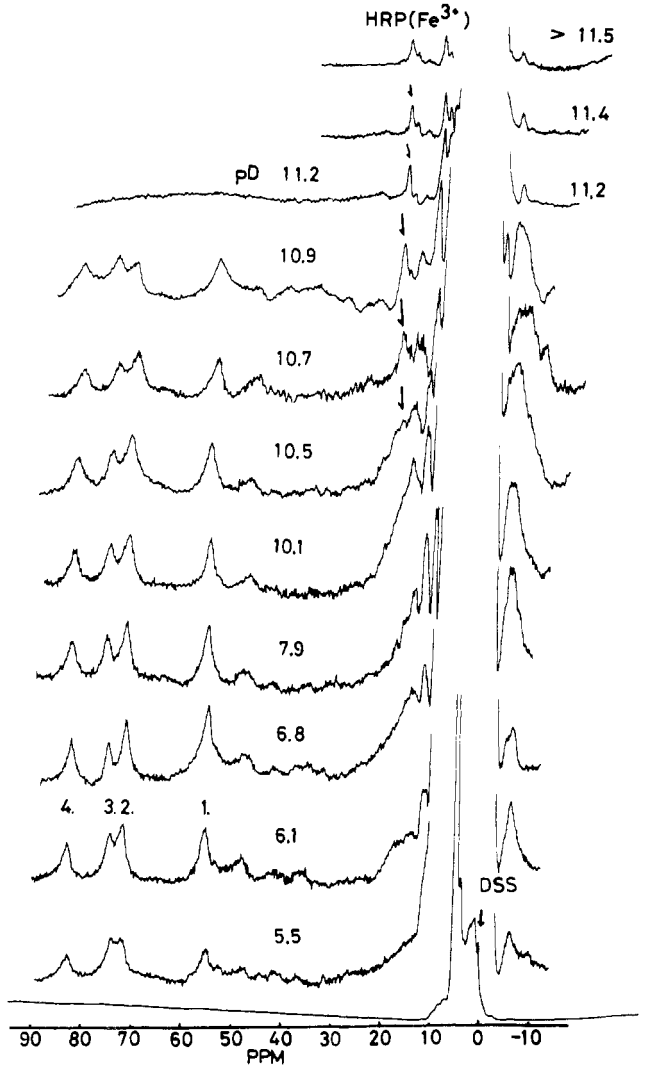

FIGURE 1: pH dependence of the proton NMR spectra of ferric horseradish peroxidase at 22 °C. The hyperfine shifted peaks 1–4 of the heme peripheral methyls are shown. The spectrum above pH 11.2 is typical of the heme enzyme in ferric low spin state and the two heme methyl peaks are seen at 18 and 11 ppm at pH 11.2.

1970), the hyperfine shifted proton NMR resonances of the heme peripheral groups are considered to provide us with useful information on the alkaline transition of ferric hemoproteins.

It has recently been becoming clear that proton high resolution nuclear magnetic resonance spectroscopy is quite a relevant tool for characterizing the ligand binding nature and detecting the conformational change in the heme environment of hemoprotein under the physiological conditions (Wüthrich, 1970). Although the signals located beyond the diamagnetic range (0 to 10 ppm from DSS) as a result of hyperfine interaction with the paramagnetic heme iron have been extensively examined to gain insight into the geometrical and electronic structures of the heme, most of the studies so far reported were concentrated on the ferric low spin derivatives of hemoproteins such as cyanometmyoglobin and cytochrome *c*, in which paramagnetically shifted peaks are separated by about 10 to 40 ppm from diamagnetic spectral region. For ferric high spin derivatives of hemoprotein, on the other hand, the NMR studies have not been extensively carried out possibly because of some difficulties in observing much more broadened signals with large hyperfine shift (50 to 110 ppm from DSS). This is especially serious for the protein samples with limited quantity and concentration. Recently we have been able to investigate the NMR of ferric high spin heme enzymes by the use of the 220-MHz pulsed Fourier transform method with the aid of the quadrature phase detection technique. We have studied here the structure of the heme environment of HRP by monitoring the pH dependence of the heme peripheral methyl signals of both the native high spin enzyme and its low spin derivatives. Preliminary results and discussion of this study have been previously reported (Iizuka et al., 1976).

Materials and Methods

Horseradish peroxidase (Sigma type VI, isoenzyme B+C) was directly dissolved in ^2H_2O and used for the NMR measurement without further purification. The cyanide and azide complexes of ferric HRP were prepared by adding an excess of these ligands (1:5) to the HRP solution. The enzyme concentration was about 2 mM in each of the samples. The p^{2}H value was adjusted by careful addition of NaO^{2}H or ^{2}HCl successively to the enzyme solution in the NMR sample tube in alkaline or acidic p^{2}H region. The value of p^{2}H was the direct reading of the pH meter (Radiometer) equipped with a micro-combination glass electrode (Ingold).

The 220-MHz proton NMR spectra were recorded at 22 °C in a pulsed Fourier transform mode (PFT) by a Varian Associates HR-220 spectrometer equipped with a Nicolet TT-100 PFT instrument. The quadrature phase detection (QPD) method was used for the detection of the heme ring methyl signals in ferric enzyme with short pulse width (20 μs = 48° pulse). The spectra were obtained by 4K data points transformation of 40 kHz spectral width after ca. 8192–16 384 pulses with a repetition time of 0.05 s. Proton NMR chemical shifts were referenced with respect to the resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and expressed in parts per million (ppm) assigning positive values to lower field resonance.

Results

The spectra of native HRP and the cyanide complex were measured at several pHs in the region between pH 3 and 12, and for the azide complex between pH 4 and 10.

Native Ferric High Spin HRP. The spectrum of the enzyme changed depending on the pH value of the enzyme solution in three steps at pH 4, 6, and 11. The spectra of the enzyme at several pHs between pH 5.5 and 12 are illustrated in Figure 1. The four peaks which are possibly assigned to the heme peripheral methyl signals are seen at 54.6, 71.3, 75.0, and 82.0 ppm at pH 6.8. On raising the temperature, these signals showed upfield shift obeying Curie's law. Although the spectrum of the enzyme was almost pH independent between pH 7 and 10, it changed progressively from the ferric high spin spectrum to the ferric low spin-one above pH 10.5. New signals which may be assigned to the heme ring methyl resonance of alkaline HRP (ferric low spin form) are simultaneously observed at 18.1 and 11.2 ppm with the signals of the residual native enzyme at pH 10.9. Above pH 11.2, the peaks of the native high spin enzyme have all faded away from their original positions. This change of the spectrum was reversible with respect to raising or lowering of the pH value of the enzyme solution.

The acid–alkaline transitions of horse heart metmyoglobin observed with the proton 220-MHz NMR are illustrated in Figure 2a. At pH 6.7, the four heme ring methyl signals are seen at 53.0, 72.8, 86.5, and 92.9 ppm. These peaks are characteristic of acid metmyoglobin in ferric high spin form. On raising the pH of metmyoglobin solution, all the signals shifted

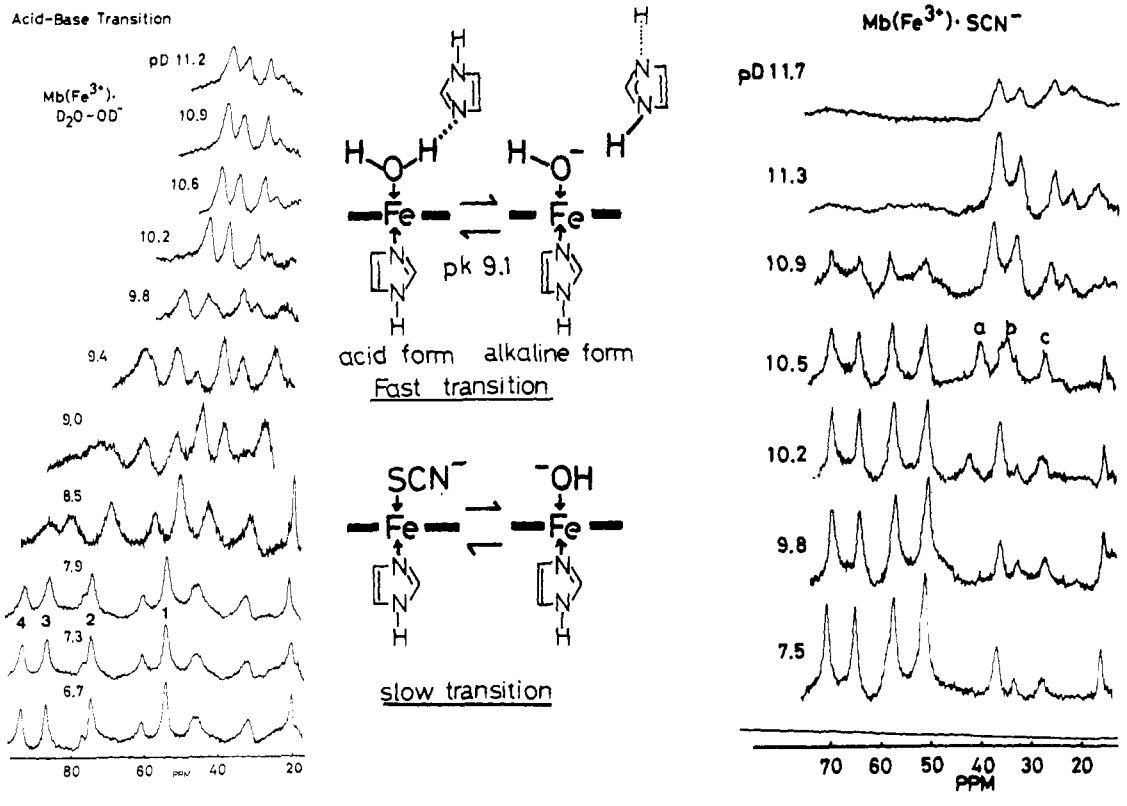

FIGURE 2: (a) Acid-alkaline transition of horse heart ferric myoglobin observed by the 220-MHz proton NMR at 22 °C. The spectra at the low field side are shown. pH dependence of the signal positions of four heme peripheral methyl peaks 1–4 gives the titration curves with pK = 9.1. (b) pH dependence of the proton NMR spectra of thiocyanate complex of horse heart ferric myoglobin. The peaks at 51.0, 57.7, 65.9, and 71.0 ppm seen at pH 7.5 and at 22 °C are the four heme ring methyl signals. The spectrum at pH 11.3 is almost the same as that of the metmyoglobin at corresponding pH (alkaline form). a and b indicate that alkaline transition and the ligand exchange from the external thiocyanate to hydroxyl anion in metmyoglobin are fast and slow in the ligand exchange, respectively, on the 220-MHz proton NMR time scale.

to the higher field side and the four methyl peaks were located between 25 and 40 ppm above pH 11, characteristic of alkaline metmyoglobin (Iizuka and Morishima, 1975). From the plot of the chemical shift of the heme ring methyl signals against pH, the titration curves with pK = 9.1 were obtained for the acid-alkaline transition of metmyoglobin. As Figure 2b shows, the other type of pH dependence was observed for the thiocyanate complex of metmyoglobin in which the coordinated water molecule is already replaced by the external ligand SCN^-. In this high spin ferric complex, four heme methyl peaks are seen at 51.0, 57.7, 65.9, and 71.0 ppm at pH 7.5. The pH dependence of this complex is quite distinguishable from that of the aquometmyoglobin, since no shifts of the heme methyl peaks are noted on raising pH but these four methyl peaks decrease their intensity above pH 10.5. Above pH 11.0 the spectra of this complex are almost the same as that of alkaline metmyoglobin at a corresponding pH value, indicating that the thiocyanate ligand is replaced by hydroxide anion from its coordination site at high pH region.

The spectral change around pH 6 of HRP was not as drastic as the one observed at pH 11. Around pH 6 all the hyperfine shifted signals showed small but significant upfield or downfield shifts by about 1 ppm depending on the pH of the enzyme solution. In Figure 3, plotted in detail, are the signal positions of the four heme ring methyl peaks against the pH value. Analysis of these titration curves afforded pK = 5.9 for this transition.

Cyanide Complex of HRP. The spectrum of the cyanide

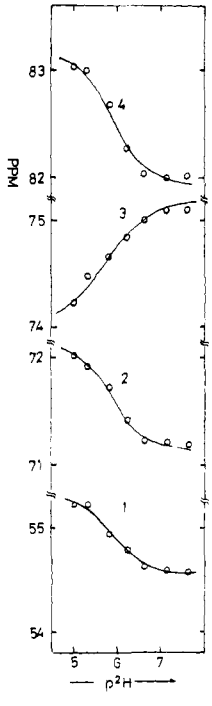

FIGURE 3: A plot of the signal positions of four heme ring methyl peaks of horseradish peroxidase against pH. The pH region between 5 and 7.5 is depicted in detail. The titration curves with the reflection point at pH 5.9 are noted.

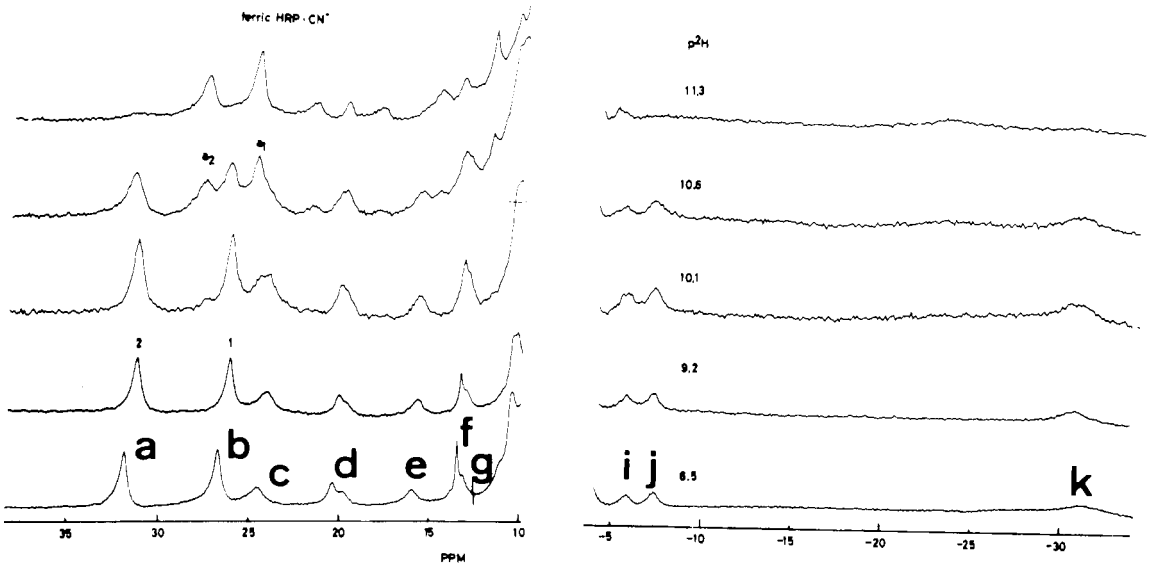

FIGURE 4: pH dependence of the NMR spectra of the cyanide complex of HRP at 22 °C. The hyperfine shifted signals of the heme prosthetic groups are illustrated. The spectral change at pH 11 is a drastic one as observed in Figure 1 for the native enzyme at pH 11.

complex of HRP in ferric low spin state changed in three steps in the same pH regions around pH 4, 6, and 11 as encountered for the native enzyme in ferric high spin state. The hyperfine shifted proton NMR spectra of cyano-HRP have been previously reported by Williams et al. (1975), who observed the pH-dependent shift of the heme ring methyl signals by about 1 ppm around pH 6. By monitoring the pH dependence of the hyperfine shifted signals, we also confirmed the result of Williams et al. (1975). We observed furthermore a striking and reversible spectral change around pH 11 for the cyanide complex of HRP as illustrated in Figure 4. The cyanide complex gives many resolved peaks of the hyperfine shifted resonance. The peaks 1 and 2 at 24.8 and 31.7 ppm at pH 9.2 which are assigned to the two heme ring methyl signals (Williams et al., 1975) decrease their intensity with the raising of the pH of the enzyme solution above 10.5. Concurrently new signals a_1 and a_2, probably the heme ring methyl signals, appear at 24.8 and 27.1 ppm. It is to be noted here that the spectrum of the cyanide complex of HRP in alkaline solution is quite different from that of the native enzyme at corresponding pH region (ferric low spin spectrum of the native alkaline-HRP), indicating that these new signals a_1 and a_2 result from the so termed alkaline-cyano-HRP.

Azide Complex of HRP. Figure 5 shows the pH dependence of the hyperfine shifted NMR spectra for the azide complex of HRP. The spectrum is characteristic of the ferric high spin state of the native enzyme above pH 7.5, indicating that HRP forms no complex with azide in this pH region. However, by lowering the pH of the enzyme solution down to pH 4, all the signals at the low-field side moved continuously to the upper field side to finally give the spectrum characteristic of ferric hemoprotein in thermal mixture of high spin and low spin state (Wüthrich, 1970; Iizuka and Morishima, 1975). From this pH dependence of the shift of four heme methyl peaks, the curves with the sigmoidal behavior with a reflection point at pH 5.8 were obtained. This pH dependence of azide-HRP is to be compared with that of the azide complex of metmyoglobin, which is illustrated in Figure 6. In this case the spectrum is almost pH independent between pH 5 and 11 and gives three peaks of the heme ring methyl group at 25.1, 27.7, and 33.1 ppm at 22 °C, as previously reported (Morishima and Iizuka, 1974).

Discussion

The proton NMR spectra of ferric HRP and its complexes with cyanide and azide presented in the former section are reasonably considered to come from the protons on and near the heme by the hyperfine contact and/or pseudocontact interactions with odd electrons of the ferric heme iron. The exact assignment of each peak is not clear at present.[2] However, the signals resulting from the heme peripheral methyls can be readily assigned from their signal intensity.

Ligand Binding Property and the Heme-Linked Ionizable Group of HRP. Accessibility of external ligand in hemoprotein has been extensively studied in ferric and ferrous states for various ligands by the spectrophotometric method in kinetic and pH variation experiments. It has recently been revealed that the slow ligand exchange process frequently encountered for hemoprotein in solution is most evidently manifested in the paramagnetically shifted NMR spectrum of the heme peripheral group where two separate sets of the signals or a set of averaged signals are observed depending upon whether the rate of the reversible process is slow or fast respectively on the NMR time scale (Wüthrich, 1970; Iizuka and Morishima, 1975). For example, on the addition of external cyanide in half-saturating amounts to the solution of metmyoglobin, two sets of the hyperfine shifted signals come from the ferric high spin metmyoglobin and ferric low spin cyano-metmyoglobin. Both of them are simultaneously seen with the signal intensity of each set of signals depending on the concentration of the ligand because of the slow exchange between two species. This slow chemical exchange on the NMR time scale between the parent protein and its complex with the ligand is normally the case even for other ligands such as F^-, SCN^-, and $HCOO^-$ (ferric high spin ligands) and N_3^-, imidazoles, and amines (ferric low spin ligands) in most of the ferric hemoproteins. In spite of this slow ligation process, azide complex of HRP showed an unusual feature of the fast chemical exchange between the native enzyme and the azide complex in acidic pH region. As illustrated in Figure 5, HRP in the presence of azide showed continuous and reversible spectral shift from the ferric

[2] The NMR signal assignment of HRP is in progress (I. Morishima, T. Inubushi, and T. Iizuka, in preparation).

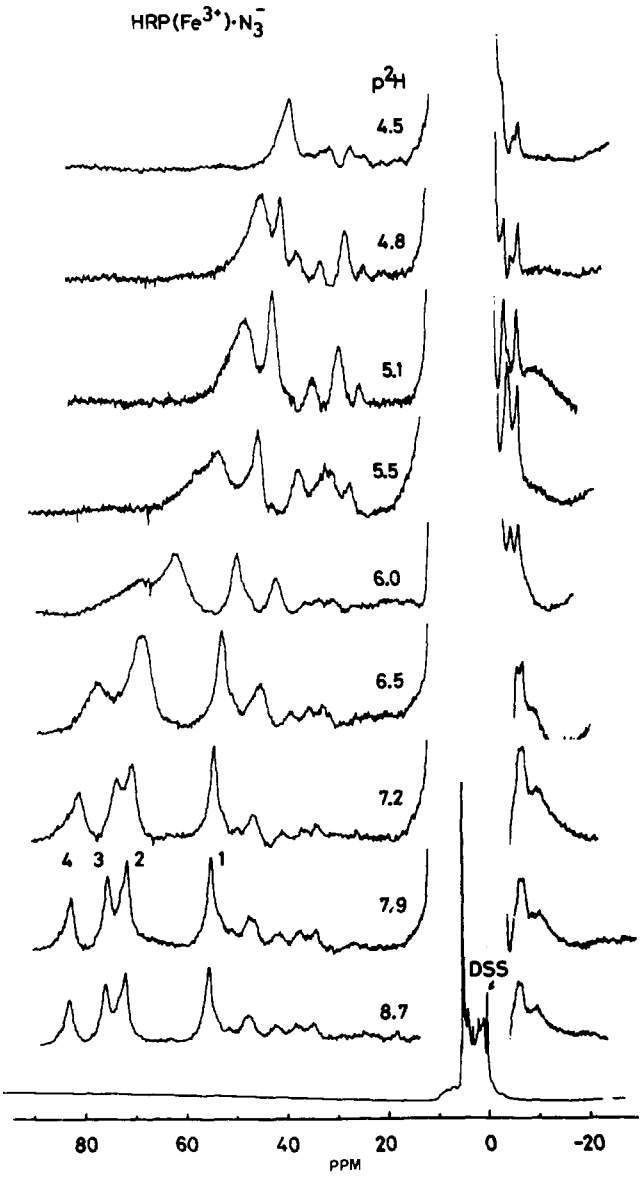

FIGURE 5: pH dependence of the proton NMR spectra of azide complex of horseradish peroxidase at 22 °C. The ratio of the molar concentration of the heme and the ligand is 1:5. The spectrum at pH 7.9 is characteristic of the native enzyme at corresponding pH. By plotting the pH-dependent shift of the heme ring methyl signals, the curves with sigmoidal behavior of reflection point at pH 5.8 were noted.

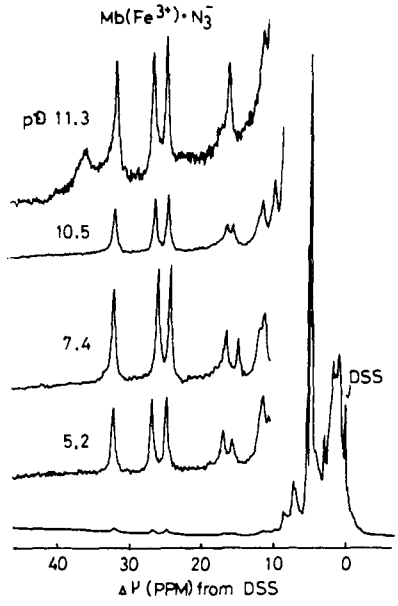

FIGURE 6: pH dependence of the proton NMR spectra of metmyoglobin azide at 22 °C. The spectrum of the azide complex is almost pH independent in the pH region between 5 and 11. The signal which appeared at 35 ppm above pH 11 is unambiguously assigned to myoglobin hydroxide.

high spin spectrum of the native enzyme to the predominantly ferric low spin one with the lowering of the pH of the enzyme solution. This may suggest that the lifetime of the azide in the iron coordination position is much shorter than the 220-MHz proton NMR time scale (10^{-4} s). It is also likely that a proton participates as a reactant in the entry of the external azide to the heme iron of HRP.

It has frequently been pointed out that the pH dependence of the equilibrium constant for the complex formation of HRP with the external ligands at neutral and acidic pH regions arises from the influence of structural factors on reactivity of the heme, probably from the change of micro-environmental structure of the heme prosthetic group (George and Lyster, 1958; Hayashi et al., 1976). Recently Yamada and Yamazaki have suggested the presence of a heme linked ionizable group which is important in ligand binding reactions of HRP (Yamada and Yamazaki, 1974, 1975). For example, they interpreted the shift of the pK value of this heme linked ionizable group upon formation of the carbon monoxide complex of the ferrous enzyme in terms of the interaction between the iron-bound ligand and this heme-linked ionizable residue. The presence of the heme-linked ionizable group in HRP has been suggested by many investigations including the peptide mapping sequential studies (Chance, 1952; Phelps et al., 1971; Ellis and Dunford, 1968; Welinder and Mazza, 1975). The present results of the pH-dependent shift of NMR spectra observed in the native enzyme and the cyanide complex around pH 6 may reflect the pK value of this ionizable group in the protein moiety. It is likely that protonation of this group may disturb the electronic structure of the heme ring slightly through its direct or indirect interaction with the central heme iron, and hence the spectral changes of the hyperfine shifted resonances are noted in this pH region.

From the above results and discussion, it is tempting to assume that the pH-dependent fast entry of azide to the heme iron of HRP is associated with the proton uptake of the heme-linked ionizable group since the azide binds to HRP with the midpoint at pH 6 by lowering the pH of the enzyme solution. It must be noted here that pK values of this ionizable group were 5.9 and 6.3 as determined for the native enzyme and the cyanide complex, respectively, which are almost close to the value observed in the complex formation of HRP with azide. Moreover, the virtue of its pK value may allow us to suggest that the group seems to be histidine residue in which the protonated nitrogen could be located remote from the heme iron in neutral solution, while in acidic condition the close nitrogen is protonated to give a site of specific hydrogen bonding with the azide at the coordination sphere. A question, however, bears on the different behavior of the pH dependence of the azide complexes between HRP and myoglobin. In the case of metmyoglobin the distal histidine is visualized by x-ray crystallographic study.In the latter case, azide binds to the ferric heme iron at any pH between pH 5 and 10 to produce a stable complex and consequently no such change in the spectra as seen for HRP was observed by pH variation. One possible explanation for this different behavior in HRP and metmyoglobin could be drawn from the difference of the spacial

location or orientation of the distal base in these two hemoproteins. We have examined by the 220-MHz proton NMR the effect of various external ligands such as amines and imidazoles that are known to form a stable complex with metmyoglobin. The presence of these ligands does not alter the hyperfine shifted proton NMR spectrum of ferric HRP, indicating that the enzyme does not form complexes with these external ligands. Hence, it is likely that the heme of HRP is more tightly embedded in the hydrophobic crevice than in metmyoglobin and that some amino acid residue of the protein chain sterically hinders the entry of the external ligand to the heme iron of HRP.

Alkaline Ionization of HRP. The proton NMR spectra of the hyperfine shifted resonances of HRP changed drastically from the ferric high spin spectrum to the ferric low spin spectrum around pH 11 (Figure 1). This pH-dependent spectral change of HRP is compatible with the results of magnetic susceptibility measurement (Theorell and Ehrenberg, 1951) and optical absorption study (Ellis and Dunford, 1969). These authors reported that the ferric enzyme converts itself from the high spin state to the low spin state depending on the pH of the solution with pK = 11. It has been customarily postulated, without any proof, that this spin state change of HRP is attributable to the simple protolytic reaction of the iron bound water molecule on the analogy to other ferric hemoproteins such as methemoglobin and metmyoglobin (Ellis and Dunford, 1969; Yamada and Yamazaki, 1974). In the case of the metmyoglobin, pH-dependent change of optical absorption spectra from the high spin spectrum to the predominantly low spin spectrum was accounted for by the presence of two components, i.e., acid and alkaline forms, which carry H_2O and OH^- as an iron axial ligand, respectively. It is also suggested that this ionization of the water molecule coordinated to the heme iron in metmyoglobin occurs from the simple one-step proton transfer which accompanies deprotonation of the distal histidine (Antonini and Brunori, 1971; Ilugenfriz and Schuster, 1971; Iizuka and Morishima, 1975). In the NMR observation of this alkaline ionization of metmyoglobin (Figure 2a), the signals of the heme side group showed marked upfield shift with the raising of the pH of the solution. Also, the position of these peaks at alkaline pH appears to be an average of acidic and alkaline forms, indicating that the chemical exchange time between both forms is short beyond the NMR detection of each signal. On the other hand, in the thiocyanate complex of metmyoglobin in which the water ligand is already replaced from the coordination position by the external ligand, the ligand exchange between thiocyanate and hydroxide anions at pH 10 is slow on the NMR time scale. Hence, the signals are separately observed for both forms at the midpoint of this pH-dependent change (Figure 2b). Consequently, it appears reasonable to relate the rapid chemical exchange between the acidic and the alkaline forms of metmyoglobin to the proton uptake of the distal histidine, which is followed by the ionization of the iron bound water molecule. On the contrary, the pH-dependent features of the NMR spectrum of native HRP are quite different from the case of metmyoglobin but rather similar to that of thiocyanate complex of metmyoglobin in which the high spin and the low spin forms of the enzyme can be separately observed by the proton NMR. This indicates that the chemical exchange time between two forms of HRP is longer than 10^{-4} s and that the rate is too slow to attribute the alkaline ionization of HRP to a simple protolytic reaction of the iron bound water molecule. As noted in the former section, the cyanide complex of metmyoglobin was clearly separated in the NMR spectrum from the residual uncomplexed metmyoglobin at an insufficient saturation of the ligand. This indicates that the rate of ligand exchange between the water molecule and the cyanide is slower than 10^{-4} s. The slow exchange between the high spin and the low spin forms of HRP in alkaline solution may allow us to expect that so-called acid–alkaline transition of HRP is attributable not to the simple proton transfer from the iron bound water molecule to a heme-linked group, but to a coordination of an internal ligand of the protein side chain to the heme iron. Some amino acid residue from the apoprotein such as histidine might occupy the sixth coordination position to afford the typical ferric low spin spectrum of the hyperfine shifted resonances above pH 11. The small hyperfine shift of the heme ring methyl peaks of the alkaline HRP suggests that fairly strong field ligand such as a nitrogenous base occupies the coordination position of the heme iron of HRP.

It has been well established in the studies of the denaturation of hemoglobin and its constituent chain that methemoglobin is spontaneously converted into the low spin form called hemichrome whose sixth iron ligand is considered to be histidine side chain (E-7) but not the water molecule (Rachmilewitz, 1974; Rachmilewitz et al., 1971; Peisach et al., 1975). Along with this line, the direct coordination of the distal base to the heme iron appears to be responsible for the alkaline transition of HRP. It should be noted here that the ESR spectrum of alkaline HRP (g = 2.94, 2.08, 1.63) has much resemblance with those of imidazole complex of metmyoglobin (g = 2.91, 2.26, 1.53) and hemoglobin hemichrome (g = 2.8, 2.26, 1.67) (Peisach et al., 1971, 1975; Rachmilewitz et al., 1971) and that the low spin visible spectrum of HRP at high pressure is quite similar to that of the hemichrome (Ogunmola et al., 1977). In addition to these results, we have found that the NMR spectrum of hemoglobin hemichrome (heme methyl signals at 13 and 20 ppm) is quite similar to that of alkaline HRP (Morishima et al., 1977). These observations are in favor of the present model of the pH-induced coordination of the distal histidine. The present model of alkaline ionization of HRP is also compatible with the result of the pH-jump experiment by Epistein and Schejter (1972), who suggested that the half-time for the reaction is in milliseconds and that the rate is much slower than those measured for metmyoglobin, implying the conformational change of the enzyme. This conformational change of the heme vicinity of the enzyme at pH 11 seems to be manifested in the drastic change of the NMR spectrum of the cyanide complex of HRP at pH 11 as illustrated in Figure 4. It is well established that the hyperfine shifted proton NMR spectrum of hemoprotein is quite sensitive to the nature of the iron ligand and the heme environmental structure. Therefore, the spectral change at pH 11 for the cyano-HRP is reasonably attributable to the conformational change of the enzyme, since the spectrum of the cyanide complex at high pH differs from that of alkaline HRP in ferric low spin state, indicating that the external cyanide is still bound to the heme iron above pH 11. It is likely that alkaline transition of HRP is triggered by ionization of a protein side chain, possibly histidine, followed by the conformational change of the heme environment, leading to the direct coordination of an amino acid residue to the heme iron.

In the present study we have dealt with the NMR spectral change of HRP occurring at pH 6 and 11. In addition to these observations, we have also obtained NMR evidence of the pH titration in the lower pH region. In the cases of the cyanide complex and the native HRP, the heme methyl signals shifted to the upper or lower field side by about 3–5 ppm depending on pH of the enzyme solution, and titration curves with pK = 4.3 and 4.2 were obtained by monitoring the shift for the cyanide complex and the native enzyme, respectively. In Figure

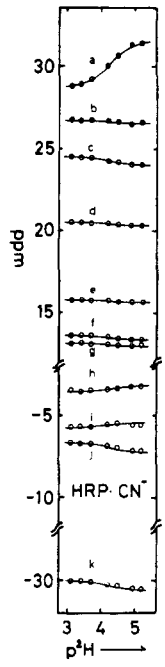

FIGURE 7: A plot of the signal position of each peak of the hyperfine shifted resonances for the cyanide complex of horseradish peroxidase. The pH region between 3 and 5 is illustrated.

7, pH-dependent shift of the hyperfine shifted signals is illustrated for the cyanide complex. These results appear to correspond with the result of kinetic study of the cyanide and the fluoride complexes of HRP (Ellis and Dunford, 1968). One possible explanation for such spectral change may be the change in the electronic structure of the heme due to the proton uptake of the heme propionate group. The detailed discussion on this point remains open to further studies.

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