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PROTON NUCLEAR MAGNETIC RESONANCE STUDIES OF COMPOUNDS I AND II OF HORSERADISH PEROXIDASE

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SUMMARY: Proton high resolution nuclear magnetic resonance spectra have been successfully obtained for compounds I and II of horseradish peroxidase (HRP) at various temperatures and pH. The paramagnetically shifted peaks of heme peripheral methyl protons were well resolved to show a high and a low spin hemes having ferryl irons for compounds I and II, respectively. The spectrum was also observed in solution of horse metmyoglobin to which H_2O_2 was added. The electronic formulations of the hemes in compound II of HRP and "ferryl" myoglobin appear to be just alike as judged from their spectra. It is revealed from the proton spectra that the π-cation radical on the heme ring can be ruled out as a source of oxidizing equivalent in compound I of HRP. The pH dependent shifts for these intermediates afforded an evidence for a heme-linked ionizable group with pK=5.6 as the case for the native ferric HRP.

The enzymic cycle of horseradish peroxidase (HRP) proceeds via sequential intermediates, compounds I and II, which are two and one oxidizing equivalents above the native ferric enzyme, respectively (1,2). Although a number of suggestions have been presented concerning the formal states of the heme iron and its ligands in these intermediates (3-6), no compelling evidence has been evoked to account for their characteristic physicochemical properties. Magnetic susceptibility measurements of compounds I and II of HRP showed that they are paramagnetic with Bohr magnetons of 3.99 and 3.53, respectively (7). Mossbauer spectral measurements gave the same iron isomer shift for these HRP compounds, showing the ferryl Fe(IV) state of the heme iron (8). The electronic spectrum of compound I resembled to that of a porphyrin cation radical, from which the suggestion was drawn that compound I contains a ferryl F(IV) and porphyrin π-cation radical (9).

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We wish to report here first successful observation of both compounds I and II of HRP at various temperatures and pH, which shows that these compounds are fairly stable under appropriate condition and are in ferryl high and low spin states, not being the porphyrin π-cation radical. The presence of a heme-linked ionizable group with pK=5.6 for these HRP compounds is also shown on the basis of pH dependent behaviors of these NMR spectra.

MATERIALS ANS METHODS: HRP (isoenzyme c) was purchased from TOYOBO Co. Ltd., as a lyophilized sample (Type G-C-1, RZ 3.4). The concentration of HRP was determined spectrophotometrically by using an absorptivity of 102 cm^{-1} mM^{-1} at 403 nm and pH 7.0. p-Cresol and indole propionic acid (IPA) were obtained from Nakarai Chemical Co., Inc. and were used without further purification. The buffer systems employed at various pH ranges were: 25 mM borate between pH 7.5 and 10.5, and 0.1 M citric acid and 0.2 M phosphate between pH 4.0 and 7.2.

Proton NMR spectra were recorded at 220 MHz with a Varian HR-220 spectrometer equipped with a Nicolet TT-100 attachment in a pulse Fourier transform mode. Quadrature phase detection method was used to cover wide range of spectra with pulse repetition time of 0.05 s. To obtain the spectra of compounds I and II of HRP, the following procedure was employed (10). The enzyme was pretreated with a stoichiometric amount of H_2O_2 to remove the endogenous oxidizable substrate. A 0.2 ml of 3.0 mM HRP ^2H_2O solution at desired pH was pipetted into an NMR sample tube and then 20 μl of 30 mM H_2O_2 was deposited on the enzyme solution. The spectrum of compound I was taken immediately after this procedure. The compound II was formed by subsequent addition of p-cresol or IPA in a stoichiometric amount to the compound I solution. The concentration of H_2O_2 was determined by using an absorptivity of 72.4 $M^{-1}cm^{-1}$ at 203 nm.

RESULTS: We have obtained the NMR spectra of HRP at various intervals after the addition of H_2O_2 to the enzyme solution. Figure 1(A) shows the initial spectrum of native ferric HRP at pH 7.0 and 20 °C, where paramagnetically shifted regions are depicted (11). The four heme ring methyl peaks are seen at 83.1, 75.8, 72.1 and 55.3 ppm from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Figure 1(B) shows the representative spectrum of the green HRP species recorded after the addition of H_2O_2 to the enzyme solution at pH 7.0. The proton NMR peaks due to the native enzyme are replaced by an entirely different spectrum in the same paramagnetically shifted region. The signals at 76.2, 72.2, 59.5 and 51.2 ppm in figure 1(B) were assigned to the four heme ring methyl peaks of the green species. Initially, the relative intensity of

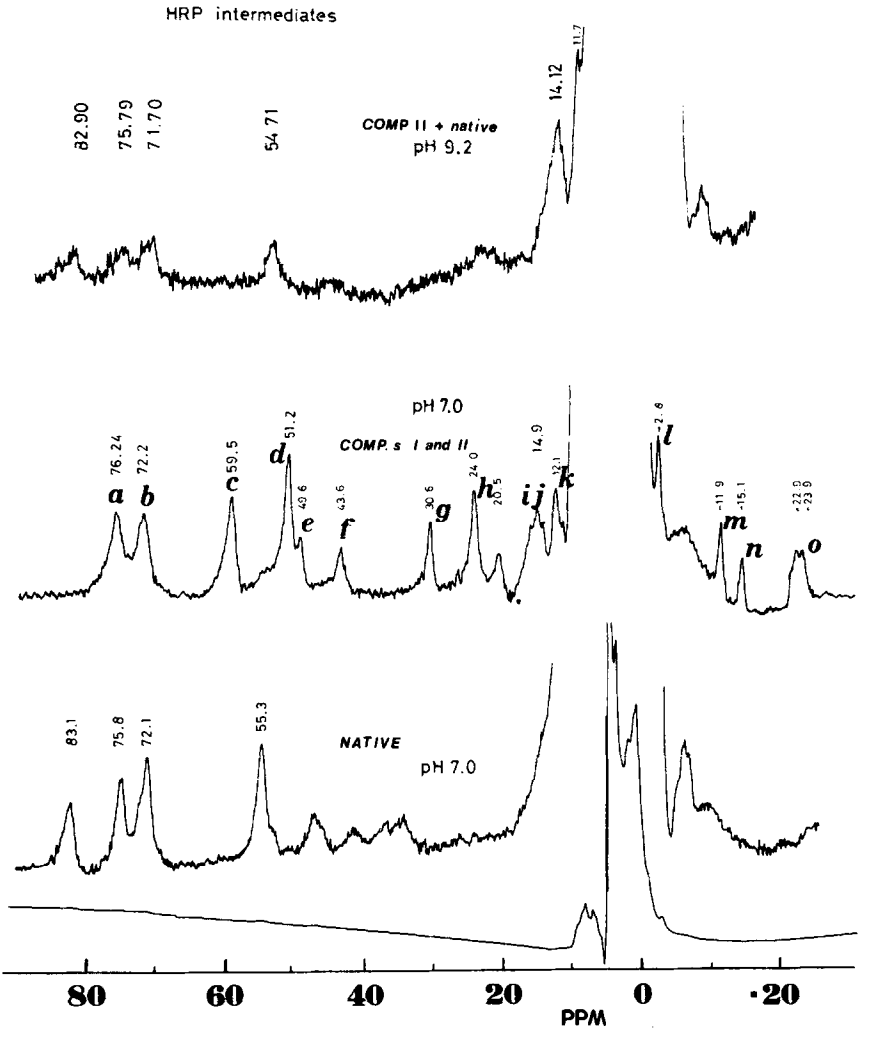

Figure 1. 220 MHz pulse Fourier transfom proton NMR spectra of horseradis peroxidase (HRP) and its reaction intermediates at 20 °C. Chemical shifts are referenced with respect to DSS.
(A) Ferric native HRP, 3.0 mM enzyme solution at pH 7.0.
(B) The spectrum of compound I recorded immediately after the addition of H_2O_2 to the above HRP solution. It took about four minutes to collect 4k transients of the spectrum. The compound II spectrum is contaminated at 14.9 ppm (peaks i and j).
(C) HRP compound II. The spectrum was recorded at pH 9.2 just after the addition of p-cresol to the compound I solution. The peaks located between 50 and 85 ppm are due to recovered ferric native enzyme. The peaks i and j at 14.12 ppm are assigned to compound II. The spectrum of compound I (spectrum B) also converted to the spectrum C in one hour without addition of p-cresol.

the peak at 14.9 ppm(i,j) increased in time and then gradually reduced.

Within ten to twenty minutes this whole spectrum gradually disappeared

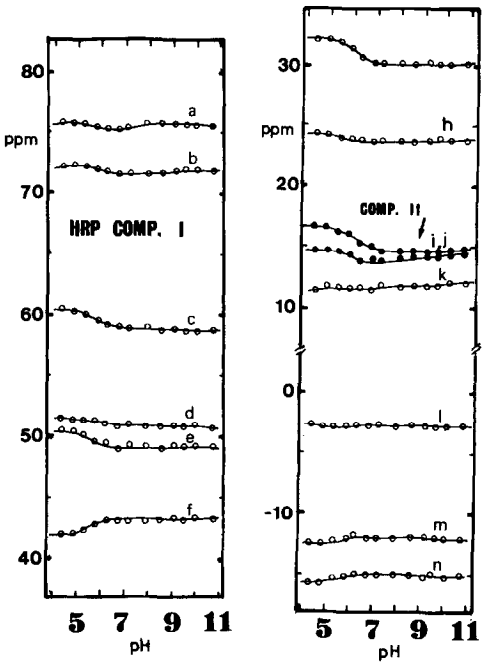

Figure 2. The pH dependence of each proton peak of HRP compounds I and II in figure 1(B) at 20 °C. pH-titration curves with pK=5.6 are noted.

and after one hour the spectrum of the native ferric HRP was recovered. This spectral change appears to be consistent with development and decay of compound I of HRP, which was confirmed by the absorption spectrum of the solution. The addition of H_2O_2 followed by subsequent addition of p-cresol or IPA to the HRP solution afforded the red species which also gradually decayed back to the native brown enzyme. The spectrum of this HRP red species (compound II) was recorded at pH 9.2 immediately after the addition of p-cresol and is illustrated in figure 1(C). The same spectrum was also obtained by the addition of IPA to the compound I solution at pH 9.2. In this figure, new NMR peaks at 14 ppm, which appear to correspond to the peaks i and j in figure 1(B) and exhibited typical Curie's law behavior of the temperature dependence, are seen separately from those of the recovered native HRP. The peaks were assigne to the two of four heme ring methyl resonances of the HRP red species

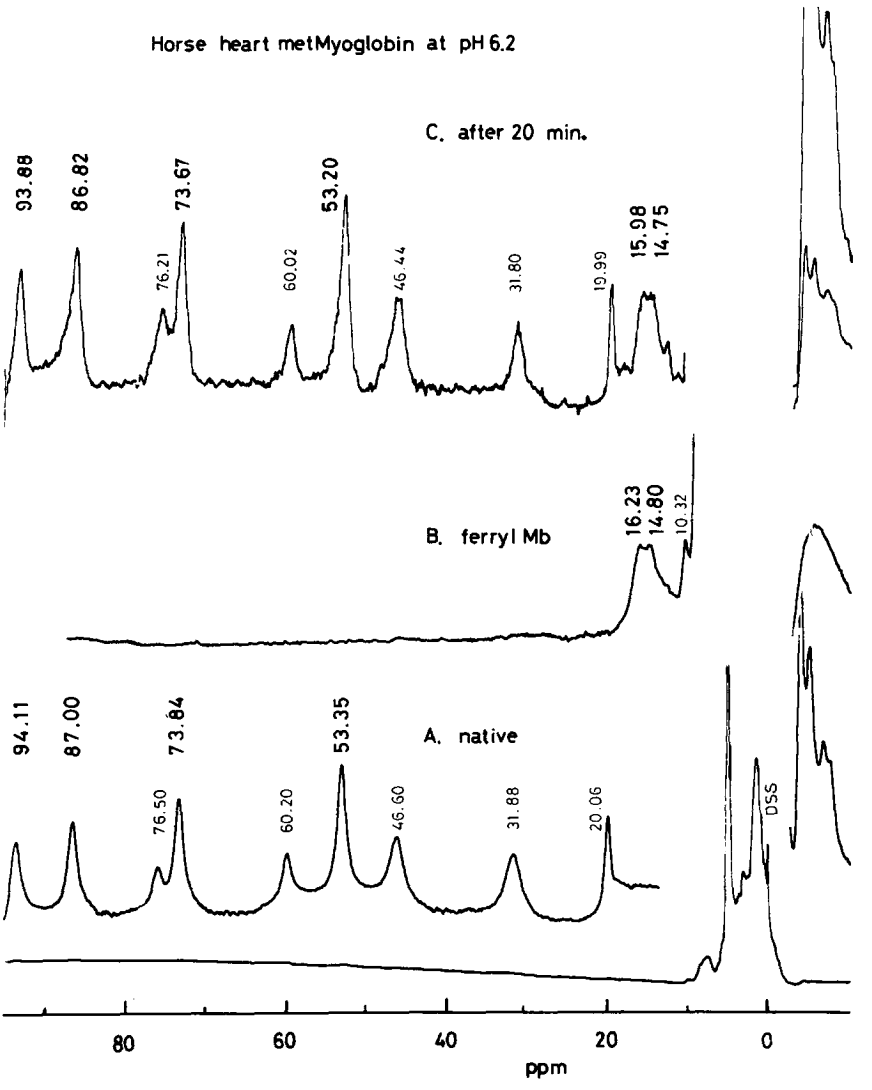

Figure 3. 220 MHz pulse Fourier transform proton NMR spectra of horse metmyoglobin and its H_2O_2 adduct at pH 6.2 at 20 °C.
(A) native horse metmyoglobin spectrum.
(B) ferryl myoglobin. The spectrum was taken after adding four fold exces of H_2O_2 to above metmyoglobin solution.
(C) The spectrum of myoglobin taken 1.5 hour after adding H_2O_2 to the metmyoglobin solution.

(compound II). When another mole of H_2O_2 was added to the solution of regenerated native HRP, the green HRP species was again reproduced and gave rise to the same spectrum as figure 1(B).

The pH-dependent features of the spectrum of Fig. 1(B) are also

depicted in figure 2. The pH titration curves show the possible presence of a heme-linked ionizable group with pK=5.6.

The NMR spectrum was also recorded for horse metmyoglobin in the presence of four fold excess of H_2O_2 between pH 6 and 8 at 20 °C (12). Figure 3 exemplifies the spectra measured immediately (spectrum B) and 1.5 hour (spectrum C) after the addition of H_2O_2 to the metmyoglobin solution at pH 6.2 in the 0.2 M phosphate buffer. The spectrum of the native protein is also illustrated (spectrum A) in this figure. The signals at 16.2 and 14.8 ppm of the spectrum B were assigned to the two of the four heme ring methyl peaks of "ferryl" myoglobin. As spectra (B) and (C) show, the ferryl myoglobin gradually decayed back to the parent protein within twenty or thirty minutes after the addition of H_2O_2.

DISCUSSION: The proton spectra obtained after the addition of H_2O_2 to HRP consisted of those of HRP green and red species, their relative intensities being varied with the time interval. The peaks at 14.9 ppm grew up in time at the expence of the signals a-h and l-o in Fig. 1(B). From the above results, we have unambiguously assigned the spectra (B) and (C) in Fig. 1 to the compounds I and II of HRP, respectively. As Fig. 1 shows, the spectra of compounds I and II are contaminated with those of compound II (peaks i and j) and regenerated native ferric enzyme respectively. We have also assigned the spectrum in figure 2(B) to so called ferryl myoglobin which is the transient species obtained on adding H_2O_2 to metmyoglobin (13). It is of interest to note that the NMR spectrum of ferryl myoglobin is similar to that of compound II of HRP. This indicates that the electronic formulations of the hemes in these transient species of HRP and myoglobin are alike.

Felton et al. have reported the proton NMR spectra of ferryl porphyrins produced by electrochemical oxidation of ferric hemin derivatives (14). The singly oxidized tetraphenylporphyrin chloride, $FeTPPCl^+$, showed β-pyrrole proton signals at 68.6 ppm which, in time,

progressed to 79.4 ppm, characteristic of ferric FeTPPCl. From this result and magnetic susceptibility measurement of the oxidized porphyri they claimed that $FeTPPCl^+$ is in ferryl iron state with a spin of S=2 (high spin). This spectral change from Fe(IV) to Fe(III) states of FeTPPCl is likely consistent with our present observation that the heme peripheral methyl signals exhibited small upfield bias on going from ferric HRP to compound I. This may allow us to expect that compound I of HRP is in ferryl high spin state. The proton spectrum of compound I further throw a strong argument against the π-cation radical on the heme ring as a source of oxidizing equivalent in compound I. If we suppo that compound I of HRP is such a species as ferryl iron with π-cation radical on the heme, spin densities at various carbon atoms on the porphyrin ring may be drastically altered from the native ferric state. This may result in dramatic change in the paramagnetically shifted NMR spectra of HRP, and proton signals may be too much broadened. Nevertheless, no such changes as the line broadening and the large paramagnetic shift of the peaks were encountered for compound I of HRP, indicating that the one of two oxidizing equivalents of compound I is likely retained in a protein moiety of the enzyme as suggested by the recent EPR studies of HRP compound I (15). The drastic paramagnetic shift change between compounds I and II of HRP or ferryl myoglobin reminds us of the similar change in the proton NMR spectra of ferric high spin (S=5/2) and low spin (S=1/2) hemoproteins (10). The small paramagnetic shifts of the heme peripheral group in compound II of HRP and ferryl myoglobin are best understood in termes of ferryl low spin state (S=1) of the heme iron.

The identities of the heme axial iron ligands are presently unknow for compounds I and II of HRP and ferryl myoglobin as well. Presumably they involve an oxygen atom originating from H_2O_2 (16). The possible candidates for the axial iron ligands are H_2O, OH^- and =O (metal-oxo),

either of which we have no confirmative evidence to choose at present. It is also of interest to note that pH titration behaviors of the heme side methyl proton signals for compounds I and II are indicative of the presence of a heme-linked ionizable group, presumably distal histidine by the virtue of its pK value (5.6) as the case for native ferric HRP (11). The hydrogen bond bridge between this distal ionizable group and the sixth iron-bound ligand appears to be responsible for such pH dependent shifts of the heme side methyl proton peaks.

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