Proton Nuclear Magnetic Resonance Spectra of Compounds I and II of Horseradish Peroxidase†

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ABSTRACT: Enzymatic reaction intermediates of horseradish peroxidase, compounds I and II, were studied by high-resolution nuclear magnetic resonance spectroscopy at 220 MHz. The heme peripheral proton peaks were successfully obtained in the downfield region of 50 to 80 ppm from 4,4-dimethyl-4-silapentane-5-sulfonate for compound I and of 10 to 20 ppm for compound II at pH 9.2. This indicates that no isoporphyrin appears in the catalytic cycle of the enzyme. Temperature dependences of the spectra also were determined for these compounds between 7 and 32 °C. With increasing temperature, all the peaks in the downfield region for compound I shifted upfield, obeying the Curie law. These results suggest that the Fe atoms in compounds I and II are in ferryl high- and low-spin states, respectively. The spectrum was also observed in solutions of horse metmyoglobin to which hydrogen peroxide (H_2O_2) was added. The electron formulations of the hemes in ferrylmyoglobin and compound II are just alike, as judged from their spectra. Evidence was found against a π-cation radical on the heme ring as a source of the oxidizing equivalent in compound I.

The enzymatic cycle of horseradish peroxidase (HRP)[1] proceeds via key catalytic reaction intermediates called compounds I and II, which retain 2 and 1 oxidizing equiv, respectively, above the native ferric enzyme. The enzymatic reaction is well characterized (Chance, 1952; George, 1953; Hewson and Dunford, 1976), and the current status of structural studies of the enzyme and these intermediates has been reviewed on several occasions (Dunford and Stillman, 1976; Brill, 1966; Yamazaki and Yokota, 1973; Yonetani, 1970). Although many suggestions have been advanced concerning the source of the oxidizing equivalents and the electron formulations of the hemes in compounds I and II, no compelling evidence has been obtained to account for their characteristic properties. Magnetic susceptibility measurements of compounds I and II of HRP showed that they are paramagnetic with effective Bohr magnetons of 3.99 and 3.53, respectively (Theorell and Ehrenberg, 1952; Ehrenberg, 1966). These values are expected only for the spin contribution of three or two unpaired electrons, suggesting that compounds I and II of HRP are in formal iron states of V and IV, respectively. Mossbauer spectra of compounds I and II gave the same iron isomer shift, indicating tetravalent irons for these compounds (Moss et al., 1969). An ESR signal of compound I of HRP, characteristic of a free radical, was recently obtained (Aasa et al., 1975). It is thus suggested that 1 oxidizing equiv of compound I is retained on an amino acid residue near the heme iron, which is similar to the case of complex ES (compound I) of cytochrome *c* peroxidase (CCP) (Yonetani et al., 1966). The absorption spectrum of HRP compound II is a normal porphyrin type, but that of the compound I resembles that of a porphyrin π-cation radical, leading to the suggestion that compound I contains a π-cation radical on the heme ring as a source of the oxidizing equivalent (Dolphin et al., 1971; Dolphin and Felton, 1973).

High-resolution proton nuclear magnetic resonance (NMR) spectra have been obtained for many hemoproteins, including HRP and its complexes with cyanide and azide (Williams et al., 1975; Morishima et al., 1977; Wuthrich, 1970). In these studies the paramagnetic shifts of protons on and near the heme have been shown to be sensitive to the iron oxidation state and spin state. It is of interest to extend these NMR studies to the reaction intermediates of the heme enzyme. We have performed here an intensive NMR study of compounds I and II of HRP, because these transient species are sufficiently stable under appropriate conditions to allow their NMR characterization.

Materials and Methods

HRP was purchased from Toyobo Co., Ltd., as a lyophilized sample [Type G-I-C, isoenzyme c, purity index (RZ) = 3.4] and was used without further purification. The concentration of HRP was determined spectrophotometrically by using an absorptivity of 1.02×10^5 M^{-1} cm^{-1} at 403 nm and pH 7.0 (Schonbaum and Lo, 1972). The solutions of the enzyme were made at pH 9.2 by employing 20 mM borate-HCl–deuterium oxide (^2H_2O) buffer. The pH value was a direct reading of a pH meter (Radiometer) with a microcombination glass electrode (Ingold). Deuterium oxide used for the NMR measurements was a commercial product of Merk. Oxidizable substrates, *p*-cresol and indolepropionic acid, and the buffer components were all reagent grade.

Proton NMR spectra were recorded at 220 MHz on a Varian HR-220 spectrometer equipped with a Nicolet TT-100 accessory in a pulse Fourier transform mode. The quadrature phase detection method was used to cover a wide range of the spectra. A pulse repetition time of 0.05 s was used to collect the spectrum. Proton chemical shifts were referenced with respect to the residual water proton signal in the ^2H_2O solution of HRP which is, in turn, referenced to the resonance of DSS, assigning a positive value to the low-field resonance. The chemical shift of the water protons is 4.75-ppm downfield from the resonance of DSS.

The following was a typical procedure to obtain the NMR spectra of the compounds I and II of HRP. The enzyme was pretreated with a stoichiometric amount of H_2O_2 to remove

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[1] Abbreviations used are: HRP, horseradish peroxidase; CCP, cytochrome *c* peroxidase; NMR, nuclear magnetic resonance; ESR, electron-spin resonance; DSS, sodium 4,4-dimethyl-4-silapentane-5-sulfonate; FeTPPCl, tetraphenylporphyrin chloride.

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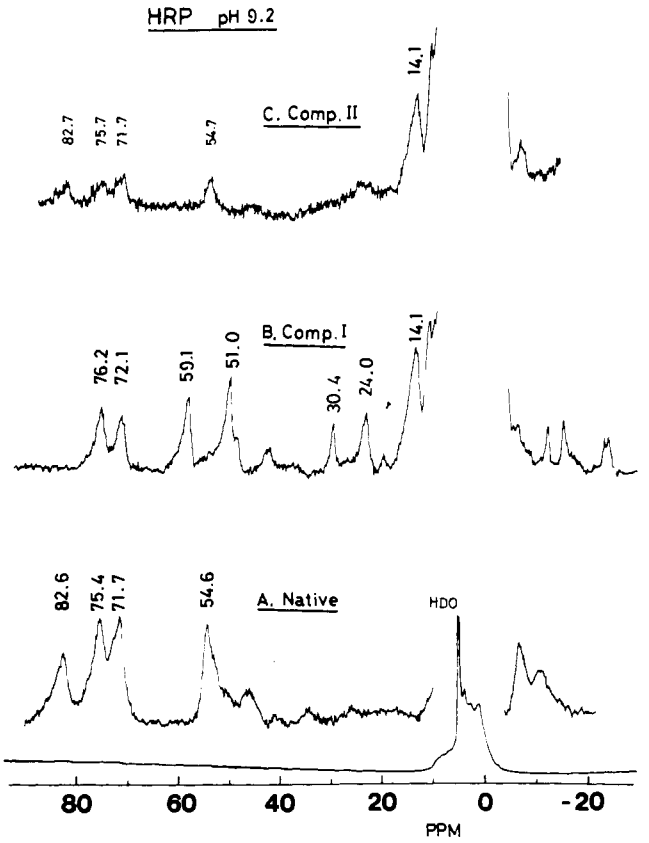

FIGURE 1: 220 MHz proton NMR spectra of horseradish peroxidase and its reaction intermediates at 20 °C. All the samples are in 20 mM borate buffer at pH 9.2: (A) Ferric native horseradish peroxidase. (B) Compound I. Immediately after the addition of H_2O_2 to the HRP solution, 4K transients of the spectrum were collected. The spectrum is contaminated with that of compound II. (C) Compound II. The spectrum was recorded just after adding *p*-cresol (oxidizable substrate) to the compound I solution. The peaks located at 50 to 85 ppm are due to the native recovered enzyme.

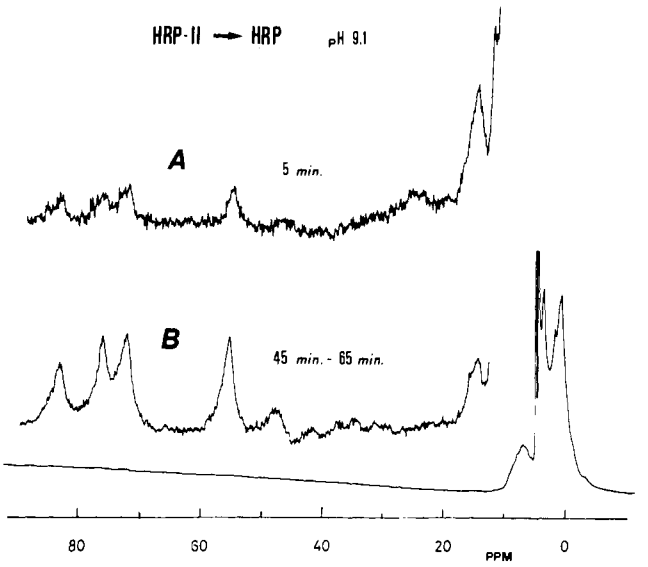

FIGURE 2: Time progression of the NMR spectrum of compound II of HRP at 20 °C. The spectra taken in about 5 (B) and 50 (A) min after adding *p*-cresol to the compound I solution are illustrated.

endogenous oxidizable substrate. An HRP solution (0.2 mL, 3.0 mM) in 20 mM borate ^2H_2O buffer at pH 9.2 was pipetted into an NMR sample tube and then 20 μL of 30 mM H_2O_2 was deposited with a microliter syringe on the enzyme solution. After immediate mixing of the solutions, the spectrum of compound I (intense green solution) was recorded within 1 to 4 min. It took about 1 to 4 min to collect 1K or 4K transients of the spectrum after the addition of H_2O_2 to the enzyme solution. The spectrum of compound II (red solution) was also recorded immediately after a subsequent addition of *p*-cresol or indolepropionic acid in a stoichiometric amount to the compound I solution. The concentrations of H_2O_2 and *p*-cresol were determined spectrophotometrically at 230 and 270 nm by using absorptivities of 72.4 and 1.7×10^3 M^{-1} cm^{-1} (Herngton and Kynatson, 1957), respectively.

Results

Figure 1A shows the spectrum of native ferric HRP at pH 9.2 and 20 °C, where the paramagnetically shifted region of the spectrum is depicted. The four heme peripheral methyl proton peaks are seen at 82.6-, 75.4-, 71.7-, and 54.6-ppm downfield from DSS (Morishima et al., 1977). This spectrum of HRP is entirely replaced by a different spectrum on adding H_2O_2 to the enzyme solution. Figure 1B shows a spectrum of an intense green solution of HRP recorded immediately after the addition of H_2O_2 to the HRP solution at pH 9.2 and 20 °C. The peaks at 76.2, 72.1, 59.1, and 50.9 ppm were tentatively assigned to those of the four heme ring methyl protons of the HRP green species (compound I). Within 10 to 20 min these signals of the green species lost their intensities with a concomitant increase in the intensity of the peak at about 14 ppm, and within 1 or 2 h after the addition of H_2O_2 the spectrum of the native ferric enzyme was spontaneously recovered.

A subsequent addition of *p*-cresol or indolepropionic acid to the intense green solution of HRP (compound I solution) afforded a red solution of HRP. The spectrum of this red species was immediately recorded and is illustrated in Figure 1C. A new peak at 14.1 ppm is seen separately from those of recovered native enzyme. The peak was tentatively assigned to the signal of two of the four heme peripheral methyl protons from the integrated intensity of the red species (compound II). As Figure 2 shows, the HRP red species gradually decayed back to the native enzyme. In this figure, the spectra taken at 5 (A) and 50 (B) min after the addition of the oxidizable substrate to the solution of the HRP green species are illustrated. When another mole of H_2O_2 was added to the recovered enzyme solution, an intense green solution was again formed and gave rise to the same spectrum as Figure 1B.

In addition to the above observations, the NMR peaks of the green compound, the red compound, and the native brown enzyme were simultaneously observed in the spectrum at a transient time after the addition of H_2O_2, which is illustrated in Figure 3.

Figure 4 shows the NMR spectra of the green compound of HRP at 14 and 32 °C. The peaks of the red compound are also seen at 14 ppm in the spectra. Figure 5 shows the temperature dependence of these peaks between 7 and 32 °C. With raising the temperature, all the peaks of the green compound in the downfield region shifted upfield, obeying the Curie law, whereas that of the red compound showed small or little temperature dependencies. It is also noted in Figure 4 that an improvement of the spectral resolution occurs with the raising temperature.

The spectrum was also recorded for horse metmyoglobin solution to which a fourfold excess of H_2O_2 was added.[2] Figure

[2] The proton NMR spectrum of ferryl myoglobin was initially observed for kangaroo myoglobin, details of which will be published elsewhere (Ueda, M., Ogawa, S., and Morishima, I.).

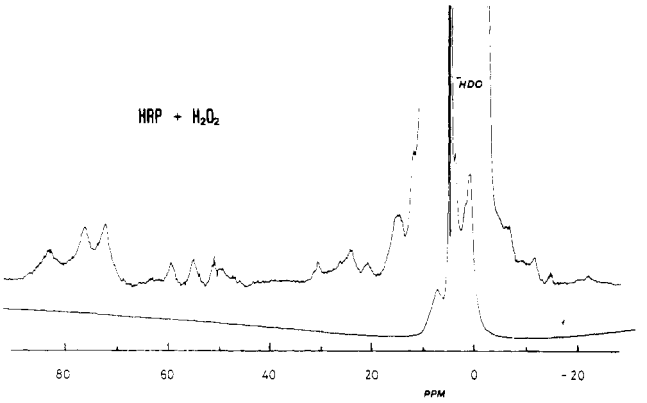

FIGURE 3: Time progression of the NMR spectrum of compound II of HRP at 20 °C. In about 30 or 40 min after the addition of H_2O_2 to the HRP solution, NMR peaks of the recovered native HRP and the compounds I and II are simultaneously seen at their positions.

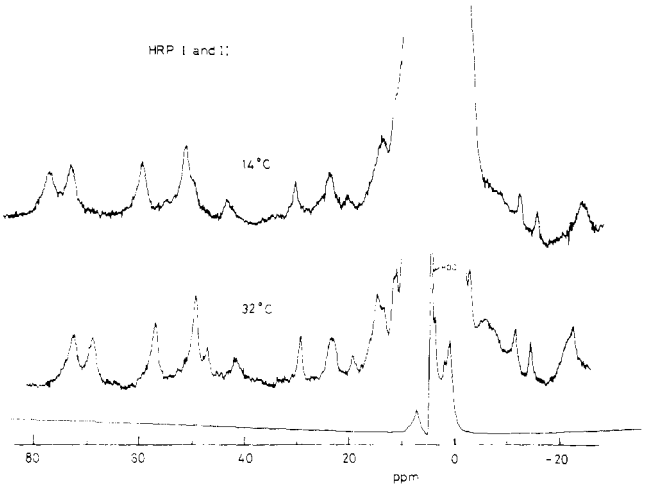

FIGURE 4: Proton NMR spectra of compound I of HRP at 14 and 32 °C. The peaks for the compound II are also seen in the spectra at 14 ppm. With raising temperature, an improvement in the spectral resolution is seen.

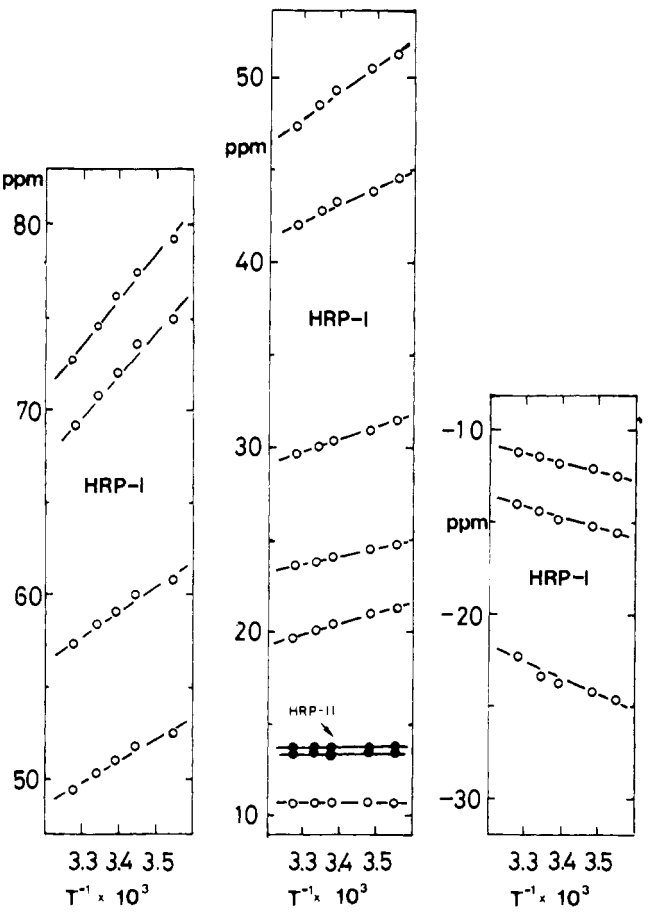

FIGURE 5: Temperature dependence of the NMR peaks for the compounds I and II of HRP. The peaks for compound I show a marked temperature-dependent shift, obeying the Curie law.

6 shows the spectra recorded at 20 °C immediately and at 1.5 h after the addition of H_2O_2 to the metmyoglobin solution in 0.2 M phosphate buffer at pH 7.8. The spectrum of the native protein is also illustrated in the figure. All the peaks of the native protein disappeared on adding H_2O_2, and new peaks concomitantly appeared at 16.1 and 14.8 ppm. These peaks are tentatively assigned to signals of the two heme ring methyl groups of ferrylmyoglobin (King and Winfield, 1963; Fox et al., 1974; Yonetani and Schleyer, 1967). With increasing temperature these proton peaks of ferrylmyoglobin shifted upfield, obeying the Curie law. We have also measured the spectrum of ferrylmyoglobin at pH 7.0 and 6.2. The spectra at these pH's are the same as that at pH 7.8, though the decay of ferrylmyoglobin to the native protein is much faster at pH 6.2 and 7.0 than at pH 7.8, as indicated by the spectral time progression.

Discussion

We have presented above the time progression of the NMR spectra of the green and red species of HRP, where the proton peaks to these transient species and the recovered native enzyme were concomitantly detected after the addition of H_2O_2 to the enzyme solution. Such a progression of the spectrum is an indication of a slow chemical exchange rate on the NMR time scale among these HRP species under the condition ex-

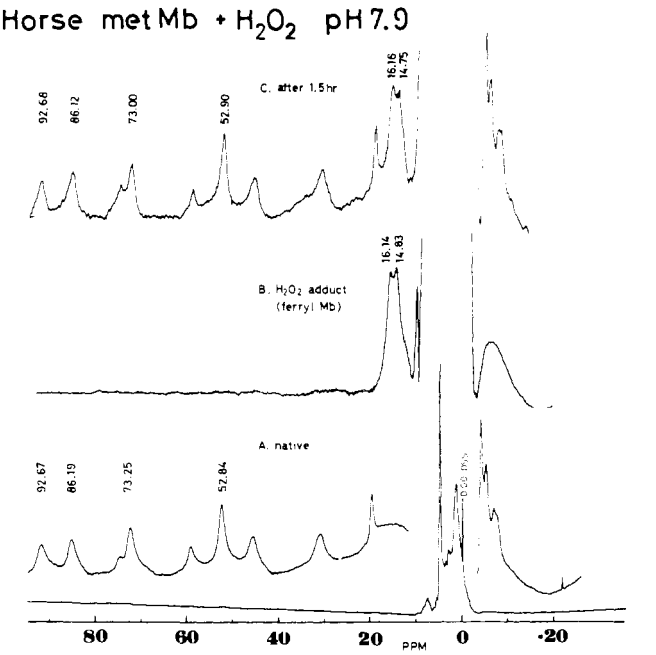

FIGURE 6: 220 MHz proton NMR spectra of horse ferrimyoglobin and its H_2O_2 adduct at 20 °C and at pH 7.9 in 0.2 M phosphate-^2H_2O buffer: (A) Native ferrimyoglobin. (B) Ferrylmyoglobin. The spectrum was taken immediately after the addition of a fourfold excess of H_2O_2 to the metmyoglobin solution. (C) Time progression of the ferrylmyoglobin. The spectrum was recorded 1.5 h after the addition of H_2O_2. The peaks for the native ferrimyoglobin were gradually recovered with a concomitant decrease in the intensities of the ferrylmyoglobin peaks.

amined here and is consistent with the decay and development of the compounds I and II of HRP. The latter was confirmed independently by optical absorption spectra of the intense green and the red solutions of HRP, which agreed well with those of the compounds I and II obtained by others (Schonbaum and Lo, 1972; Blumberg et al., 1968; Roman and Dunford, 1972). Thus, we unambiguously assigned the spectra B and C in Figure 1 to those of compounds I and II of HRP. The spectra of compounds I and II in this figure are contaminated with those of the compound II and the recovered native enzyme as minor fractions, respectively. We have also assigned spectrum B in Figure 6 to that of ferrylmyoglobin, which is a transient species obtained on adding H_2O_2 to metmyoglobin solution (Fox et al., 1974; King and Winfield, 1963; Yonetani and Schleyer, 1967). It is of interest to note that the spectrum of ferrylmyoglobin is similar to that of the compound II of HRP. This indicates that the electron formulations of the hemes in these transient species are just alike.

The NMR peaks of the heme ring methyl protons of compound I are observed in the downfield region of 40 to 80 ppm from DSS and show a marked upfield shift, obeying the Curie law, with raising temperature, suggesting that compound I may be in a ferryl high-spin state ($S = 2$) of the heme. Felton et al. recently reported the proton NMR spectra of ferrylporphyrins produced by an electrochemical oxidation of ferric hemin derivatives (Felton et al., 1976). The singly oxidized tetraphenylporphyrin chloride ($FeTPPCl^+$) showed the β-pyrrole proton signal at 68.6 ppm which in time shifted to 79.4 ppm of the parent ferric hemin (FeTPPCl) position. They also measured the magnetic susceptibility of $FeTPPCl^+$ and concluded that the oxidized product of the ferric hemin is in ferryl high-spin state ($S = 2$). These results suggest that the chemical shift of the heme peripheral protons in the ferryl high-spin state is relatively small when referenced to the resonance of the ferric porphyrin. Such a change of the proton NMR spectra from a ferric high-spin to a ferryl high-spin state of the model hemin is likely consistent with the present observation for HRP that the heme ring methyl proton shifts show a small upfield bias on going from ferric high-spin HRP (native) to compound I. It should also be noted that the spectrum of compound I is different from that of compound II. The large difference of these spectra between the compounds I and II cannot be explained in terms of the same spin state of ferryl hemes. Despite the fact that Mossbauer spectra of compounds I and II have given the same iron isomer shift and suggested low-spin hemes with ferryl irons for these compounds (Moss et al., 1969; Harami et al., 1977), a ferryl high-spin heme in compound I of HRP appears to be responsible for the present NMR observations. A suggestion was also made that the primary compound of peroxidase may be an iron complex with mesohydroxyporphyrin containing a saturated carbon at this position (Brill and Williams, 1961), though the NMR spectrum of compound I of HRP is characteristic of a normal type of protoporphyrin, providing evidence against such an isoporphyrin in the enzymatic cycle.

From the similarity of optical absorption spectra of compound I and an oxidized product of manganooctaethylporphyrin, Dolphin et al. suggested that 1 oxidizing equiv of compound I is retained on the heme ring as a π-cation radical (Dolphin et al., 1971; Dolphin and Felton, 1973). The present NMR spectrum of compound I throws a strong argument against the π-cation radical as a source of the oxidizing equivalent. If we suppose that compound I is such a species as ferryl iron with π-cation radical on the heme ring, spin densities at various carbon atoms on the heme may be drastically altered from those of the native enzyme. This may result in dramatic change in the paramagnetically shifted NMR spectra, and proton signals of the heme peripheral group may be too much broadened. Nevertheless, neither line broadening nor a large shift of the proton peaks was encountered for compound I, indicating that 1 oxidizing equiv of the compound I is not retained on the heme ring as a π-cation radical. In the case of the complex ES (compound I) of CCP, a narrow ESR signal of a free-radical type at $g = 2.00$ was obtained (Yonetani et al., 1966). The spin concentration of this free radical was estimated to be about 1 equiv per enzyme hematin unit and is attributed to the formation of a stable and reversible free radical on an aromatic amino acid residue of the enzyme located near the heme iron. It is thus concluded that 1 oxidizing equiv of the complex ES is retained in an amino acid residue near the heme but not in the heme ring. The most exciting ESR signal for compound I of HRP was recently obtained to show the presence of a free radical in the protein moiety (Aasa et al., 1975). The broadened ESR signal is likely due to an electron-spin relaxation of a free radical enhanced by the nearby heme iron. It then follows that the structural characteristic of compound I of HRP is similar to that of complex ES of CCP. We are thus forced to conclude that 1 oxidizing equiv in compound I of HRP is retained on an amino acid residue near the heme but not on the heme ring as a π-cation radical. Of particular interest is the analysis of optical absorption spectra of oxidized metalloporphyrin. Although several different spectra are observed depending upon the central metal ion, porphyrin substitution, and counterion, a proper comparison of the spectra should be made after a detailed characterization of the transition bands for these porphyrin derivatives.

The drastic change in the NMR spectra on going from compound I to compound II reminds us of the spectral change for ferric hemoprotein from a high to low spin state; in ferric hemoproteins, the heme peripheral methyl proton peaks were observed in the downfield region of 40 to 100 ppm from DSS for the high-spin state and of 10 to 40 ppm for the low-spin state. Thus, the small paramagnetic shift of the heme peripheral methyl proton peaks for compound II and ferrylmyoglobin is best understood in terms of ferryl low-spin hemes. This is consistent with the interpretations of Mossbauer (Moss et al., 1969), magnetic susceptibility (Theorell and Ehrenberg, 1952; Ehrenberg, 1966), and resonance Raman (Rakhit et al., 1976; Felton et al., 1976) measurements of compound II. It is also of interest to assume that the iron's sixth ligands are different between compound I and compound II, because a spin-state change of the central heme iron is usually incurred by changing the nature of the axial iron ligand. In compounds I and II, one of the iron ligands may be a proximal histidylimidazole (Henry and Mazza, 1974; Brill and Sandberg, 1968; Critchlow and Dunford, 1972), while the other could be an oxygen atom derived from H_2O_2, as evidenced for compound I of chloroperoxidase (Hager et al., 1972). The possible sixth iron ligands in compounds I and II are H_2O, OH^- (Yamada and Yamazaki, 1974), and O= (oxometal) (Jones and Dunford, 1977), either of which we have no confirmative evidence to choose at present. It may at least be safe to say that the ligands of compounds I and II are of weak- and strong-field types, respectively, judging from the paramagnetic shifts of the heme peripheral methyl proton peaks.

Acknowledgments

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References

Aasa, R., Vanngard, T., and Dunford, H. B. (1975), *Biochim. Biophys. Acta 391,* 259.

Blumberg, W. E., Peisach, J., Wittenberg, B. A., and Wittenberg, J. D. (1968), *J. Biol. Chem. 243,* 1854.

Brill, A. S. (1966), *Comp. Biochem. 14,* 447.

Brill, A. S., and Sandberg, H. E. (1968), *Biochemistry 7,* 4254.

Brill, A. S., and Williams, R. J. P. (1961), *Biochem. J. 78,* 253.

Chance, B. (1952), *Arch. Biochem. Biophys. 41,* 404.

Critchlow, J. E., and Dunford, H. B. (1972), *J. Biol. Chem. 247,* 3714.

Dolphin, D., and Felton, R. H. (1973), *Acc. Chem. Res. 7,* 26.

Dolphin, D., Forman, A., Borg, D. C., Fajer, J., and Felton, R. H. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 614.

Dunford, H. B., and Stillman, J. S. (1976), *Coord. Chem. Rev. 19,* 187.

Ehrenberg, A. (1966), *Hemes Hemoproteins, Proc. Collog., 3rd, 1966,* 331.

Felton, R. H., Owen, G. S., Dolphin, D., Forman, A., Borg, D. C., and Fajer, J. (1973), *Ann. N.Y. Acad. Sci. U.S.A. 206,* 504.

Felton, R. H., Romans, A. Y., Yu, N.-T., and Schonbaum, G. R. (1976), *Biochim. Biophys. Acta 434,* 82.

Fox, J. B., Jr., Nicholas, R. A., Ackerman, S. A., and Swift, C. E. (1974), *Biochemistry 13,* 5178.

George, P. (1953), *Biochem. J. 55,* 220.

Hager, L. P., Doubek, D. L., Silverstein, R. M., Hargins, J. H., and Martin, J. C. (1972), *J. Am. Chem. Soc. 94,* 4364.

Harami, T., Maeda, Y., Morita, U., Trautwein, A., and Gonser, U. (1977), *J. Chem. Phys. 67,* 1164.

Henry, Y., and Mazza, G. (1974), *Biochim. Biophys. Acta 371,* 14.

Herington, E. F. G., and Kynatson, W. (1957), *Trans. Faraday Soc. 53,* 138.

Hewson, W., and Dunford, H. B. (1976), *J. Biol. Chem. 251,* 6043.

Jones, P., and Dunford, H. B. (1977), *J. Theor. Biol. 69,* 457.

King, N. K., and Winfield, M. E. (1963), *J. Biol. Chem. 238,* 1520.

Morishima, I., Ogawa, S., Inubushi, T., Yonezawa, T., and Iizuka, T. (1977), *Biochemistry 16,* 5109.

Moss, T., Ehrenberg, A., and Bearden, A. J. (1969), *Biochemistry 8,* 4151.

Rakhit, G., Spiro, T. G., and Ueda, M. (1976), *Biochem. Biophys. Res. Commun. 71,* 803.

Roman, R., and Dunford, H. B. (1972), *Biochemistry 11,* 2076.

Schonbaum, G. R., and Lo, S. (1972), *J. Biol. Chem. 247,* 3353.

Theorell, H., and Eherenberg, A. (1952), *Arch. Biochem. Biophys. 41,* 442.

Williams, R. J. P., Wright, P. E., Mazza, G., and Ricard, J. R. (1975), *Biochim. Biophys. Acta 412,* 127.

Wuthrich, K. (1970), *Struct. Bonding 8,* 53.

Yamada, H., and Yamazaki, I. (1974), *Arch. Biochem. Biophys. 165,* 728.

Yamazaki, I., and Yokota, K. (1973), *Mol. Cell. Biochem. 2,* 39.

Yonetani, T. (1970), *Adv. Enzymol. 33,* 309.

Yonetani, T., and Schleyer, H. (1967), *J. Biol. Chem. 242,* 1974.

Yonetani, T., Schleyer, H., and Ehrenberg, A. (1966), *J. Biol. Chem. 241,* 3240.