Nuclear Magnetic Resonance Studies of Porphyrin π-Cation Radical in Ruthenium(II)-Substituted Horseradish Peroxidase and Some Implications for the Electronic State of Peroxidase Compound I†

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ABSTRACT: One-electron-oxidized products of ruthenium(II) mesoporphyrin and deuteroporphyrin and of horseradish peroxidase (HRP) reconstituted with these Ru(II) porphyrins were studied by proton NMR, electron spin resonance (ESR), and optical absorption spectroscopies. Ru(II)HRP was readily oxidized by K_2IrCl_6 to form a porphyrin π-cation radical, which afforded well-resolved hyperfine-shifted proton NMR spectra similar to that of HRP compound I. This suggests that the radical has an electronic structure similar to that of HRP compound I. The heme peripheral proton resonances for these Ru(II) porphyrin π-cation radicals, in which the metal center is in a diamagnetic state, were not seriously broadened and exhibited large downfield or upfield shifts. Non-Curie law behaviors for these signals were interpreted in terms of a thermal equilibrium between $^2A_{1u}$ and $^2A_{2u}$ π-radical states, as has been done for the model Ru(II) porphyrin π-cation radicals. By analogy to the Ru(II)HRP porphyrin π-cation radical, HRP compound I may be in the $^2A_{1u}$ state admixed with the $^2A_{2u}$ state. The mechanism of the enhancement of electron spin relaxation in compound I was also discussed with reference to the unusually sharp proton NMR signals and extremely broadened ESR signal of compound I.

Peroxidases and catalase (CAT) are ferric protoporphyrin IX containing enzymes that decompose hydrogen peroxide and organic peroxides. Their catalytic cycles involve oxidation of the heme prosthetic group to produce the reaction intermediates referred to as compound I and compound II (Keilin & Mann, 1937; Theorell, 1941; Chance, 1952; George, 1953). Two-electron oxidation of the resting ferric enzyme generates the green compound I, and its 1-equiv reduction yields the red compound II. Mössbauer results are consistent with an iron(IV) configuration in both compounds (Maeda & Morita, 1967; Harami et al., 1977; Moss et al., 1969; Schulz et al., 1979). After several years of debates over the electronic structure of the compound I on the basis of a variety of spectroscopic measurements for both the compound I and its model metalloporphyrin π-cation radicals, the second oxidation equivalent in compound I is now best described as a porphyrin-centered π-cation radical (Dolphin et al., 1971, 1973; Fajer et al., 1973, 1974; Felton et al., 1973; La Mar & de Ropp, 1980; La Mar et al., 1981; Roberts et al., 1981). In doubly oxidized cytochrome *c* peroxidase (CCP), on the other hand, the second oxidation equivalent has been assigned as a protein-free radical on the basis of electron spin resonance (ESR) and electron nuclear double resonance (ENDOR) spectra (Yonetani et al., 1966; Hoffman et al., 1979).

In recent years, porphyrin π-cation radicals formed in horseradish peroxidase (HRP) and CAT have been again subjected to π electronic structural characterization. One of the prevalent arguments in such studies is the distribution of the electron spin density on the porphyrin, from which the symmetry of the π-radical orbital is characterized (Fajer et al., 1970; Fajer & Davis, 1979). Dolphin and his co-workers, who first proposed a ferryl (Fe^{4+}) heme π-cation radical complex as a model for compounds I of HRP and CAT, found two types of model metalloporphyrin π-cation radicals having visible spectra similar to those of either HRP or CAT compound I, where the spectral difference has been ascribed to electron abstraction from either one of two nearly degenerate highest occupied π orbitals (a_{1u} or a_{2u}) of the porphyrin (Dolphin et al., 1971, 1973; Fajer et al., 1973, 1974; Felton et al., 1973). In CAT where the heme iron is ligated by tyrosyl phenolate, the oxidation is postulated to occur from an a_{1u} orbital ($^2A_{1u}$ radical), whereas HRP compound I, with a histidyl imidazole ligand, is supposed to be a $^2A_{2u}$ radical. Recent ESR and ENDOR results (Schulz et al., 1979; Roberts et al., 1981) appear to support the ferryl-$^2A_{2u}$ radical profile predicted for HRP compound I, if allowance is made for a weak exchange interaction between electron spins on iron(IV) and on the porphyrin radical. This electron spin coupling has been proposed to result in enhanced electron spin relaxation, leading to extremely broadened ESR spectra (Schulz et al., 1979) and eventually nonbroadened ^1H NMR spectra of HRP compound I (Morishima & Ogawa, 1978; La Mar & de Ropp, 1980; La Mar et al., 1981).

Recently, we have found that the π-cation radicals of ruthenium(II)- or cobalt(III)-octaethylporphyrin complex where the metal center is in a diamagnetic state can afford unbroadened ^1H NMR spectra, from which electron distributions on the porphyrin periphery, especially at the meso carbon, can be deduced and, thereby, the symmetry of the π-cation radical orbitals is readily discerned (Morishima et al., 1983, 1984). It was also suggested from the non-Curie law behavior of the meso proton signal that these radicals are in a thermal admixture of the nearly degenerate $^2A_{1u}$ and $^2A_{2u}$ states and further that the radical species with a $^2A_{2u}$-type absorption spectrum is more $^2A_{1u}$ like than the one having a $^2A_{2u}$-type spectrum. These results seem to contradict the prevailing dogma regarding $^2A_{1u}/^2A_{2u}$ assignments for metalloporphyrin π-cation radicals based on visible spectral data (Dolphin et al., 1971, 1973; Fajer et al., 1973, 1974; Felton et al., 1973). Rutter et al. (1983) have also reached a similar argument by a different approach, in which compound I prepared from mesoheme-substituted HRP having an absorption spectrum

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characteristic of $^2A_{1u}$ porphyrin π-cation radical exhibits ESR and Mössbauer spectra similar to those of native HRP compound I. We further suggested that the mixing of the nearly degenerate radical states may induce enhanced electron spin relaxation, responsible for unbroadened NMR spectra and eventually broadened ESR spectra.

Now it seems of interest to see the proton NMR of Ru(II) porphyrin π-cation radicals reconstituted in HRP. We have reported (Morishima & Ogawa, 1978) for the first time the unbroadened hyperfine-shifted proton NMR spectrum of HRP compound I, which was tentatively interpreted in terms of the ferryl high-spin ($S = 2$) porphyrin rather than ferryl low-spin ($S = 1$) porphyrin π-cation radical on the basis of the NMR data available at that time for ferryl porphyrin complexes (Felton et al., 1973). This ferryl complex was later shown to be erraneous and reassigned as the ferric porphyrin π-cation radical (Phillippi & Goff, 1982; Goff & Phillippi, 1983). Meanwhile, La Mar and his co-workers (La Mar et al., 1980, 1981) described the similar proton NMR spectrum of deuteroheme-substituted HRP compound I and suggested that the spectral feature could be reasonably interpreted in terms of the porphyrin π-cation radical. However, it was uncertain that these NMR studies provide direct evidences for the occurrence of a π-cation radical intermediate. Although Yamazaki and his co-workers (Yamazaki et al., 1980, 1982) recently reported and characterized the π-cation radical of diamagnetic metal-substituted porphyrins such as magnesium- and zinc-substituted HRP, these radicals did not exhibit well-resolved ESR or ^{1}H NMR spectra, and their detailed electronic structures have not been directly defined so as to compare with that of native HRP compound I. On the other hand, our preliminary NMR study of the porphyrin π-cation radical in Ru-substituted HRP (Morishima et al., 1983) has directly proved that the hyperfine-shifted resonances of HRP compound I result mostly from the π-cation radical center rather than from the paramagnetic iron center. Therefore, Ru(II)-substituted HRP is expected to disclose in more details the electronic structure of the π-cation radical in HRP compound I. Herein, we present the detailed proton NMR studies of Ru porphyrin π-cation radical incorporated in HRP and discuss them in the light of its unique electronic structure and the possible account for the NMR line narrowing for the HRP compound I.

Materials and Methods

Ruthenium(II) carbonylmesoporphyrin dimethyl ester [MPDME·Ru(II)·CO] and ruthenium(II) carbonyldeuteroporphyrin dimethyl ester [DPDME·Ru(II)·CO] were prepared by a variant of the previous method (Tsutsui et al., 1971). The corresponding protoporphyrin complex was not obtained due to the rigorous synthetic conditions. Meso-d_4-MPDME and 2,4-d_2-DPDME were prepared by the literature methods (Kenner et al., 1973). Oxidation of MPDME·Ru(II)·CO and DPDME·Ru(II)·CO to π-cation radicals was performed by adding Br_2 to the CD_2Cl_2 solution at −20 °C. The free base of the Ru porphyrin was generated from the corresponding ester by hydrolysis in 1% KOH–methanol. The Ru porphyrin was incorporated into apoHRP according to the method described (Kaneko et al., 1980; Kuwahara et al., 1982).

Isoenzyme c of HRP was purchased from Toyobo Co. as a salt-free lyophilized powder (G-I-C, RZ = 3.3), and apoHRP was prepared by the HCl–butanone method. For NMR measurement, the Ru-reconstituted HRP (RuHRP) was dissolved in 0.1 M phosphate buffer at pH 7.0 and oxidized by K_2IrCl_6, which was stored in weakly acidified solution (Kaneko et al., 1980; Kuwahara et al., 1982).

Proton NMR spectra at 300 MHz and deuterium NMR spectra at 46.1 MHz were recorded with a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Optical absorption spectral measurements were made on a Hitachi 330 spectrometer.

Results

Porphyrin π-Cation Radicals of Natural Porphyrin Ruthenium(II) Complexes. It has been known that oxidation behaviors of ruthenium(II) porphyrin compounds characteristically depend upon their axial ligand: ring oxidation occurs for the carbonyl complex, while metal oxidation does for other ligand complexes (Brown et al., 1973, 1975; Barley et al., 1981). As the case for carbonmonoxy Ru(II) OEP and TPP, the natural porphyrin analogues, Ru(II) monocarbonylmesoporphyrin dimethyl ester [MPDME·Ru(II)·CO] and Ru(II) monocarbonyldeuteroporphyrin dimethyl ester [DPDME·Ru(II)·CO], formed π-cation radicals by bromine oxidation (Morishima et al., 1983). Both oxidized products afforded absorption spectra characteristic of a porphyrin radical that has absorption maxima at 626 and 386 nm for mesoporphyrin π-cation radical and at 634 and 389 nm for deuteroporphyrin derivative (Dolphin et al., 1971, 1973; Fajer et al., 1973, 1974; Felton et al., 1973). These radicals gave a single ESR signal with $g = 2.003$ and 11-G peak to peak width at 77 K. However, their ESR spectra were not detectable at room temperature, possibly due to enhanced electron spin relaxation. This prompted us to measure their proton NMR spectra.

Figure 1 shows ^{1}H NMR spectra of these radicals, $[MPDME·Ru(II)·CO]^{+\cdot}Br^-$ (**1**) and $[DPDME·Ru(II)·CO]^{+\cdot}Br^-$ (**2**), in methylene chloride at −20 °C. The paramagnetically shifted proton peaks for **1** and **2** are surprisingly sharp and well resolved, with a poor S/N ratio for the spectrum of **2** owing to its lower stability. In both spectra, four porphyrin peripheral methyl signals are clearly resolved in the downfield region: 79.6, 76.2, 71.3, and 68.4 ppm for **1** and 92.8, 81.6, 76.4, and 49.8 ppm for **2**. Four meso proton signals are located in the upfield region: −34.6, −35.2, −37.0, and −38.2 ppm for **1** and −62.3, −69.5, −76.0, and −80.3 ppm for **2**. The assignment of the meso proton signals was confirmed by ^{2}H NMR measurement for selectively mesodeuterated **1**, as shown at the upper line in Figure 1. The resonances in the 10–30 ppm region in both spectra possibly arise from the ethyl and the propionate groups at the heme periphery. In the ^{1}H NMR spectrum of **2**, the pyrrole 2,4-proton signals, of which signal positions are expected to sensitively reflect spin densities at the pyrrole β-carbons, cannot be unambiguously assigned, probably due to their shifts in the crowded diamagnetic region or extreme broadening. In fact, in the ^{2}H NMR spectrum of 2,4-deuterated **2**, the 2,4-$^2H_2$ resonances were clearly resolved at 1.7 ppm (the spectrum is not shown).

The large hyperfine shifts of the heme peripheral proton signals are consistent with extensive spin delocalization in the π system, as expected for a porphyrin π-cation radical. Recently, Hanson et al. (1981) presented the charge iterative extended Hückel (IEH) molecular orbital calculations for ferryl porphyrin π-cation radical models. Because of the negligible mixing between the porphyrin and iron orbitals, the calculated unpaired spin density profiles for iron(IV) porphyrin π-cation radical complexes may serve to substantiate the present NMR results of the ruthenium porphyrin π-cation radicals, in which the metal is diamagnetic. In particular, the calculated spin density distribution in the deuteroporphyrin $^2A_{1u}$ and $^2A_{2u}$ radicals without imidazole ligand appears to parallel the NMR hyperfine shifts for **2**; no appreciable spin density resides at the 2,4-carbons (vide infra). Furthermore, in the $^2A_{1u}$ deutero radical, the asymmetry of the spin dis-

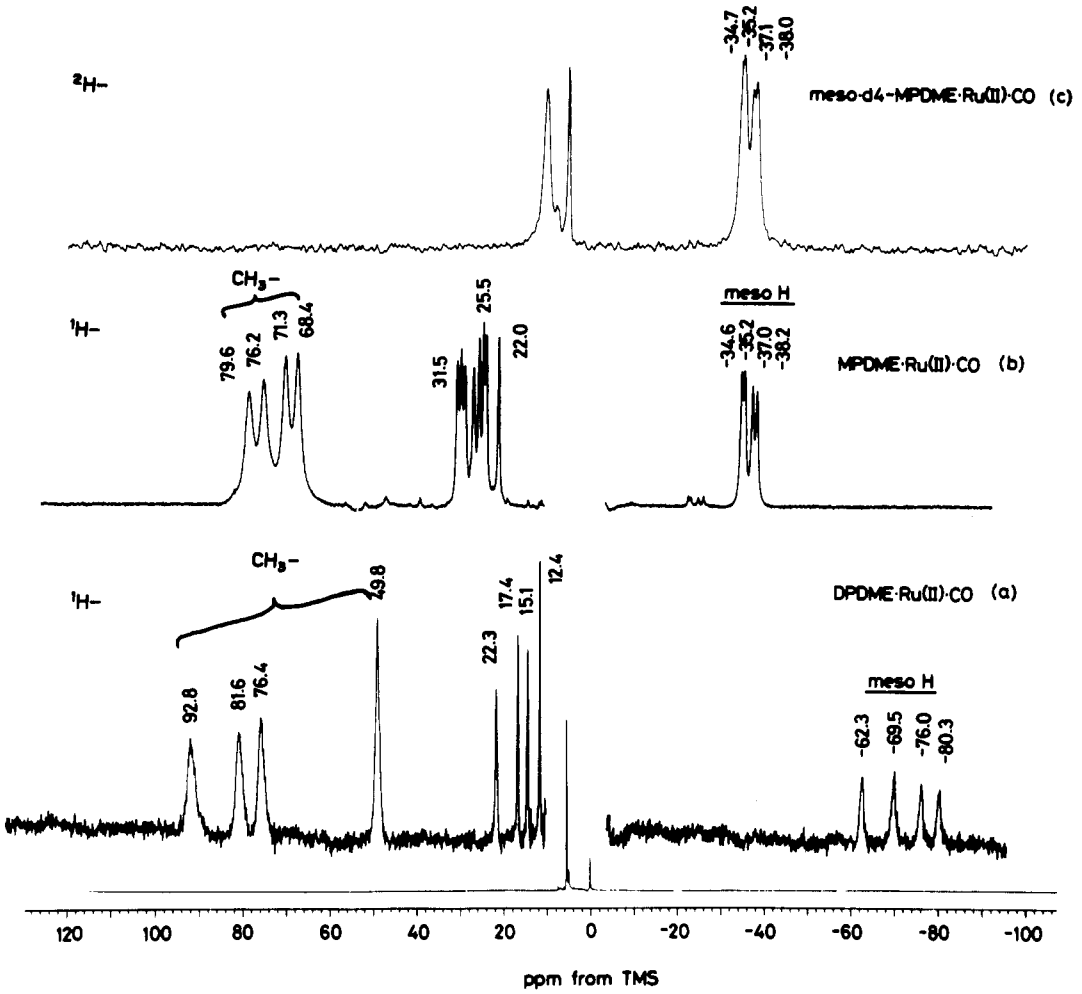

FIGURE 1: Proton NMR spectra of π-cation radicals of (a) DPDME·Ru(II)·CO and (b) MPDME·Ru(II)·CO at −20 °C in CD_2Cl_2. (c) Deuterium NMR spectrum of meso-$^2H_4$-MPDME·Ru(II)·CO π-cation radical in CH_2Cl_2.

tribution is remarkable; substantial amounts of spin density are placed at three of the four pyrrole β-carbons connected with methyl groups, and the remaining one bears a small amount of spin density. This MO result appears to correspond to the present finding of three large downfield methyl shifts (92.8, 81.6, and 76.4 ppm) and one relatively small shift (49.8 ppm) for **2**, which seems to verify that present hyperfine-shifted NMR spectra are induced by porphyrin π-radical spin density.

However, one may still suspect that the hyperfine-shifted NMR spectra in Figure 1 arise from the Ru(III) paramagnetic complex. Hence, to ensure that the hyperfine-shifted signals in Figure 1 do not come from the Ru(III) porphyrin complex, we tried to observe the proton NMR spectra of MPDME·Ru(III)·$(py)_2$ and DPDME·Ru(III)·$(py)_2$, which are formed from the corresponding Ru(II) complexes by Br_2 oxidation (Brown et al., 1973), 1975; Chow & Cohen, 1971). As shown in Figure 2, the resulting NMR spectra are quite different from those for the oxidized products of Ru(II) monocarbonyl analogues, where methyl signals with a narrower line are observed around 40 ppm and meso H signals are not located in the upfield region. These findings rule out the possibility that the paramagnetic Ru(III) species is responsible for the spectra in Figure 1.

It has been established that an isotropic ^1H or ^2H NMR hyperfine shift for the radical system is related to the π-*electron spin density* at the carbon to which the proton or deuterium is attached. Then, the observed hyperfine shifts of the meso H for **1** and **2** and pyrrole ^2H for **2** are translated into π spin density at the meso carbons and pyrrole β-carbons,

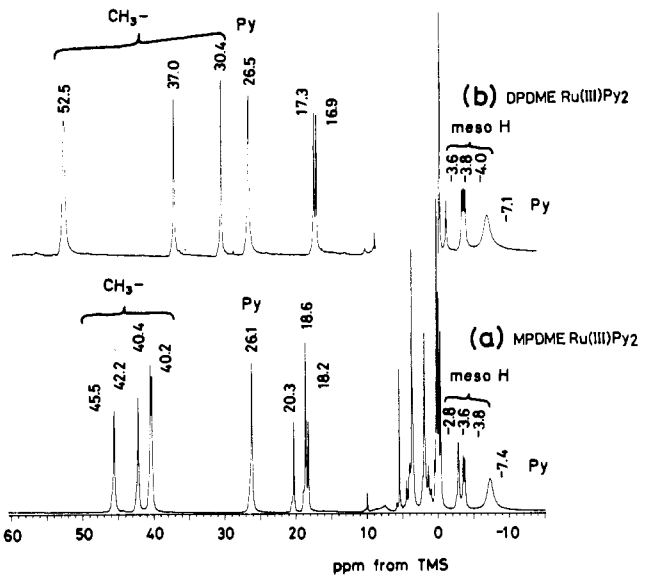

FIGURE 2: Proton NMR spectra of (a) MPDME·Ru(III)·$(py)_2$ and (b) DPDME·Ru(III)·$(py)_2$ at 0 °C in CD_2Cl_2.

respectively, through the McConnel equations (Jesson, 1973; Kreilick, 1973; McConnell, 1956). Spin densities were estimated as 0.026–0.029 for the meso carbon of **1** and 0.043–0.053 for the meso carbon and 0.001 for the pyrrole β-carbon of **2**. Small spin density on the 2,4-carbons for **2** appears reasonably consistent with the IEH calculation

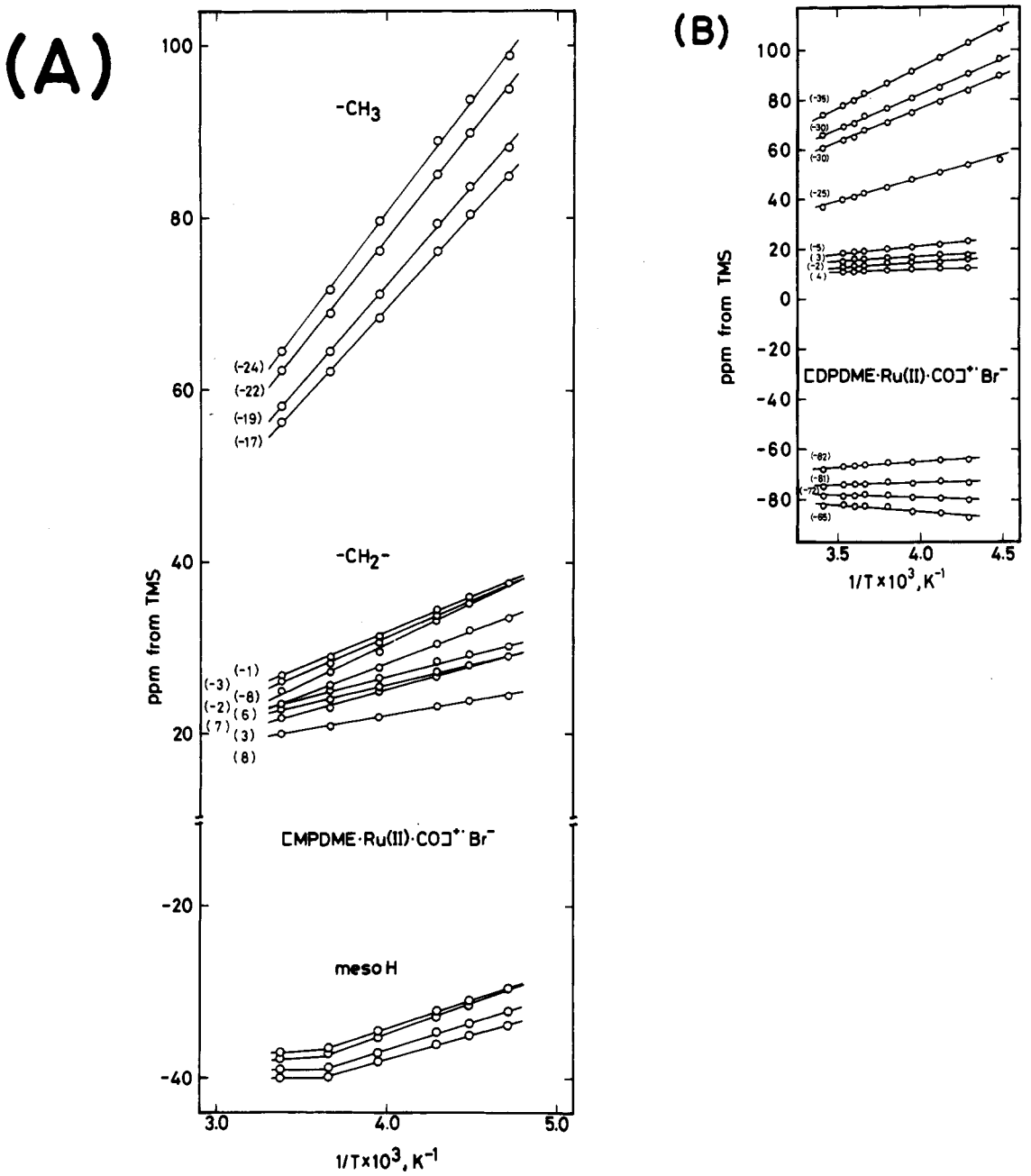

FIGURE 3: Temperature dependence of the proton NMR spectra of porphyrin π-cation radicals of (A) MPDME·Ru(II)·CO and (B) DPDME·Ru(II)·CO between 10 and −40 °C.

(Hanson et al., 1981), as stated above. However, the spin density on the meso carbon is inconsistent with the spin distributions for both a_{1u} (−0.14) and a_{2u} (0.28) radicals, which were obtained from unrestricted Hartree–Fock selfconsistent field calculation (Loew et al., 1977; Loew & Herman, 1980) and, therefore, could not be accounted for if **1** and **2** are in a single spin state.

This suggestion is more evidently confirmed by the temperature dependence of the proton NMR spectra of **1** and **2**. In Figure 3 is illustrated the temperature dependences of the hyperfine-shifted proton resonances of **1** and **2** in the form of the Curie plot. Inspection of the figure shows that the unusual non-Curie law behaviors are seen for the meso proton resonances for both radicals. The temperature dependences of the methyl and the methylene proton resonances do not appear to follow the normal Curie law behavior, since their extrapolations to $1/T = 0$ are beyond the diamagnetic shift region. All these unusual results suggest that both radicals, **1** and **2**, are in the thermal admixture of the $^2A_{1u}$ and $^2A_{2u}$ states, as the case for $[OEP\cdot Ru(II)\cdot CO]^{+\cdot}$ reported previously (Morishima et al., 1984).

Porphyrin π-Cation Radicals of Ruthenium(II)-Substituted HRP. Ru(II) monocarbonylmesoporphyrin [MP·Ru(II)·CO] and Ru(II) monocarbonyldeuteroporphyrin [DP·Ru(II)·CO] were incorporated into apoHRP to form the corresponding Ru-substituted peroxidases, HRP(MP·Ru·CO) and HRP(DP·Ru·CO), respectively. Upon addition of the oxidant, K_2IrCl_6, the absorption spectra of the Ru peroxidases turned into characteristic ones having featureless bands in the visible region, which are similar to that of HRP compound I. Figure 4 shows the optical spectral changes upon successive addition of K_2IrCl_6 for HRP(MP·Ru·CO) and HRP(DP·Ru·CO). The isosbestic points are clearly seen during the spectral titration. The spectral changes were reverted by the addition of the substrate indolepropionic acid (IPA), which serves as an electron donor. The oxidation of Ru peroxidase by K_2IrCl_6

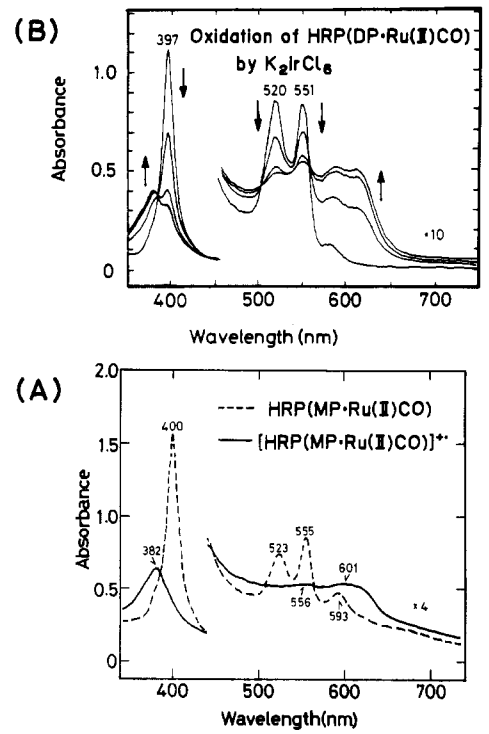

FIGURE 4: (A) Absorption spectrum of the porphyrin π-cation radical of HRP(MP·Ru·CO) and (B) the spectral change of HRP(DP·Ru·CO) upon successive addition of K_2IrCl_6 at 10 °C in 0.1 M phosphate buffer at pH 7.0.

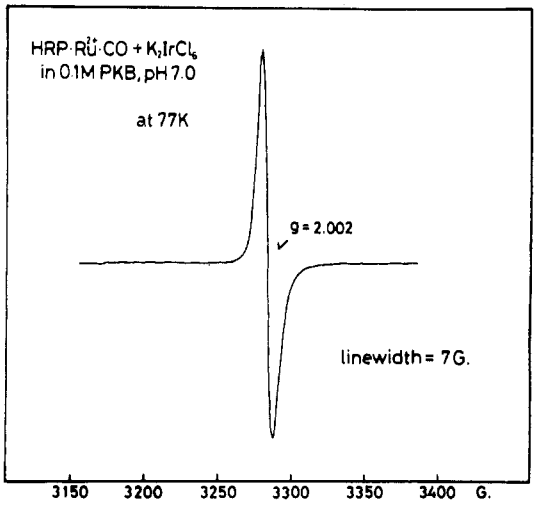

FIGURE 5: ESR spectrum of the oxidized product of HRP(DP·Ru·CO) at 77 K in 0.1 M phosphate buffer at pH 7.0.

was also confirmed by measuring an ESR spectra at 77 K, which afforded a single absorption at $g = 2.00$ with 7-G width (Figure 5). These results clearly show that one oxidizing equivalent is held as a porphyrin radical, as was the case for Mg and Zn peroxidases (Kaneko et al., 1980; Kuwahara et al., 1982). However, it is now of interest to note that the absorption spectrum of the oxidized Ru peroxidase is different from those of Zn and Mg peroxidases, which closely resemble the spectrum of CAT compound I.

In Figure 6 are illustrated the proton NMR spectra of Ru meso- and deuteroporphyrin π-cation radicals in HRP, [HRP(MP·Ru·CO)]+· (3) and [HRP(DP·Ru·CO)]+· (4), together with those of the compound I for native HRP and deuteroheme-reconstituted HRP. In the spectrum of 3, the four heme methyl signals are clearly resolved at 74.9, 71.6, 64.0, and 60.1 ppm. Several single-proton resonances, probably coming from the heme peripheral ethyl and propionate CH_2 groups, are also observed in the 60–10 ppm region.

Although it is not possible to directly compare the spectrum of 3 with that of mesoHRP compound I, of which the proton NMR spectrum is not available due to its instability, the NMR spectrum of 4 can be directly compared with that of deuteroHRP compound I that was previously reported by La Mar and de Ropp (1980). The NMR spectrum of 4 shows large average hyperfine shifts for the methyl substituents (125.0, 93.6, 90.5, and 54.3 ppm) compared with that of 3. This result seems to correspond to the NMR observation of a large downfield shift of methyl group of deuteroHRP compound I (98.2, 85.5, 69.8, and 46.9 ppm) relative to protoHRP compound I (71.0, 67.0, 54.3, and 46.2 ppm). In this spectral region, some minor signals are observed, presumably due to the methyl groups for the normally oriented porphyrin in HRP, since the previous NMR result suggested that deuteroporphyrin is predominantly inserted as a 180°-rotated form around porphyrin meso α–γ axis into apoHRP (La Mar et al., 1978, 1980).[1] Furthermore, it is worthy to note that the two single-proton signals at −19.8 and −30.7 ppm for 4 were clearly assigned to the 2,4-pyrrole protons by using selectively 2,4-deuterated 4. The relatively large upfield shift of 2,4-H's for 4 is noticeably consistent with that of deuteroHRP compound I (−28 and −37 ppm), of which signal positions were pointed out as the probe for the electronic structure of the porphyrin π-cation radical (La Mar et al., 1981). These spectral features are quite identical with that for deuteroHRP compound I (La Mar & de Ropp, 1980). All the hyperfine-shifted proton signals disappeared upon addition of IPA.

In order to directly assign the meso H signal, we measured the proton spectrum of [HRP(MP·Ru·CO)]+· reconstituted with meso-$^2H_4$-MP·Ru(II)·CO. However, no significant difference was observed upon deuterium labeling, indicating that the meso H peaks are not resolved in the spectrum of the π-cation radical of RuHRP. Moreover, we attempted to observe the meso deuterium NMR resonances, which is more suitable than proton NMR for the studies of the paramagnetic species, because the deuterium spectrum does not experience unwanted signal broadening which is often encountered in the proton spectrum. To our regret, no deuterium resonances from meso-$^2H_4$ were observed, presumably due to an enhanced quadrupolar relaxation effect on these deuterium resonances for HRP with a M_r of 42 000, which has a large rotational correlation time.

The temperature dependence of the resolved hyperfine-shifted resonances for 3 is exhibited in Figure 7 in the form of a Curie plot. The apparent intercepts at $1/T = 0$ for the straight lines are given in parentheses on the left. It is worthy to note that the extrapolations are beyond the diamagnetic region, as the case for the model compound, 1.

The pH dependence of the spectrum of 3 was also examined. The resulting features resembled the pH-dependent NMR spectral change for HRP compound I (Morishima & Ogawa, 1978b), and the pH titration curves show the possible presence of a heme-linked ionizable group with pK = 6, which may correspond to that reported for HRP compound I (Hayashi & Yamazaki, 1978).

Discussion

The electronic structure of the compounds I of HRP and CAT is now formulated as a ferryl low-spin (Fe^{IV}) porphyrin

[1] In the NMR spectrum of Mb substituted by DP·Ru·CO, two methyl peaks arising from Val E11 were observed at −7.4 and −7.5 ppm from H_2O, suggesting the presence of two heme isomers rotating around the meso α–γ axis in the protein.

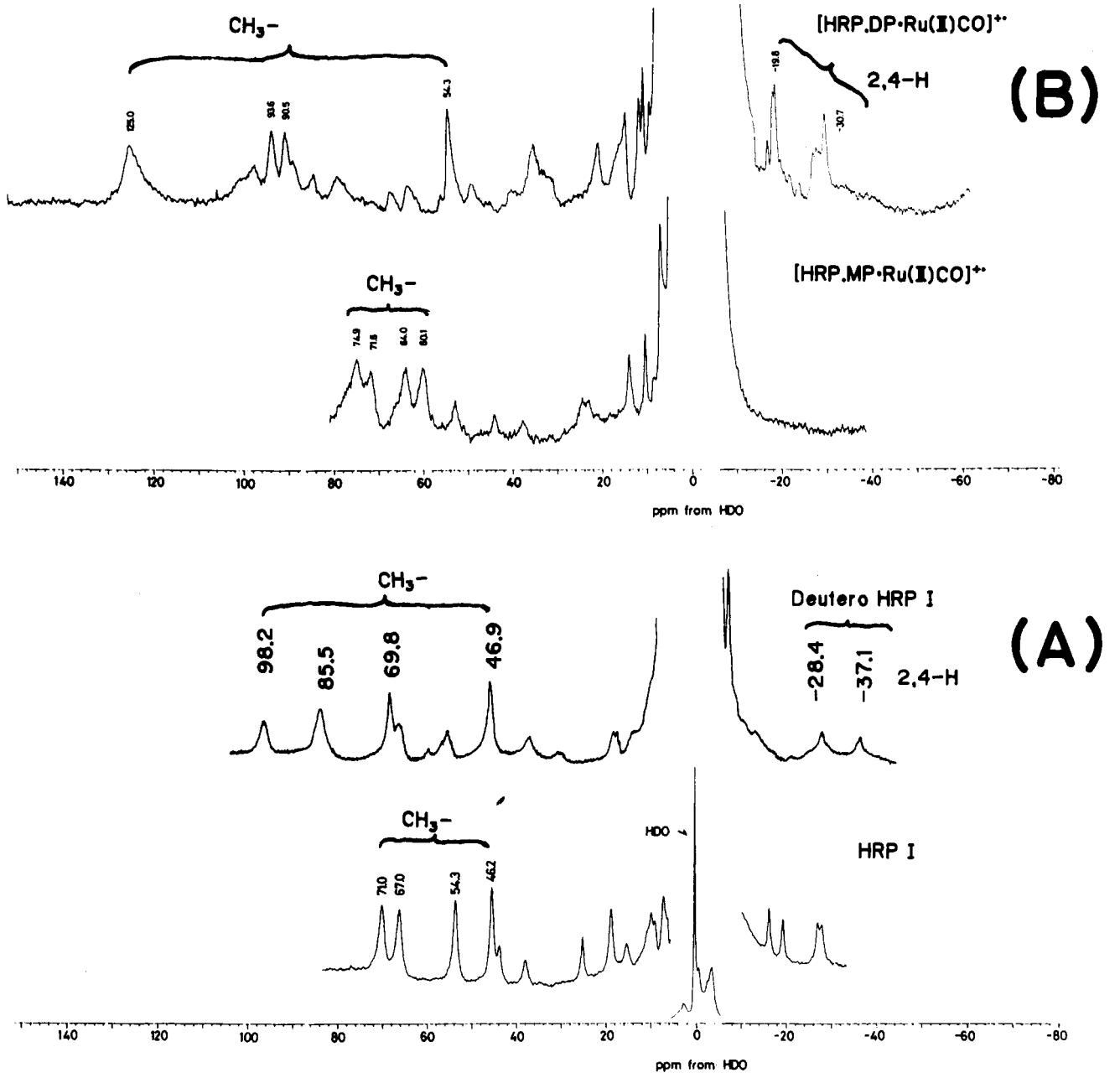

FIGURE 6: Proton NMR spectra of (A) HRP compound I and deuteroheme HRP compound I and (B) the porphyrin π-cation radicals of HRP(MP·Ru·CO) and HRP(DP·Ru·CO) at 10 °C in 0.1 M phosphate buffer at pH 7.0.

π-cation radical. The Mössbauer results (Maeda & Morita, 1967; Harami et al., 1977; Moss et al., 1969; Schulz et al., 1979) on the compound I of various peroxidases confirm an Fe^{IV}, spin $S = 1$, $(t_{2g})^4$ configuration of the heme iron, characterized by an isomer shift in the range of $\delta_{Fe} = 0.02$–0.13 mm/s and a quadrupole splitting of $E_Q = 1.0$–1.6 mm/s. Furthermore, reactions carried out with ^{18}O- and ^{17}O-labeled peroxides established a single oxygen ligand in the primary compounds of chloroperoxidase and HRP (Browett & Stillman, 1981). Thus, an oxyferryl center appears to be a common feature for all compounds I. However, the optical absorption spectra of the various compounds I show quite different features; CAT compound I shows a strong absorption band around 680 nm, whereas HRP compound I does not absorb in this region. By analogy to the absorption spectra of the model compounds, Dolphin and his co-workers (Dolphin et al., 1971, 1973) suggested that the differences in the absorption spectra among various peroxidases are associated with the spin distribution on the porphyrin ring in the π-cation radical; the CAT compound I spectrum is indicative of a $^2A_{1u}$ porphyrin π-radical, while HRP compound I is of a $^2A_{2u}$ radical type. The assignment of porphyrin radical symmetry on the basis of the visible spectra has been accepted for the past decade.

However, our recent proton and deuterium NMR studies of Ru(II) and Co(III) octaethylporphyrin π-cation radicals (Morishima et al., 1984) suggested that the visible absorption spectrum does not serve as a diagnosis for characterization of the electronic structure of the π-cation radical. We focused on the spin density at the meso positions of Ru(II) or Co(III) complexes of a highly symmetric porphyrin OEP radicals (D_{4h}) and revealed that the porphyrin π-cation radicals are in a mixing state of nearly degenerate $^2A_{1u}$ and $^2A_{2u}$ states depending on temperature, axial ligand, and counteranion, regardless of the absorption spectral features. In other words, two distinct states are thermally admixed for the π-cation radical, and the mixing is modulated by temperature and ligand. It has been well established that the salient feature

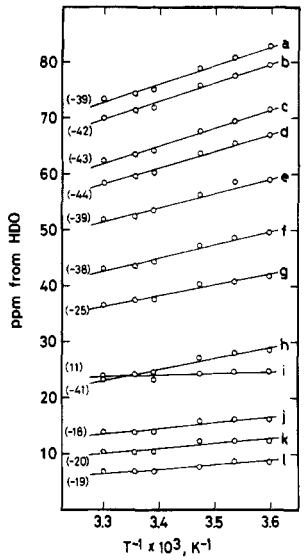

FIGURE 7: Temperature dependence of the proton NMR spectrum of the porphyrin π-cation radical of HRP(MP·Ru·CO).

of the $^2A_{2u}$ porphyrin π-cation radical is distribution of a substantial amount of positive spin density at the meso carbons and the pyrrole nitrogens, while the radical orbital in the $^2A_{1u}$ cations has a node at these atoms (D_{4h} symmetry) (Fajer & Davis, 1979). Since the isotropic hyperfine shift of the meso proton is proportional to the meso carbon spin density, the typical $^2A_{2u}$ radical is expected to induce a large upfield contact shift for the meso proton, and the $^2A_{1u}$ radical exhibits a downfield bias because of a negative spin density due to the electron correlation effect. If we assume that the a_{1u} orbital possesses slightly higher energy than the a_{2u} orbital, the thermal mixing of the $^2A_{2u}$ state increases with a raise in temperature to afford more positive spin density at the meso position, eventually inducing more upfield isotropic shift. Indeed, the temperature dependence of the meso H signal for $[OEP·Ru·CO]^{+\bullet}$ appears to satisfy this prediction. The present study thus shows that this is also the case for the natural porphyrin analogues that do not have a full D_{4h} symmetry (Figure 1).

The quantitative analysis showed that the mixing of $^2A_{1u}$ and $^2A_{2u}$ radicals readily occurs due to a relatively small energy gap between these two radical states (Morishima et al., 1984), and each content proportion is significantly affected by slight structural perturbations such as the porphyrin substituents or axial ligand binding. For example, the 100% $^2A_{2u}$ state observed in TPP·Mg π-cation radical is inverted to 100% $^2A_{1u}$ for the OEP analogue (Dolphin et al., 1971, 1973; Fajer et al., 1973, 1974; Felton et al., 1973). This suggests that introduction of substituents to the meso position affects preferentially the energy level of the a_{2u} orbital because of large spin density at the meso carbon in the $^2A_{2u}$ radical but not in the $^2A_{1u}$ one. The effect of the porphyrin 2,4-substituents on the ground-state occupancy could be also expected to be manifested as a difference in a $^2A_{1u}$ content between $[MPDME·Ru·CO]^{+\bullet}$ (**1**) and $[DPDME·Ru·CO]^{+\bullet}$ (**2**). We then tried to determine the proportion of the $^2A_{1u}$ and $^2A_{2u}$ states in this radical admixture model from the observed isotropic shifts for the meso protons of **1** and **2**, by following the procedure reported previously (Morishima et al., 1984). The small upfield shifts for the meso H in **1** and **2** relative to the predicted shift for the pure $^2A_{2u}$ radical (−250 ppm) could be reasonably accounted for if we assume that the $^2A_{2u}$ state is mixed to the predominant $^2A_{1u}$ state. When the limiting shifts for typical states are estimated as −250 ppm for the $^2A_{2u}$-state radical and 110 ppm for the $^2A_{1u}$-state radical deduced from the proton ESR coupling constant for the radicals in the corresponding states, $[TMP·Zn(II)]^{+\bullet}ClO_4^-$ ($^2A_{2u}$) (Fajer et al., 1974) and $[OEP·Mg(II)]^{+\bullet}ClO_4^-$ ($^2A_{1u}$) (Fajer & Davis, 1979), the $^2A_{2u}$ content amounts to about 30 % for **1** and about 40 % for **2**. This suggests that deuteroporphyrin π-radical **2** is more admixed with the $^2A_{2u}$ state than mesoporphyrin π-radical **1**. It is also expected that the axial ligation may affect significantly the ground-state occupancy of the porphyrin radical, because of a substantial spin density on the central metal in the $^2A_{2u}$ state, but not in the $^2A_{1u}$ state. This effect seems to be also manifested in the RuHRP π-cation radical, which will be discussed in the following.

Ru-substituted HRP was readily oxidized by K_2IrCl_6 to form the porphyrin π-cation radical, as confirmed by the absorption and ESR spectra. The resultant visible spectrum bears a striking resemblance to that of native HRP compound I, which is in contrast to the case of porphyrin π-cation radical in Mg- and ZnHRP's (Kaneko et al., 1980; Kuwahara et al., 1982). However, as mentioned above, this could not be taken as an evidence to indicate the $^2A_{2u}$ electronic state. In fact, the unusual non-Curie law behavior of the hyperfine-shifted proton NMR resonances for **3** (Figure 7) could be explained by assuming that the π-cation radical in the protein is not in a single electronic structure, but in an admixture of the two different electronic states, $^2A_{1u}$ and $^2A_{2u}$, as was the case for the model Ru porphyrin π-cation radicals. This suggestion is strongly supported by our previous finding (1984) that an imidazole adduct of the Ru porphyrin radical, which mimics the structure of the porphyrin π-Cation radical in RuHRP, induced a downfield bias of the hyperfine shifts for the meso deuterium NMR signal from −28.6 to 11.5 ppm. According to our previous analysis based on the isotropic shift of the meso-^{2}H signal, the spin density on the meso carbon significantly changes from 0.024 to −0.001 upon binding of imidazole. Recent IEH calculations by Hanson et al. (1981) suggested that the imidazole binding to the porphyrin radical slightly alters the spin density at the meso position for both $^2A_{1u}$ and $^2A_{2u}$ radicals, but the spin density change is not so significant as the present case. Quite a small magnitude of negative π spin density for the imidazole adduct of Ru porphyrin π-cation radical may suggest that the binding of histidyl imidazole to the Ru porphyrin π-cation radical in HRP more favors a $^2A_{1u}$ state in the thermally mixing electronic state. Our failure to resolve the meso H resonance in the proton spectrum of RuHRP π-cation radical is reasonably consistent with its small hyperfine shift in the crowded diamagnetic envelop due to a small π spin density in the $^2A_{1u}$ state. These considerations lead us to reexamination of the electronic structure of porphyrin π-cation radical in HRP compound I.

We note here the similarity of the hyperfine-shifted proton NMR spectra, especially the heme methyl and 2,4-proton resonances between $[HRP(DP·Ru·CO)]^{+\bullet}$ and deuteroHRP compound I. Keeping in mind that π radical spin mainly contributes to the hyperfine-shifted resonances in HRP compound I rather than the iron-centered one (Morishima et al., 1983), the NMR spectral similarity of both spectra with respect to the upfield-shifted 2,4-proton resonances and relatively large methyl shifts may allow us to suggest that the electronic structure of the porphyrin radical of native HRP compound I may have a close resemblance to that of RuHRP π-cation radical; the predominant $^2A_{1u}$ state admixed to some extent with the $^2A_{2u}$ state. This assignment for HRP compound I is in disagreement with an early suggestion of an electronic hole in the a_{2u} orbital on the basis of optical (Dolphin et al.,

1971, 1973; Fajer et al., 1973, 1974; Felton et al., 1973) and ENDOR (Roberts et al., 1981) measurements. Although Rutter et al. (1983) explained the ENDOR frequency arising from the porphyrin radical as the result of the spin distribution in the $^2A_{2u}$ state on the basis of the visible spectral feature, their result could be alternatively explained by assuming HRP compound I to be in the predominant $^2A_{1u}$ state, since the experimentally estimated average spin densities on porphyrin nitrogen and carbon are very close to MO theoretical values calculated for an $^2A_{1u}$ radical. Moreover, the meso proton coupling by the ENDOR experiment appears closer to the MO results for the $^2A_{1u}$ state if we assume that the coupling constant with meso H has a negative sign (Roberts et al., 1981). In addition, consistency of the MCD spectrum of HRP compound I with that of $[OEP{\cdot}Co(III)]^{2+}{\cdot}2ClO_4^-$ (Browett & Stillman, 1981), which was found to be in the mixing state of 70% $^2A_{1u}$ and 30% $^2A_{2u}$ from our previous analysis (Morishima et al., 1984), also apparently supports our present assignment for the electronic structure of porphyrin π-cation radical in HRP compound I.

We now reexamine the NMR spectrum of HRP compound I, in which the heme methyl proton signals are located at 50–80 ppm downfield from H_2O. Such a downfield contact shift requires spin density of the order of 0.01–0.02 at the β-carbons. The IEH MO calculations for imidazole-bound Fe(IV) protoporphyrin $^2A_{2u}$ cation (Hanson et al., 1981) revealed that the pyrrole β-carbons are essentially devoid of unpaired spin density (0.002), while the $^2A_{1u}$ radical places a relatively large density at this position (0.047–0.001). It was also shown that the iron orbital contribution to the spin density at the heme periphery is at most 0.003 and 0.004. Therefore, the proton NMR spectral feature of the native HRP compound I could be reasonably explained in terms of the a_{1u} states admixed to some extent with the a_{2u} state. It is also to be noticed in the spectrum of HRP compound I that the meso H signal was not detectable (La Mar & de Ropp, 1980; La Mar et al., 1981; Morishima & Ogawa, 1978). A failure to detect the meso proton resonance has been interpreted, but not proven yet, as due to substantial shifts and concomitantly extensive line broadening resulting from large π spin density at the meso carbon for the $^2A_{2u}$ radical. However, if we assume that HRP compound I is in $^2A_{1u}$ state mixed to some extent with $^2A_{2u}$ state, undetectable hyperfine-shifted meso-H signal could simply be ascribed to its concealment in the crowded protein signal envelope, as is the case for RuHRP radical. This suggestion is supported by a recent finding by La Mar and his co-workers that the extremely broadened ^2H NMR signals for meso-$^2H_4$ compound I were observed in the diamagnetic region.[2] Furthermore, the failure to detect a hyperfine-shifted exchangeable NH resonance for the axial histidyl imidazole of HRP compound I also appears to correspond to a small π spin density placed on the central iron, eventually on its axial imidazole, in the $^2A_{1u}$ state (La Mar et al., 1982).

The electronic structure of porphyrin π-cation radical in compound I significantly relates with its unusual magnetic property; sharp proton NMR and extremely broadened ESR signals due to an enhancement of the electron spin relaxation. Schulz et al. (1979) have explained the relaxation enhancement as a result of the electron coupling between the spin $S = 1$ of the ferryl iron and the $^2A_{2u}$ radical on the porphyrin, which places a substantial amount of spin density on the central iron. Several lines of evidences including ESR (Schulz et al., 1979), ENDOR (Roberts et al., 1981), and Mössbauer (Schulz et al., 1984) data have apparently supported this model. However, it must be noted that the exchange coupling constant, $|J| \sim 4$ K, is relatively small and is plausibly explained by the lack of overlap between the magnetic orbitals of the Fe^{IV} and of the porphyrin radical. Furthermore, in the present case of Ru porphyrin π-cation radicals in HRP where the metal center is diamagnetic [Ru(II), d^6, $S = 0$], the unusually sharp resonances in their proton NMR spectra should also demand an enhanced electron spin relaxation mechanism other than the metal spin–radical spin coupling. More recently, we suggested that mixing of the nearly degenerate $^2A_{1u}$ and $^2A_{2u}$ porphyrin radical states may induce enhanced electron spin relaxation, eventually leading to sharp proton NMR spectra of $[OEP{\cdot}Ru(II){\cdot}CO]^{+\bullet}$, $[OEP{\cdot}Co(III)]^{2+\bullet}$ (Morishima et al., 1983, 1984), and $[TPP{\cdot}Ru(II){\cdot}CO]^{+\bullet}$.[3] This reminds us of the fact that the proton hyperfine splitting of the nearly degenerated hydrocarbon free radicals is markedly temperature dependent and their ESR resonances are broadened (Vincow, 1968). This effect has been successfully interpreted in terms of the hypothesis that the energy difference between the ground and lowest lying excited configurations is of the order of kT (200 cm^{-1}). Measured splittings are then statistical averages of the values in the ground and thermally populated excited states and should be very dependent on temperature. This seems to be quite compatible with the present case. As to the electron relaxation, Kivelson (1966) suggested that the most important contribution to the spin–lattice relaxation for this system may arise from an Orbach-type relaxation process, which is effective due to a presence of the low-lying excited electronic state.

Lastly, we note that oxidation of the ruthenium(II) myoglobin (Mb) did not yield the corresponding π-cation radical, in accord with the fact that Mb affords compound II (King & Winfield, 1963) but not compound I. The same conclusion was obtained by the use of Zn- or Mg-substituted HRP and Mb. This implies that a characteristic heme environmental structure of HRP may serve to stabilize the porphyrin π-cation radical through the heme proximal or distal side, or combination of both. Studies to elucidate the structural origin are in progress in our laboratory.

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