activity of human erythrocyte membrane protein extract. The findings suggest that protein incorporation is facilitated by the preexistence of bilayer disruptions (defect) in the lipid mixture, whereas the activity of the CB binding protein incorporated increases with reduction of lipid fluidity. Both effects are apparently based on the physical properties rather than the chemical preference of the phospholipids.

Registry No. CB, 14930-96-2; POPC, 6753-55-5; DLPE, 55252-82-9; DMPC, 13699-48-4; cholesterol, 57-88-5.

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Chemical Nature of the Porphyrin π Cation Radical in Horseradish Peroxidase Compound I†

R. Rutter, M. Valentine, M. P. Hendrich, L. P. Hager,* and P. G. Debrunner

ABSTRACT: The electron paramagnetic resonance (EPR) and Mössbauer properties of native horseradish peroxidase have been compared with those of a synthetic derivative of the enzyme in which a mesohemin residue replaces the natural iron protoporphyrin IX heme prosthetic group. The oxyferryl π cation radical intermediate, compound I, has been formed from both the native and synthetic enzyme, and the magnetic properties of both intermediates have been examined. The optical absorption characteristics of compound I prepared from mesoheme-substituted horseradish peroxidase are different from those of the compound I prepared from native enzyme [DiNello, R. K., & Dolphin, D. (1981) *J. Biol. Chem. 256*, 6903–6912]. By analogy to model-compound studies, it has been suggested that these optical absorption differences are due to the formation of an A_{2u} and an A_{1u} π cation radical species, respectively. However, the EPR and Mössbauer properties of the native and synthetic enzyme and of their oxidized intermediates are quite similar, if not identical, and the data favor an A_{2u} radical for both compounds I.

Peroxidases and catalases are ferric protoporphyrin IX containing proteins that react with hydroperoxides to produce an oxidized enzyme intermediate referred to as compound I (Keilin & Hartree, 1951; George, 1952, 1953; Chance, 1952). Titration of compound I with ferrocyanide has shown it be 2-equiv oxidized above the native resting enzyme state. The compounds I formed by horseradish peroxidase (HRP)[1] and chloroperoxidase (CPO) have been subjected to a great deal of study in recent years (LaMar & de Ropp, 1980; Schulz et al., 1979; Roberts et al., 1981a,b; Rutter & Hager, 1982). These studies indicate that one of the two oxidation equivalents

† From the Departments of Biochemistry (R.R. and L.P.H.) and Physics (M.V., M.P.H., and P.G.D.), University of Illinois, Urbana, Illinois 61801. *Received December 8, 1982; revised manuscript received April 21, 1983.* Supported by grants from the National Institutes of Health to L.P.H. (GM-07768) and P.G.D. (GM-16406).

[1] Abbreviations: HRP, horseradish peroxidase; M-HRP, mesoheme-substituted horseradish peroxidase; CPO, chloroperoxidase; Cat, catalase; EPR, electron paramagnetic resonance; ENDOR, external nuclear double resonance; EDTA, ethylenediaminetetraacetic acid.

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in compound I is stored as a low-spin ferryl iron ($Fe^{III} \rightarrow Fe^{IV}$) (Moss et al., 1969), while the other oxidation equivalent is stored as a porphyrin-centered π cation radical (Schulz et al., 1979). However, the distribution of the radical spin density on the porphyrin system has been a matter of debate.

Dolphin and co-workers (Dolphin et al., 1971; DiNello & Dolphin, 1979, 1981; Dolphin & Felton, 1974; Felton et al., 1971; Fajer et al., 1970) first proposed a ferryl heme cation radical complex as a model for the primary compounds of HRP and catalase (Cat). They were able to oxidize several synthetic metalloprophyrins to a stable π radical state. Depending on the particular combination of solvent, metalloporphyrin, and counterion, they found two types of model-compound radicals having visible spectra similar to those of either HRP or Cat compounds I. Molecular orbital calculations suggest that the highest filled orbitals of the porphyrin, a_{1u} and a_{2u}, are comparable in energy; thus, DiNello & Dolphin (1981) argued that oxidation could remove an electron from either one of them, depending on conditions. Since the a_{2u} orbital has been shown to have electron density (Loew & Herman, 1980) on the pyrrole nitrogen while the a_{1u} orbital has none, they further argued that HRP compound I type radicals that show nitrogen hyperfine splitting have the unpaired electron in the a_{2u} orbital, whereas Cat compound I type radicals have the unpaired electron in the a_{1u} orbital (Dolphin et al., 1971). A measurement of the magnetic properties of the model compounds, in particular by ENDOR, provided a sensitive test that substantiated these assignments (Dolphin et al., 1971).

The recent analysis of the Mössbauer, EPR, and ENDOR spectra of HRP compound I agrees qualitatively with the model of an A_{2u} porphyrin cation radical if allowance is made for a weak exchange interaction between the radial spin, $S' = {}^1/_2$, and the spin, $S = 1$, of the oxyferryl iron (Schulz et al., 1979; Roberts et al., 1981a,b). So far, however, no A_{1u} radical state has been identified in a heme protein. According to DiNello & Dolphin (1981), a Cat compound I type visible absorption spectrum is the hallmark of this state. In comparison with the A_{2u} radial, the exchange interaction for the A_{1u} state is expected to be weaker, since the a_{1u} orbital is further removed from the iron and has no overlap with any of its orbitals. A Cat compound I type spectrum has been reported for CPO compound I (Palcic et al., 1980), but EPR measurements revealed a broad, unusual signal extending from $g = 2$ to $g = 1.73$ (Rutter & Hager, 1982). The magnetic properties of CPO compound I are compatible with an exchange interaction that is stronger than that in HRP compound I, a result that favors an A_{2u} instead of the predicted A_{1u} radical.

In order to minimize any differences that could arise from comparisons between two different proteins, we now compare HRP compound I with a HRP preparation in which a mesoheme prosthetic group has been substituted for the native iron protoporphyrin IX. The horseradish peroxidase mesoheme compound I (M-HRP) shows a visible absorption spectrum that has characteristics of both the A_{1u} and A_{2u} ground states (DiNello & Dolphin, 1979). This finding argues that some of the M-HRP compound I molecules must have an electron hole in the a_{1u} orbital while others must have an electron hole in the a_{2u} orbital. In this paper, we record physical properties of M-HRP compound I as studied by electron paramagnetic resonance, Mössbauer, and low-temperature visible absorption spectroscopies. In spite of the fact that the visible absorption spectrum of M-HRP compound I is different from that of HRP compound I, we find the EPR and Mössbauer properties to be quite similar if not identical.

Materials and Methods

Crude HRP was obtained from Sigma Chemical Co. and purified to an R_z (absorbance at 403 nm/absorbance at 278 nm) value of 3.4 by the method of Shannon et al. (1966). Apo-HRP was prepared by the Teale (1959) procedure as modified by Tamura et al. (1972). Apo-HRP was reconstituted with a 1.2–1 molar excess of mesohemin. Reconstituted M-HRP was further purified by chromatography on a 3 cm by 7 cm column of CM-52 cellulose. The column was eluted with a linear gradient prepared by mixing 1.0 L of 0.005 M sodium acetate, pH 4.4, and 1.0 L of 0.1 M sodium acetate, pH 4.4. The purified M-HRP was then dialyzed against two 1000-fold volumes of high-purity distilled water. Ordinary laboratory deionized water was distilled, filtered through charcoal and Millipore filters, distilled from potassium permanganate, and then distilled a final time.

Compound I was prepared by adding a 1.5–1 molar excess of peracetic acid to the reconstituted enzyme cooled to −20 °C in 40% ethylene glycol. The pH and buffers were adjusted prior to the cooling step to assure that the final proton activity was equivalent to a pH of 6.5 (Maurel et al., 1975).

Iron-57 mesohemin was prepared by the Adler procedure from $^{57}FeCl_2$ and mesoporphyrin (Adler & Kampers, 1970). The mesoporphyrin was purchased from Strem Chemical Co. The purity of the synthesized mesohemin was monitored by the pyridine hemochromogen assay (Falk, 1964).

Peroxide concentrations were determined by allowing a measured aliquot of the peroxide solution to react with excess iodide to form triiodide, which has an absorption coefficient of 2.55×10^4 M^{-1} at 353 nm (Cotton & Dunford, 1973). A trace of HRP was used to catalyze the formation of I_3^- in this assay system.

Copper–EDTA standards were prepared by mixing a 10-fold molar excess of EDTA disodium salt with oven-dried copper chloride. Sodium hydroxide was added to the EDTA–copper complex to form the tetraanion of EDTA.

EPR data were taken on a Bruker ER200 X-band spectrometer equipped with an Oxford helium-flow cryostat and interfaced to an LSI computer system. Mössbauer spectra were accumulated in the constant acceleration mode. The ^{57}Fe-enriched samples were maintained at low temperature in a Janis cryostat. The spectrometer was calibrated periodically with a metallic iron absorber, and all Doppler shifts are referenced to that standard at 300 K. Analysis of the spectra was based on a spin Hamiltonian model. Computer simulations (Münck et al., 1973) were generated and the parameters iteratively adjusted to best match the data.

Visible absorption spectra were recorded in a Cary 14 spectrophotometer modified with a cryostat that permitted spectral measurements at 4.2 K. Samples were placed in 1-mL plastic cuvettes and frozen in liquid nitrogen before being placed in the instrument.

Results

The EPR spectrum of ferric M-HRP shown in Figure 1a strongly resembles that of native HRP; it contains at least two species with different rhombic splittings, and the average of the low-field g values is less than 6, the value expected for a pure spin $S = {}^5/_2$ state. As illustrated in Figure 1b, the spectrum can be simulated reasonably well by two-components, a and b, satisfying the spin Hamiltonian

$$H = D\left[S_{z^2} - \frac{S(S+1)}{3}\right] + E(S_{x^2} - S_{y^2}) + \mu\beta H\cdot\mathbf{g}\cdot S \quad (1)$$

Table I: Parameters Used in EPR Simulation of M-HRP (Figure 1b)

component	percentage	D (K)[a]	E/D	g_x[b]	g_y[b]	g_z[b]	g_x^{eff}[c]	g_y^{eff}[c]	g_z^{eff}[c]
a	57	20	0.0292	1.925	1.925	1.988	5.09	6.43	1.96
b	43	20	0.0286	1.848	1.848	1.976	4.90	6.16	1.95

[a] Assumed value. [b] Refers to the spin $S = {}^5/_2$ representation, eq 1. [c] Refers to the effective spin $S^{eff} = 1$ representation of the lowest Kramers doublet.

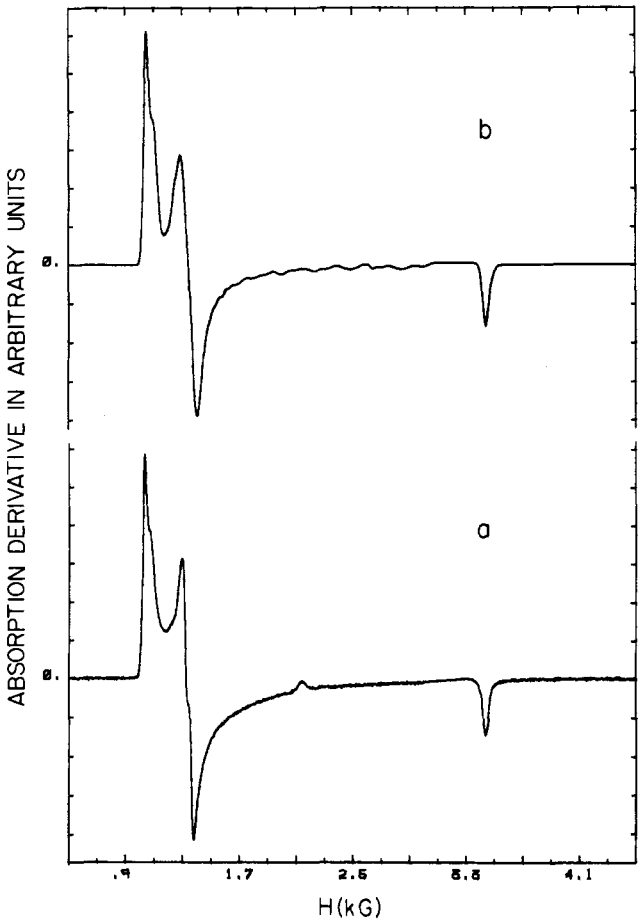

FIGURE 1: EPR spectrum of ferric mesoheme horseradish peroxidase. The lower trace (a) is the experimental derivative at 3 K, frequency 9.43 GHz, microwave power 100 μW, 10-G peak modulation at 100 kHz, sweep rate 8 G/s, and time constant 0.5 s. The upper trace is a simulation based on eq 1 as discussed in the text. Two species a and b are assumed with relative intensities of 57 and 43%, respectively, having the parameters listed in Table I.

with $S = {}^5/_2$ and the parameters listed in Table I. The zero field splitting was taken to be $D = 20$ K as measured for the benzohydroxamic acid complex of horseradish peroxidase, $D = 20.3 \pm 0.6$ K (Colvin et al., 1983). At 3.8 K, only the lowest of the three Kramers doublets described by eq 1 is populated; its effective g values follow from eq 1 with the parameters listed in Table I (see last column, table I). In order to account for the low effective g values deduced from Figure 1, we allow $\mathbf{g}$ in the $S = {}^5/_2$ representation of eq 1 to have axial symmetry with components less than the spin-only value $g_s = 2.0023$. Maltempo & Moss (1976) suggested an admixture of an excited spin quartet to the sextet ground state as an explanation of the unusually low effective g values of peroxidase and other heme proteins. The line shape in Figure 1b is obtained with an intrinsic Gaussian width of 14 and 30 G for components a and b, respectively. Species a has in addition a Gaussian distribution of E/D with standard deviation of 0.0046.

The Mössbauer spectra of M-HRP shown in Figure 2 consist of a six-line pattern that is characteristic of the lowest

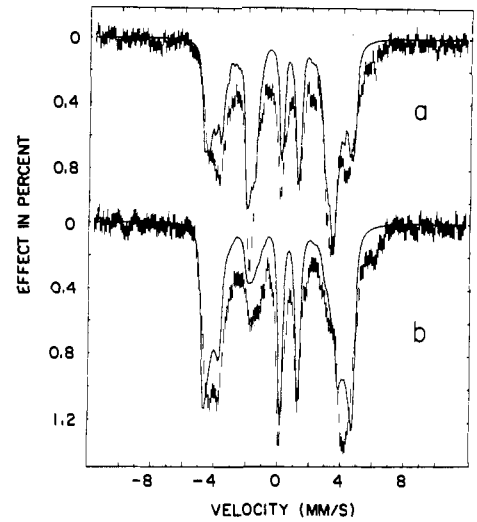

FIGURE 2: Mössbauer spectra of ferric mesoheme horseradish peroxidase at 4.2 K. A field of 320 G is applied either perpendicular (a) or parallel (b) to the direction of the γ-ray beam. The solid lines are simulations based on eq 1 and 2 with the parameters $D = 20$ K, $E/D = 0.035$, $\mathbf{g} = 1.92$, 1.92, and 2, $\mathbf{A}/(g_n\mu_n) = -17.3$, −17.3, and −18 T, quadrupole splitting $\Delta = 1.5$ mm/s, isomer shift $\delta_{Fe} = 0.41$ mm/s, and line width (FWHM) $\Gamma = 0.25$ mm/s.

Kramers doublet of high-spin ferric heme proteins. Even a weak magnetic field strongly affects the shape of the spectrum as a comparison of the traces in Figure 2 indicates. The pattern is similar to, but not as well resolved as, that of the benzohydroxamic acid complex of HRP, a further indication that the sample is inhomogeneous. We nevertheless attempted to simulate the Mössbauer data, assuming a single species. The parameters are adjusted to match the major features of the spectra, but not surprisingly, the resulting simulations fail to reproduce the details, as shown by the solid lines in Figure 2. The calculations are based on the spin Hamiltonian (eq 1), augmented by the magnetic and electric hyperfine interaction $\hat{H}_I$:

$$\hat{H}_I = \hat{S}\cdot\mathbf{A}\cdot\hat{I} + (eQV_{zz}/4)[\hat{I}_z^2 - I(I+1)/3] \qquad (2)$$

Here A represents the magnetic hyperfine interaction and $-V_{zz}$ is the major component of the electric field gradient tensor, which are both assumed to be axial and to be aligned with the zero field splitting. No meaningful improvement is obtained when these constraints are dropped. Although a single species thus cannot reproduce the spectrum adequately, the values of $A_x = A_y$ and the quadrapole splitting $\Delta = eQV_{zz}/2$ can be determined within ±3%.

When ferric M-HRP samples are reacted with a slight stoichiometric excess of hydrogen peroxide or peracetic acid, the ferric M-HRP EPR signals disappear, and a new signal is formed at $g = 1.99$. Figure 3 shows the EPR spectrum of M-HRP compound I on a broad (inset) and a centered scan. The complete absence of a ferric M-HRP EPR signal in the compound I preparation (see Figure 3, inset) demonstrates that the reaction has gone to completion. Double integration of the derivative spectrum (Figure 3) and comparison with the Cu–EDTA standard yield a value of 0.8 ± 0.05 spins per heme

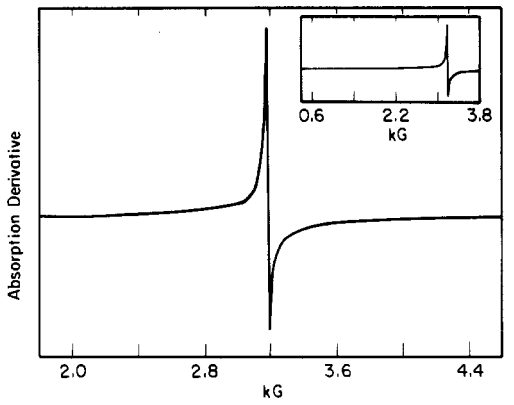

FIGURE 3: EPR spectrum of mesohemin-substituted horseradish peroxidase compound I. The lower trace is the absorption derivative at 3.5 K, microwave frequency 9.41 GHz, microwave power 0.3 mW, 10-G field modulation at 100 kHz, sweep rate 8 G/s, and time constant 0.2 s. The inset is the whole scan of the same sample under identical spectrometer conditions with a 1.5 times lower gain.

group. Visible absorption spectra recorded for diluted aliquots from the same samples demonstrate that no compound II is present.

The EPR signal associated with M-HRP compound I is reminiscent of that reported for HRP compound I. The signal has broad sloping wings on both the high- and low-field sides of the main feature centered at $g = 1.99$.

We adopt the spin-coupling model used earlier in the interpretation of HRP I (Schulz et al., 1979) and add to eq 1 the Hamiltonian $H_{S,S'}$:

$$H_{S,S'} = -S\cdot\mathbf{J}\cdot S' + \mu\beta g S'\cdot H \qquad (3)$$

which represents the coupling of a radical with spin $S' = {}^1/_2$ to the spin $S = 1$ of the ferryl heme iron and to an external field H. The eigenstates of the coupled system then are three Kramers doublets, the lowest of which gives rise to the observed EPR spectrum.

If the zero field splitting parameter D is the dominant term, i.e., $D \gg |J|$, as is the case for HRP compound I, then the broad wings of the EPR spectrum above and below $g = 1.99$ are explained by positive and negative components of J_x and J_y. These conjectures are borne out by the Mössbauer data of M-HRP compound I illustrated in Figure 4. The isomer shift, $\delta_{Fe} = 0.08 \pm 0.03$ mm/s, and the quadrapole splitting, $\Delta = 1.25 \pm 0.02$ mm/s, are typical of the spin $S = 1$ Fe^{IV} state of heme proteins. The fact that a weak magnetic field affects the spectrum at 4.2 K proves, on the other hand, that the ferryl spin is not isolated but rather part of a Kramers doublet resulting from exchange interaction of the iron with a radical. Mössbauer simulations using this model with an isotropic exchange are shown as solid lines in Figure 4. We assume slow spin fluctuation and let J have a Gaussian distribution about the mean $\langle J \rangle = -4$ K with standard deviation $\sigma_J = 1.75$. The simulation matches the data well; thus, the basic model and the approximate magnitude of J are reasonable. To ascertain the uniqueness of the parameters, however, would require systematic measurements as a function of field and temperature coupled with EPR simulations, a task we have not undertaken.

Figure 5 shows the visible absorption spectra of M-HRP compound I recorded at 293 and 4.2 K. At the low temperature, all of the absorption peaks sharpen, and the low-energy band shifts 5 nm to the blue. However, these are all predicted changes, and the spectra are very similar at both temperatures. This indicates that M-HRP compound I has the same electronic configuration at both room temperature and 4.2 K.

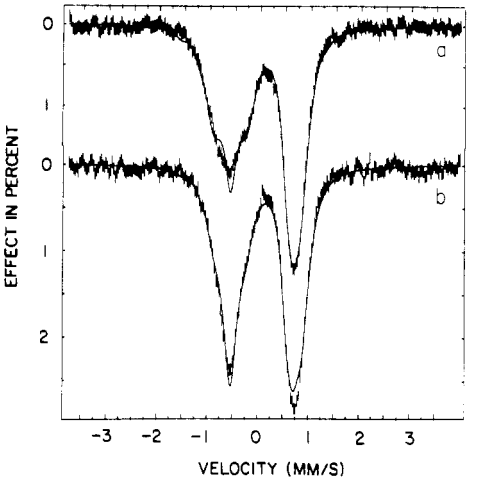

FIGURE 4: Mössbauer spectra of mesoheme horseradish peroxidase compound I at 4.2 K. A field of 320 G is applied either parallel (a) or perpendicular (b) to the γ-ray beam. The solid lines are simulations based on eq 1–3 with $S = 1$, $S' = {}^1/_2$, $D = 36$ K, $E/D = 0$, $\mathbf{g} = 2.25$, 2.25, and 1.98, $\mathbf{A}/(g_n\mu_n) = -19.3$, -19.3, and -6 T, $J = -4$ K, $\sigma_J = 1.75$, $g' = 2$, quadrapole splitting $\Delta = 1.25$ mm/s, isomer shift $\delta_{Fe} = 0.08$ mm/s, and line width (FWHM) $\Gamma = 0.25$ mm/s.

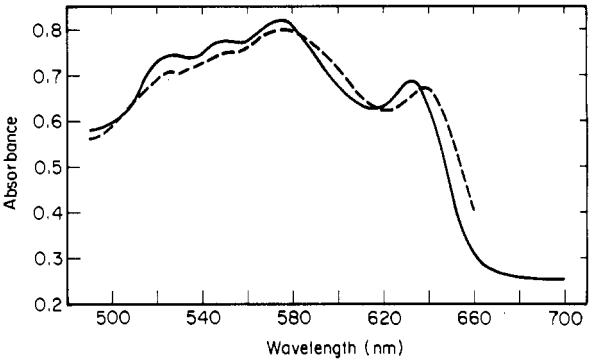

FIGURE 5: Visible absorption spectra of mesoheme horseradish peroxidase compound I at 4.2 and 293 K. The compound I samples were prepared by adding a 2-fold molar excess of peracetic acid to 50 μM M-HRP in 65% ethylene glycol and 0.05 M potassium phosphate buffer, pH 7.5. The spectra were recorded at 392 (– –) and 4.2 K (—). The room-temperature spectrum of M-HRP compound I in aqueous buffer was identical with the spectrum of this intermediate dissolved in 65% ethylene glycol and 0.5 M potassium phosphate buffer.

Discussion

A unique common feature of the primary compounds of peroxidases and catalases is their formal oxidation state of +5, i.e., 2 oxidation equiv above the resting, ferric state of the enzymes. In spite of this commonality, the optical absorption properties of the various compounds I show quite different features (see Figure 6). For example, the compounds I of catalase and CPO show a strong absorption band around 680 nm whereas HRP compound I does not absorb in this region. On the other hand, all of the Mössbauer measurements on the compounds I of various peroxidases indicate 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 (positive) quadrapole splitting of $\Delta = 1.0$–1.6 mm/s (Moss et al., 1969; Maeda, 1968; Lang et al., 1976). Furthermore, reactions carried out with ^{18}O- and ^{17}O-labeled peroxides established a single oxygen ligand in the primary compounds of chloroperoxidase (Hager et al., 1972) and horseradish peroxidase (Roberts et al., 1981). Thus, an oxyferryl center appears to be a common feature for all compounds I. These considerations lead to the conclusion that the differences that exist among peroxidases must be associated with the locali-

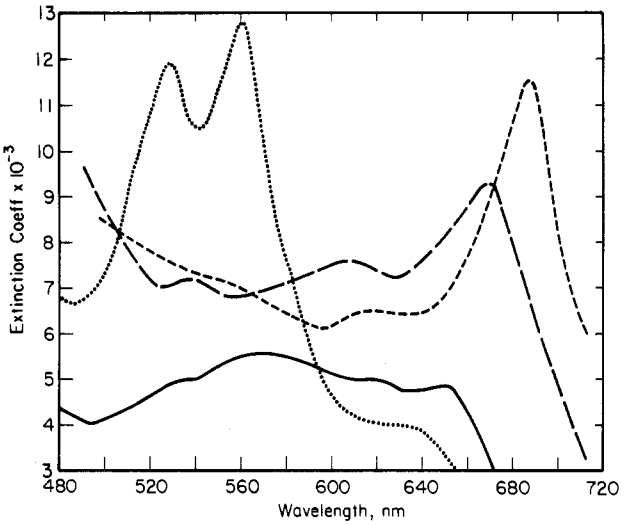

FIGURE 6: Compound I absorption spectra. The visible absorption spectra of various peroxidase compound I preparations are recorded: cytochrome *c* peroxidase (Yonetoni, 1965) (···); horseradish peroxidase (—); bacterial catalase (DiNello & Dolphin, 1981) (– –); chloroperoxidase (Palcic et al., 1980) (– · –). The compound I sample of horseradish peroxidase was prepared according to the procedure given under Methods and Materials.

zation of the second oxidizing equivalent. In compound ES of cytochrome *c* peroxidase (see Figure 6), an intermediate that is formally equivalent to the primary compounds of HRP and chloroperoxidase, magnetic resonance experiments have established the existence of a stable free radical on an amino acid residue that is sufficiently far removed from the heme to show minimal, if any, interaction with the spin $S = 1$ of the Fe^{IV} (Lang et al., 1976; Hoffman et al., 1979). On the other hand, in the primary compounds of HRP and CPO, the same spectroscopic tools indicate the presence of a porphyrin radical, which couples magnetically to the ferryl spin. Thus, the suggestion of a porphyrin π cation radical (Felton et al., 1971) is borne out for HRP compound I, for the compound I of its mesoheme analogue, and for CPO compound I (Rutter & Hager, 1982). The present study was undertaken to elucidate the nature of the spin coupling and the porphyrin radical by a comparison of the magnetic properties of HRP compound I and M-HRP compound I. DiNello & Dolphin (1981) recently observed that M-HRP compound I has a sizable optical absorption in the 640-nm region, reminiscent of the optical properties of the primary compound of catalase. By analogy to model-compound absorption spectra, they suggested that the M-HRP compound I spectrum is indicative of an A_{1u} porphyrin radical. Protoheme HRP compound I, in contrast, is placed in the A_{2u} radical class by DiNello & Dolphin, an assignment that is plausible as we will argue below. Dolphin's correlations between visible spectra and porphyrin radical symmetry are based on the optical and EPR properties of solutions of diamagnetic, highly symmetric metalloporphyrin radicals (Dolphin & Felton, 1974; Felton et al., 1971; DiNello & Dolphin, 1979; Dolphin et al., 1971). The validity of an extrapolation from these models to heme proteins remains to be demonstrated by experiment. There is reason to expect a weakening of the correlation between radical symmetry (A_{2u} vs. A_{1u}) and spectral type (HRP compound I vs. Cat compound I) in proteins as compared to those in model systems, possibly even a complete breakdown. The protoporphyrin IX found in the proteins does not have the full D_{4h} symmetry that the model compounds may have, and the open shell electrons of the Fe^{IV}, as well as the axial ligands, may profoundly perturb the electronic state of the porphyrins. Only experiments can tell whether an A_{1u} or A_{2u} orbital is still a good approximation of the porphyrin radical in the presence of the various perturbations. The EPR and Mössbauer studies discussed here focus on the magnetic interaction between the porphyrin radical and the Fe^{IV} and are (i) concerned with small energy splittings and are (ii) very sensitive to deviations from D_{4h} symmetry. Optical transitions, in contrast, involve large energies and have selection rules that pick out certain symmetries. Care must therefore be taken in the comparison of results obtained by optical and resonance spectroscopies.

We first discuss the resting state of M-HRP, which illustrates the sensitivity of EPR and Mössbauer spectra on the protein-imposed low symmetry. The similarity of the HRP and M-HRP data given under Results suggests that the mesoheme-substituted protein retains its native structure. A comparison with high-spin ferric metmyoglobin, which like HRP has a histidine axial ligand, points to a number of unusual features of the peroxidase. Both HRP and M-HRP show multiple species in frozen solution, resolved rhombic splitting, large zero field and quandrapole splitting, and a quartet admixture to the sextet ground state. The last four points also apply to cytochrome *c* peroxidase (Lang et al., 1969).

Spin admixture has been invoked to explain effective $g_\perp$ values that are, on average, smaller than 6 (Maltempo & Moss, 1976). A heme model with quartet ground state has been characterized by Reed et al., (1979). This perchlorato $Fe^{III}TPP$ has a ruffled porphyrin ring with approximate S_4 symmetry; the iron is 0.3 Å out of the porphyrin plane. Strong Fe–N pyrrole bonds are supposed to raise the antibonding $d_{x^2-y^2}$ orbital to an energy where it is no longer occupied. By analogy, one would expect the quartet state in HRP to be lowered because of relatively strong $d_{x^2-y^2}$ and weak d_z bonds.

We next turn to the primary compound, M-HRP compound I, and note that our data show very little difference from those of the regular HRP compound I in spite of the different optical absorption. As expected for a Kramers doublet, it has an EPR signal that accounts, within experimental uncertainty, for one effective spin $S' = {}^1/_2$ per heme. The signal can be observed at low temperature only; it is remarkable for its width, roughly 400 G between half-amplitude points of the integrated absorption derivative, in contrast to typical radical signals, in particular also that of compound ES of cytochrome *c* peroxidase. Schulz et al. (1979) explained the broadening in HRP compound I as a result of spin coupling between the radical, $S' = {}^1/_2$ and the spin $S = 1$ of the Fe^{IV}, expressed by the exchange interaction in eq 3. Several lines of evidence support this model. (i) The low-temperature Mössbauer spectra of HRP compound I, as well as of M-HRP compound I (see Figure 4), show magnetic hyperfine interaction typical of a Kramers half-integer spin system. Since Fe^{IV} necessarily has integer spin, the Kramers nature of the system implies the presence of a half-integer spin, the putative porphyrin radical. (ii) ENDOR measurements on HRP compound I show several nitrogen and proton resonances that must arise from a porphyrin radical, since no other known radical shows the same pattern of resonances (Roberts et al., 1981a,b). The fact that the same ENDOR resonances are observed at different *g* values of the broad EPR signal is evidence that the whole inhomogeneously broadened line is due to the porphyrin radical. Moreover, a comparison of the ENDOR frequencies with spin densities calculated for an HRP compound I model with an A_{2u} or A_{1u} porphyrin radical clearly favors the first assignment. (iii) EPR saturation-recovery studies on HRP compound I show that the longitudinal relaxation rate $1/T_1$ is dominated by an Orbach process to an excited level at 37

± 2.5 K (Colvin et al., 1983). This level can be identified with the $S_z = \pm 1$ excited state of the Fe^{IV}, which was estimated independently from Mössbauer measurements to be at ~32 K. Mössbauer, EPR, and ENDOR data on HRP compound I thus support the model of a spin-coupled complex consisting of the spin $S = 1$ Fe^{IV} and a porphyrin radical, most likely of A_{2u} symmetry. More specifically, the zero field splitting, $D = 37$ K, of the Fe^{IV} was found to be an order of magnitude larger than the exchange coupling, $|J| \sim 4$ K. It should be noted, however, that the size of the phenomenologically introduced exchange interaction **J** and the model used to reproduce the EPR line shape (Schulz et al., 1979) deserve further explanation. Given approximate wave functions of the Fe^{IV} and the porphyrin radical, it should be possible to calculate **J** and, consequently, the shape of the EPR spectrum. Such a calculation has not been carried out yet, and we therefore can give only qualitative arguments. It is easy to estimate the anisotropic spin dipolar part of $-\hat{S}\cdot\mathbf{J}\cdot\hat{S}_1'$, which is proportional to r^{-3}, the inverse cube of the distance r between the spins, but it is very difficult to estimate the typically dominant exchange mediated by the overlap of metal and ligand orbitals. The reason is that the A_{2u} radical function has no overlap with the magnetic orbitals d_{xz} and d_{yz} of the iron but only with the weakly populated $4p_z$ orbital. Admixtures of other porphyrin functions appropriate to lower than D_{4h} symmetry, and spin polarization by the $4p_z$ orbital may in fact provide the major contribution to J. The $4p_z$ population is expected to depend sensitively on the geometry of the heme group, in particular on the distances of the oxygen and nitrogen axial ligands from Fe^{IV}. None of these parameters are known, but we may attempt to estimate the $4p_z$ spin density from the hyperfine interactions of the axial ligands, as measured by ENDOR, assuming that all other contributions are negligible (Roberts et al., 1981a,b). In any case, the relatively small size of J is plausibly explained by the lack of overlap between the magnetic orbitals of the Fe^{IV} and of the porphyrin radical.

Our EPR Mössbauer results on M-HRP compound I indicate that the same spin-coupling model applies as in HRP compound I. The exchange interaction, specifically, must be at least as large, $|J| \sim 4$K, as in HRP compound I. An A_{1u} porphyrin radical is therefore quite unlikely since its spin density, localized on the pyrrole carbons, has no overlap with any of the metal orbitals and yields a dipolar coupling to the iron that is at least a factor of 2 smaller than for the A_{2u} radical. We have no explanation for the resemblance of the optical spectra of M-HRP compound I and Cat compound I but see no reason that a Cat compound I type spectrum implies an A_{1u} radical. In fact, CPO compound I, the only peroxidase compound I with a Cat compound I type spectrum, for which magnetic data are known, argues against an A_{1u} radical. EPR and Mössbauer studies on CPO compound I suggest that J is roughly equal to the zero field splitting D of the Fe^{IV} spin triplet. Although neither J nor D have been measured directly yet, D is expected to be ~35K, in analogy with the values found in HRP compound I and other Fe^{IV} heme complexes. Thus the exchange J in CPO compound I appears to be an order of magnitude larger than that in HRP compound I. If we assume that the porphyrin radical either has A_{1u} or A_{2u} character, then the magnetic properties of CPO compound I definitely favor the latter.

Registry No. Peroxidase, 9003-99-0; mesohemin, 21007-37-4.

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