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.

†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).

‡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.
in compound I is stored as a low-spin ferryl iron (Fe$^{III}$ → 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 metalloporphyrins 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, $\alpha_{1u}$ and $\beta_{2v}$, 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 $\alpha_{2v}$ orbital has been shown to have electron density (Loew & Herman, 1980) on the pyrrole nitrogen while the $\alpha_{1u}$ orbital has none, they further argued that HRP compound I type radicals that show nitrogen hyperfine splitting have the unpaired electron in the $\alpha_{2v}$ orbital, whereas Cat compound I type radicals have the unpaired electron in the $\alpha_{1u}$ 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 $\alpha_{2v}$ 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 $\alpha_{1u}$ 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 $\alpha_{2v}$ radial, the exchange interaction for the $\alpha_{1u}$ state is expected to be weaker, since the $\alpha_{1u}$ 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 spectroscopies. In spite of the fact that the visible absorption resonances, Mössbauer, and low-temperature visible absorption comparisons between two different proteins, we now compare the properties of CPO compound I are compatible with an exchange interaction for the $\alpha_{1u}$ orbital. A Cat compound I type spectrum has been shown to have electron density (Loew & Herman, 1980) on the pyrrole nitrogen while the $\alpha_{1u}$ orbital has been shown to have electron density (Loew & Herman, 1980) on the pyrrole nitrogen while the $\alpha_{1u}$ orbital is different from that of the $\alpha_{2v}$ orbital. In this paper, we record physical properties of the M-HRP compound I molecules must have an electron stored as a porphyrin-centered π radical state. As illustrated in Figure 1b, the spectrum can be simulated reasonably well by two-components, a and b, satisfying the spin Hamiltonian

$$H = D \left[ S_x^2 - \frac{S(S + 1)}{3} \right] + E(S_x^2 - S_y^2) + \mu B g \cdot S$$  \hspace{1cm} (1)
Table I: Parameters Used in EPR Simulation of M-HRP (Figure 1b)

<table>
<thead>
<tr>
<th>component</th>
<th>percentage</th>
<th>$D$ (K)</th>
<th>$E/D$</th>
<th>$g_x^b$</th>
<th>$g_y^b$</th>
<th>$g_z^b$</th>
<th>$g_x^{eff}$</th>
<th>$g_y^{eff}$</th>
<th>$g_z^{eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>57</td>
<td>20</td>
<td>0.0292</td>
<td>1.925</td>
<td>1.925</td>
<td>1.988</td>
<td>5.09</td>
<td>6.43</td>
<td>1.96</td>
</tr>
<tr>
<td>b</td>
<td>43</td>
<td>20</td>
<td>0.0286</td>
<td>1.848</td>
<td>1.848</td>
<td>1.976</td>
<td>4.90</td>
<td>6.16</td>
<td>1.95</td>
</tr>
</tbody>
</table>

*a* Assumed value.  
*b* Refers to the spin $S = 3/2$ representation, eq 1.  
*c* Refers to the effective spin $S^{eff} = 1$ representation of the lowest Kramers doublet.

![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.](image)

with $S = 3/2$ and the parameters listed in Table I. The zero field splitting was taken to be $D = 20$ K as measured for the benzoxydihydroxamic 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 $g$ in the $S = 3/2$ 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

![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, $E/D = 0.035$, $g = 1.92, 1.92$, and 2, $A/(g_\mu_B) = -17.3, -17.3$, and $-18$ T, quadrupole splitting $\Delta = 1.5$ mm/s, isomer shift $\delta = 0.41$ mm/s, and line width (FWHM) $\Gamma = 0.25$ mm/s.](image)

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 benzoxydihydroxamic 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 $\hat{H}_H$:

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

(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 $A_z = A_z$ and the quadrupole 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
are explained by positive and negative components of 
M-HRP compound I illustrated in Figure 4. The isomer 
compound I recorded at 293 and 4.2 K. At the low temper-

ature, 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 elec-

tronic configuration at both room temperature and 4.2 K.

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 in-
terpretation of HRP I (Schulz et al., 1979) and add to eq 1 
the Hamiltonian \( H_{SS} \):

\[
H_{SS} = -S \cdot J \cdot S' + \mu g S \cdot H
\]

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, \( \delta_{pp} = 0.08 \pm 0.03 \) mm/s, and the quadrupole splitting, 
\( \Delta = 1.25 \pm 0.02 \) mm/s, are typical of the spin \( S = 1 \) FeIII 
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 temper-
ature coupled with EPR simulations, a task we have not un-
dertaken.

Figure 5 shows the visible absorption spectra of M-HRP 
compound I recorded at 293 and 4.2 K. At the low temper-

ature, 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 elec-

tronic configuration at both room temperature and 4.2 K.
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 of the porphyrin radical. 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. Protomeme HRP compound I, in contrast, is placed in the A_{2g} 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_{2g} 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^{III}, 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_{2g} 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^{III} 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 quadrapole 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_{||} 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}TTP 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^2} 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^{III}, 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^{III} 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_{2g} 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

![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 (Palic et al., 1980) (---). The compound I sample of horseradish peroxidase was prepared according to the procedure given under Methods and Materials.](image-url)


