Proton Nuclear Magnetic Resonance Investigation of the Electronic Structure of Compound I of Horseradish Peroxidase*

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High field proton nuclear magnetic resonance spectroscopy was used to investigate the electronic structure of compound I of horseradish peroxidase. In view of the recent characterization of an electron spin resonance signal arising from a free radical weakly coupled to the paramagnetic iron center, we emphasize the interpretation of the NMR data in terms of the alternative location of the free radical on the porphyrin or on an amino acid side chain near the heme.

Detailed characterization of the pattern of hyperfine shifts for the heme resonances was based on the reconstitution of horseradish peroxidase with both hemin and deuterohemin specifically labeled with deuterium at selected positions. A hyperfine shifted exchangeable resonance attributable to the proximal histidyl imidazole was not observed and all resolved peaks exhibiting significant hyperfine shifts (>5 ppm) were found consistent with arising solely from the heme. Two lines of evidence directly support the location of the free radical on the heme rather than on an amino acid side chain. All previously characterized oxidation/spin states of iron porphyrins yield unique hyperfine shift patterns for both model complexes and hemoproteins, none of which resemble the pattern found for compound I; this argues both for the location of the second oxidizing equivalent on the prosthetic group and against it being centered on the iron. Moreover, the observed shift pattern is consistent with radial spin density primarily on the porphyrin. Although the heme methyl signals in compound I exhibit differential paramagnetic line broadening which could be interpreted in terms of regio-selective relaxation of the heme by an amino acid-centered free radical near the heme, individual assignment of the methyls, and the 2,4-substituent resonances demonstrates that this differential line broadening is not only clearly inconsistent with arising from a second, protein-centered free radical, but that the relaxation behavior is completely consistent with iron-porphyrin-centered paramagnetism. The fact that the relaxation behavior of compounds I of both native and deuterohemin-reconstituted horseradish peroxidase could be interpreted by the same model argues for identical electronic structure for both oxidizing equivalents in these two proteins. The observation of two partial proton resonances corresponding to a single vinyl position is taken as evidence for two nonequivalent, rotationally locked orientations arising from steric heme-protein interactions.

In spite of considerable effort directed toward the understanding of the active forms of horseradish peroxidase, neither the location of all oxidizing equivalents nor the state of ligation of the iron are known with any certainty (1). There is less disagreement at this time as to the nature of the red compound II of horseradish peroxidase, one oxidizing equivalent above the resting enzyme, which is thought to contain low-spin iron(IV) (2-4) with probable axial histidyl imidazole (5-8) and oxy anion (9, 10) (i.e., Fe(IV)=O), as fifth and sixth ligands. A model compound for this species has recently been characterized (11).

The electronic structure of the green primary compound I, HRP-I, two oxidizing equivalents above the resting enzyme, is less well understood (1, 2-4, 9, 10, 12-15). Proposals vary from simple iron(V) (12), meso-attack of iron(IV) (13), to iron(IV) with a free radical either on an amino acid side chain or the porphyrin itself (14). The absence of the characteristic isosorbyrin band (15) in the infrared has argued against the second model (16). Susceptibility data (2) are indicative of S = 3/2, and Mössbauer data (3, 4, 17) dictate low spin iron(IV), S = 1, for both HRP-I and HRP-II, indicating that the second oxidizing equivalent in HRP-I resides in a free radical. The recent characterization of the ESR spectrum of this free radical (17, 18), considerably broadened by interaction with the iron center, appears to leave only the exact location of the radical center in question. The similarity of the optical spectrum of HRP-I to those of metalloporphyrin complexes possessing singly oxidized porphyrin ligands has led to the most widely accepted proposal of an iron(IV)/porphyrin-cation radical formulation for HRP-I (16). More recently, the proton NMR hyperfine shifted resonances of HRP-I, presumed to arise from the heme, have been interpreted (19-23) in terms of a simple high spin, S = 2, iron(IV) porphyrin which excludes the porphyrin cation radical and instead requires the presence of an amino acid radical near the heme. The presence of an amino acid radical for the second oxidizing equivalent has been confirmed by both ESR and electron-nuclear double resonance for compound I of cytochrome c peroxidase (22, 23).

Proton NMR hyperfine shifts can, in principle, provide a wealth of detailed electronic and structural information about the paramagnetic center(s) in not only HRP-I, but hemoproteins in various oxidation/spin states in general (24-26).
tensive proton NMR studies of model porphyrin complexes in all possible oxidation/spin states of iron have shown that, while the hyperfine shift patterns are essentially independent of the axial ligand(s) giving rise to a given oxidation/spin state, the hyperfine shift patterns differ characteristically so that the shifts can be used to determine the most likely state of an uncharacterized complex (11, 25-33). In most cases, the hyperfine shift pattern can be rationalized on the basis of the spin-containing d orbitals on the iron and the relevant iron-porphyrin bonding (26). Moreover, although iron porphyrins in proteins exhibit much larger in-plane asymmetry or spreads in hyperfine shifts for the nonequivalent pyrroles, the average hyperfine shifts for given functional groups (i.e., 1,3,5,8-CH₃, 2,4-H, γβγδ₅-meso-H) (Fig. 1) are the same in models and proteins in the same oxidation/spin states (25, 34-38). Although proton NMR spectra of models for HRP-1 have not yet been reported, comparison of the assigned shifts for HRP-I with those of the known states (24-38) will provide a means of assessing the likelihood that any simple iron-centered oxidation/reduction state can account for the electronic structure of HRP-I.

The utilization of the ligand hyperfine shift pattern to its maximum value and minimum ambiguity, however, depends critically on first examining all NMR probes needed to characterize an oxidation/spin state (25, 26), and secondly, on the completely unambiguous assignment of the signals for each of the functional groups on the porphyrin (34, 35, 37, 38). The functional groups found to be essentially diagnostic of oxidation/spin states are primarily the methyls (1,3,5,8-CH₃) and protons (2,4-H) appended directly to the porphyrin skeleton (Fig. 1), and to a lesser degree, the H₃ and H₅'s of the 2,4-vinyls (25, 26). Thus, the proton NMR spectra of both protohemin (Fig. 1, R₁ = R₂ = vinyl) and deuterohemin (Fig. 1, R₁ = R₂ = H)-reconstituted horseradish peroxidase must be investigated (41). The proposal (19-21) for the high spin iron(IV)/amino acid radical was based on the hyperfine shift of a single functional group, which is usually insufficient for an unambiguous analysis. The unambiguous assignment of the resonances for these important functional groups requires the use of deuterium isotope labels in both protohemin and deuterohemin. The relaxation behavior of assigned hyperfine-shifted resonances in HRP-I may also shed some direct light on the possible presence of an amino acid radical. Such a radical would be expected to cause substantial dipolar broadening (42) of the heme resonances, and the degree of regioselectivity in this broadening could be analyzed in terms of the orientation of the free radical relative to the heme. The assignment of the peaks of the vinyl and propionic side chains may also yield information on the degree of oscillatory immobilization of these groups, which can be related to the nature of the heme-apporprotein contacts (37, 38, 43).

We have recently carried out detailed proton NMR analyses of the resting state of native and reconstituted horseradish peroxidase, reaching the conclusions that: the spin states contain a large admixture of S = 3/2 and S = 5/2 in the resting state (38); the heme orientation in HRP and deuterohorperoxidase differ by a 180° rotation about the a-y-meso axis (40, 44, 45); the exchangeable proton of the proximal histidyl imidazole exhibits orders of magnitude-reduced lability relative to myoglobin (8); and the propionic acid and vinyl side chains exhibit considerably reduced oscillatory mobility compared to aquo-met-myoglobin (37, 38) which is indicative of a much more constrained heme pocket in horseradish peroxidase. Preliminary studies on deuterohorperoxidase have already shown (41) that the tentative assignments of the 1,3,5,8-CH₃'s and 2,4-H's argue against a high spin iron(IV). Herein we present the detailed assignment of all resonances and interpret them in the light of the possible origins of the second oxidizing equivalents in compound I.

![Fig. 1. Structure of the heme skeleton depicting conventional numbering system. R₁ = R₂ = vinyl, protohemin; R₁ = R₂ = H, deuterohemin; R₁ = H, R₂ = vinyl, pentahemin; R₁ = vinyl, R₂ = H, isopentahemin.](image-url)
NMR of Horseradish Peroxidase Compound I

EXPERIMENTAL PROCEDURES

Horseradish peroxidase, type VI, was purchased from Sigma as a lyophilized salt-free powder; the protein is predominantly isozyme C. The detailed purification (46), activity assay (47), and electrophoretic behavior of the samples used in this study have already been published (38). The preparation of apo-horseradish peroxidase (48) and the reconstitution with deuterohemin to yield deuteroHRP (49, 50), as well as with a series of isotopically labeled protohemin and deuterohemins (27, 29, 37), have also been described earlier (38). The positions of deuteration are indicated by the square-bracketed prefix to the protein using the standard numbering scheme depicted in Fig. 1. Compounds I were generated by the addition of excess HzO2 as described elsewhere (51).

Optical spectra of HRP-I and deuteroHRP-I at 22°C in 0.1 M phosphate buffer, pH 7.0, are identical with those reported earlier (49, 51).

Solutions for proton NMR studies were 1 to 3 mM in protein in either 0.2 M NaCl, 99.8% H2O, or 90% H2O/10% D2O. The solution pH, adjusted using 0.2 M HCl or 0.2 M NaOH, was measured with a Beckman 3550 pH meter equipped with an Ingold microcombination electrode. The pH was not corrected for the isotope effect and is hence referred to as "pH."

The 360 MHz proton NMR spectra were recorded on a Nicolet NT-360 Fourier transform NMR spectrometer operating with quadrature detection. Typical spectra consisted of 5,000 to 25,000 pulses using 8,000 data points over an 80,000 Hz bandwidth (9 μs 90° pulse). The number of transients accumulated was limited by the reemergence of peaks of the resting form of the enzyme in the NMR spectrum of compound I. Spectra for native and protohemin-reconstituted HRP-I were obtained from 5-35°C. The residual water peak was suppressed by a 25-ms presaturation pulse; signal-to-noise was improved by exponential apodization which introduced 10 to 20 Hz line broadening. Peak shifts were referenced to the residual water line which in turn was calibrated against internal DDS. Chemical shifts are reported in parts per million referenced to DSS with downfield shifts positive; line widths are given in Hertz.

RESULTS

The hyperfine-shifted region of the 360 MHz proton NMR spectrum of HRP-I in "pH" 7.5 for resonance in Fig. 2 (vinyl-H, presumably 2-vinyl), as a function of temperature. The sum of the two resonances is equal to one proton.

are reported in parts per million referenced to DSS with downfield shifts positive; line widths are given in Hertz.

Fig. 3. Curie plots (observed shift versus reciprocal temperature) for the resolved resonances of HRP-I. O, 1,3,5,8-CH3s; Δ, 2,4-H; □, (presumed) 6,7-H/s; ▲, 2,4-H/s; V, downfield single proton peaks; deuteroHRP-I, O, 1,3,5,8-CH3s.

Fig. 4. Hyperfine-shifted regions of the 360 MHz proton NMR spectrum of deuteroHRP-I. Compound in 0.2 M NaCl 'H2O, 5°C, "pH" 7.5. A, deuteroHRP-I; B, [1,3-(C'H3)2]deuteroHRP-I; C, [1,5-(C2H3)2]deuteroHRP-I; D, [2,4-'H2]deuteroHRP-I. Peaks with reduced intensity due to deuteration are indicated by arrows; r, peaks for the resting enzyme.

Fig. 5. Expanded portion of the 360 MHz proton NMR spectrum of HRP-I. Compound in 0.2 M NaCl 'H2O, "pH" 7.5 for resonance in Fig. 2 (vinyl-H, presumably 2-vinyl), as a function of temperature. The sum of the two resonances is equal to one proton.
and the significantly improved signal-to-noise. Comparison of the trace in Fig. 2A with one of HRP-I in 90% H2O (not shown) reveals no new peaks for the H2O solution, indicating that there are no resolved hyperfine-shifted exchangeable resonances in the region 140 to 10 and 0 to -150 ppm from DSS. Reconstitution of HRP-I with [1,3-(C'H2)]- (Fig. 2B); [1,5-(C'H2)]- (Fig. 2C); [2,4-(α-C'H)]; (Fig. 2D); [2,4-(β-C'H)]; (Fig. 2E); and [α,β,γ,δ-meso-H]-protohemin (Fig. 2F) provide direct assignments of the individual methyls, a to d, the two vinyl H's, e and f, and the four vinyl H's, r to u.

Four single-proton signals with significant hyperfine shifts (g to j) are found in the region 20 to 40 ppm. The graph of observed shift versus reciprocal temperature is illustrated in Fig. 3. Three additional downfield peaks outside the diamagnetic region are k to m (not shown), which display only weak temperature dependence indicative of only small (<3 ppm) hyperfine contributions to the observed shifts. Uptfield peaks (not shown) exhibiting very weak temperature dependence and of indeterminate intensity, n to q, are also observed.

The 360 MHz proton NMR spectrum of deuteroHRP-I at 5°C is reproduced in Fig. 4A. The significantly reduced stability of compound I for the reconstituted protein has been noted previously (51), and leads to considerably larger peaks from the reemerging resting enzyme (38). The proton NMR traces of deuteroHRP-I reconstituted with [1,3-(C'H2)]- (Fig. 4B); [1,5-(C'H2)]- (Fig. 4C); and [2,4-(C'H)]-deutohemin, (Fig. 4D) assign the individual methyls, a to d, and the 2,4-H's, e' and f'. The temperature dependence of the methyl resonances is included in Fig. 3. Four other signals with approximate unit intensity, g to j, are observed, several of which are partially obscured by the peaks from the deuteroHRP. Although peaks comparable to k to m and/or o to q appear to be present, their characterization was not pursued due to marginal resolution and signal-to-noise.

In Fig. 5 we focus on peak f of Fig. 2 which represents a single proton. This peak is seen to be composed of two components of comparable areas but unequal width, best resolved at low temperature; as the temperature is raised, each line narrows and the shift difference decreases. Hence, one of the vinyl groups must exist in two different positions. The shifts, relative to DSS at 25°C in H2O, of resolved resonances are listed in Table I; also included are the line widths for the heme methyls and 2,4-substituent protons.

**Discussion**

**Assignment of Resonances**—Assignment of peaks b, c, and d (Fig. 4) to 3-, 5-, and 1-CH3, respectively, leads at a arising from 8-CH3. We tentatively assign e to 4-vinyl and f to 2-vinyl based on the expectation that the pyrrole exhibiting the larger methyl shift will also yield the larger vinyl shift. The narrower pair of vinyl H's, r and s, must arise from H2 (cis) and the broader, t and u, from H2 (trans) due to the closer proximity of the latter protons to either iron or porphyrin spin density (29, 37). The four single proton peaks, g to j, have line widths consistent with their origin in the propionic acid H's. The remaining peaks exhibiting temperature dependence reflect only very small (<5 ppm) hyperfine shifts. If g to j are due to 6,7-H's, then the presumed axial imidazole can exhibit only minor hyperfine shifts.

For deuteroHRP-I, a, b, and c (Fig. 4) clearly arise from 3-, 1-, and 5-CH3 so that d must be 8-CH3. Peaks e' and f' arise from 2,4-H. The four peaks, g to j, are again consistent with the expected shifts for 6,7-α-CH3's. The order of the shift magnitudes of the methyls differs in HRP-I and deuteroHRP-I in that the relative position of 1-CH3 and 3-CH3 (5-CH3 and 8-CH3) are interchanged. This pairwise interchange of methyl environments can be attributed directly to the fact that the heme orientation in HRP-I and deuteroHRP-I differ by 180° rotation about the α-γ-meso axis (44).

**Origin of the Hyperfine Shifts**—The observed hyperfine shifts arise predominantly from the contact interaction, with...
A paramagnetic state other than those listed in Table 11, as suggested earlier, and hence eliminates high spin iron(IV). Similarly, a simple low spin iron(IV) ferryl complex yields hyperfine shifts an order of magnitude smaller than observed for 2,4-H (11). The observed hyperfine shifts do not allow any differentiation between the two ground states since neither ground state places significant spin density on the peripheral pyrrole carbons. Although the average heme methyl shifts are larger in deuteroHRP-I than HRP-I, this effect could come from direct perturbations on the $a_{ds}$ orbital by the different 2,4-substituents. The adherence to the Curie law of the deuteroHRP-I shifts argues against any low temperature population of the $a_{ds}$ state.

Table II

Typical porphyrin hyperfine shifts of iron porphyrins and hemoproteins.

<table>
<thead>
<tr>
<th>Oxidation state</th>
<th>Spin state</th>
<th>Porphyrin functional group</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Fe(II)</td>
<td>1'</td>
<td>H*</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>H*</td>
<td>31, 36</td>
</tr>
<tr>
<td></td>
<td>1'</td>
<td>H*</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>H*</td>
<td>31, 36</td>
</tr>
<tr>
<td>Fe(III)</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>H*</td>
<td>36</td>
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Compound I

<table>
<thead>
<tr>
<th>Spin state</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>32</td>
</tr>
</tbody>
</table>

* CH$_3$ and H attached to peripheral pyrrole carbons.
* $H_2$, $H_2$ vinyl protons (see Fig. 1).
* Observed only in model compounds; others observed in both models and hemoproteins.

Additional evidence against an amino acid radical is provided by preliminary proton NMR studies of iron porphyrins with axial carbene ligands. The iron-carbene complex has strong resemblances to the ferryl complex, and interpretation of the optical spectra by Mansuy et al. (56) has led to the proposal that these complexes are models for HRP-I. A preliminary proton NMR spectrum of the carbene complex of deuteroporphyrin-dimethyl ester yields downfield methyl hyperfine shifts (45 ppm) and upfield (40 ppm) hyperfine shifts consistent with originating from 2,4-H. This shift pattern is quite different from any listed in Table II but bears a striking resemblance to the pattern for compound I. Although these possible model compounds would confirm that both oxidizing equivalents exist on the iron porphyrin, there is not yet available direct proof that they possess porphyrin cation radical ligands.

Optical spectroscopy has shown that, while HRP-I is indicated to exist in the $a_{ds}$ cation radical ground state (15), deuteroHRP-I, at least at very low temperatures, exhibits a spectrum more consistent with the $a_{ds}$ cation radical ground state, as also found is compound I of catalase (49). Above $0^\circ$C, the optical spectrum of deuteroHRP-I reverted to one also consistent with the $a_{ds}$ ground state (49, 51). The observed hyperfine shifts do not allow any differentiation between the two ground states since neither ground state places significant spin density on the peripheral pyrrole carbons.

The differential line broadening, however, can be completely rationalized in the case of both oxidizing equivalents on the iron porphyrin, i.e. the iron(IV)/$\tau$ cation radical. The line width, $\delta$, due to paramagnetic relaxation, can be written (42):

$$\delta = \delta_0^{-2}(t) + C(A/H)^{-2}(t')$$

(1)

where $D$ and $C$ are constants for a given system, $r$ is the electron-proton distance, $A/H$ is the scalar coupling constant, and concomitantly extensive line broadening.
A/k, the square of the contact shift, \((\Delta H/H)^2\) for the heme methyls of Horseradish Peroxidase I and deuteroHRP-I, respectively. The scalar coupling constant, \(\Delta H/H\), and the larger line widths for that latter complex were thought to provide evidence for different orbital and magnetic properties of HRP-I and deuteroHRP-I, which determine \(D\) and \(C\), and \(K\) in Equation 1 can be assumed to be the same for both complexes. This graph is illustrated in Fig. 6 for both HRP-I and deuteroHRP-I.

Initially, the different field effects of HRP-I and deuteroHRP-I and the larger line widths for that latter complex were thought to provide evidence for different orbital ground states for the porphyrin cation radicals (41). However, not only are the four data points for both compounds consistent with such straight lines, but a single line can be drawn through the data points for both complexes. This indicates that both compounds experience essentially identical dipolar as well as scalar relaxation. Since the magnetic properties of HRP-I and deuteroHRP-I, which determine \(D\), \(C\), and \(K\) in Equation 1, can be assumed to be the same, \(f(\tau)\) and \(f'(\tau)\) must be the same for the two systems (42, 57). Thus, the line width difference is consistent with a steric barrier forcing the vinyl groups into a more or less in-plane configuration, with the broader component arising from the \(H_6\) pointing toward the meso-\(H\), and the narrower component arising from \(H_6\) pointing toward the methyl. The 20 to 25% difference in line widths for the two components is consistent with the two locked forms having approximately 20–30° angles between the vinyl and heme planes.

**Acknowledgments**—We are indebted to N. L. Davis for preparation of the deuterohematin and to L. K. Hanson and J. Fajer for useful discussions and for making available data before publication.

**Note Added in Proof**—Specific deuteration of the 4-vinyl group confirms \(\epsilon\) and \(f\) as arising from 4-vinyl and 2-vinyl, respectively.

**REFERENCES**

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