Deuterium NMR Study of Structural and Dynamic Properties of Horseradish Peroxidase*

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Gerd N. La Mar‡, V. Thanabal, Robert D. Johnson§, Kevin M. Smith, and Daniel W. Parish
From the Department of Chemistry, University of California, Davis, California 95616

High field deuterium NMR spectra have been recorded for various horseradish peroxidase complexes reconstituted with hemins possessing specific 2H labels. The line width of the 2H NMR signals of deuteroheme reconstituted-horseradish peroxidase (HRP) and its cyano complex for the immobilized skeletal 2-2H and 4-2H labels yield the overall protein rotational correlation time (22 ms at 55 °C), which is consistent with expectations based on molecular weight. Meso-2H-labeled 2H-NMR signals just upfield from the diamagnetic protein envelope for HRP, and in the central portion of the protein envelope for the CN- ligated resting state HRP. Meso-2H-labeled mesohemin-reconstituted HRP exhibits a similar signal but shifted further upfield by ~10 ppm. The net upfield 2H hyperfine shifts confirm a five-coordinate structure for resting state HRP. 2H resonances for essentially rotationally immobile vinyl groups were detected in both resting state HRP and CN- ligated resting state HRP. Heme methyl-2H-labeling yields relatively narrow lines (~80 Hz) indicative of effective averaging of the quadrupolar relaxation by rapid methyl rotation. Thus the 2H line width of rapidly rotating methyls in hemoproteins can be used effectively to determine the overall protein tumbling rate. Preliminary 2H experiments in meso-2H-labeled compound I do not support large π spin density at these positions on the porphyrin cation radical, and argue for a a1g rather than a a2g orbital ground state.

Nuclear magnetic resonance spectroscopy has provided a wealth of structural information on the electronic and molecular structure of functional states of horseradish peroxidase (1–11). In many of these studies, advantage has been taken of the fact that this molecule is paramagnetic in all functional states (12, 13) by utilizing diagnostic probes developed for much better characterized model complexes (14–17). Thus the contact shift patterns of ferric high-spin complexes have demonstrated that the direction of the meso-H shift is diagnostic of the coordination number of the iron (14, 16). Studies of well-characterized proteins containing both five- and six-coordinate high-spin iron(III) have confirmed the validity of these probes in proteins (18, 19). In resting state HRP, a variety of spectroscopic probes (20–22) have been used to determine if the sixth site is occupied by a water, as in most metmyoglobins (23). Unfortunately, the meso-H signals are expected to be so broad in HRP so as to be undetectable (5, 14, 15).

The validity of the direction of meso-H contact shifts as indicators for the presence of a sixth ligand on the ferric ion, however, presupposes that the ferric ion is essentially high-spin (14–16, 24). The characterization of the spin state of HRP by 'H NMR has been approached (5) by monitoring the direction and magnitude of the pyrrole-H contact shift in deuterohemin-reconstituted HRP (R = H in Fig. 1), which is known to be upfield in low-spin systems, strongly downfield in high-spin systems, and near the diamagnetic envelope for ferric hemes with appreciable intermediate spin, S = 3/2, character (14, 17, 24). A conclusion in favor of important S = 3/2 character was deduced on the basis of the inability to identify the pyrrole 2,4-H shifts downfield outside the intense diamagnetic envelope by 'H NMR of a sample with the 2,4-position deuterated (5).

While it is now generally accepted that the second oxidizing equivalent in compound I of HRP, HRP-I, resides on the porphyrin as a cation radical, the orbital ground state for the radical is not clear (25–27). The favored a1g orbital for the radical spin exhibits the largest spin density at the four meso positions, but this has not been experimentally tested by the resolution of meso-H hyperfine coupling. The meso-Hs of HRP-I would be expected to exhibit substantial contact shifts for an a1g ground state for HRP-I. Unfortunately, meso-deuteriation did not lead to the loss of any resolved 'H signals (6), and it was concluded that, either the shifts were large and the efficient relaxation broadened the peaks beyond detection, or else the shifts were small so that the signals resonate within the diamagnetic envelope, and hence the a1g orbital ground state is not appropriate.

One approach to clarifying these outstanding questions is to locate the elusive pyrrole-H and meso-H resonances not by 'H NMR area loss due to deuteration (5, 6), but by direct 'H NMR detection of the labeled sites (19, 28). The advantage would be the ability to detect signals even within the diamagnetic envelope; hence, there is no “blind” region such as the diamagnetic envelope in 'H NMR. Moreover, since paramagnetic dipolar relaxation dominates 'H NMR signal line width, the g dependence of line widths dictates that the paramagnetic contribution to line widths (and T1s), is reduced by ~42 for 'H relative to 'H NMR (14, 15, 29). In small molecules, and for rapidly rotating methyls in proteins, these dominant advantages are realized (29). Deutrium, however, has spin I

* The abbreviations used are: HRP, horseradish peroxidase; HRP-I, compound I of horseradish peroxidase; DDS, 2,2-dimethyl-2-sila-pentane-5-sulfonate; HRP-CN, horseradish peroxidase in the CN- ligated resting state.

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‡ To whom correspondence should be addressed.
§ Present address: IBM Almaden Research Ctr., 650 Harry Rd., San Jose, CA 95120.
very severe in large proteins, rapidly leading to undetectable
lines (28). Such relaxation, in the presently applicable slow-
motion limit (and negligible asymmetry) is given by the
equation for the line width, \( \delta \).

\[
\pi \delta = T_2^{-1} = \frac{3}{80} (\epsilon' q Q / h)^2 T_2 \tag{1}
\]

Estimating the protein reorientation time for HRP as \( T_2 \sim \)
20 ns, and using a typical aromatic quadrupolar coupling
constant \( -190 \) kHz (28), the line width is expected to be
over

approximately 20 kHz, and using a typical aromatic quadrupolar coupling
constant \( -190 \) kHz (28), the line width is expected to be
over

by Equation 1, but the nature of the motion must be known
from immobile protons, oscillatory mobility for amino acid or
heme side chains, or internal rotation of heme methyls. Any
internal mobility reduces the line width below that described
by Equation 1, but the nature of the motion must be known
to extract dynamic information (31). For rapid rotation about
a single bond at an angle \( \theta \) at a rate \( \tau_\theta^{-1} \gg \tau_2^{-1} \), Equation 1
transforms to:

\[
\pi \delta = T_2^{-1} = \frac{3}{80} (\epsilon' q Q / h)^2 \cos^2\theta \tau_\theta \tag{2}
\]

which for a methyl group \( \theta = 54.4^\circ \) leads to a reduction of
line width by a factor one-ninth of that expected in the
absence of internal motion (28).

We present herein the results of a \(^1\)H study of deuterium-
labeled resting state (HRP), labeled resting state (HRP-CN),
and compound I (HRP-I), of horseradish peroxidase, aimed
at assessing the utility of such detection in large enzymes as
well as providing some answer on the nature of the iron spin
state and ligation state of the resting state, and the nature of
the porphyrin radical in HRP-I. Line width data will be
analyzed for dynamic information on HRP and HRP-CN. The
\(^1\)H NMR spectra for all three states have been reported (1-6,
9, 32). In order to introduce the needed \(^1\)H probe into the
protein environment, we make use of the same isotope-labeled
hemins used previously for \(^1\)H NMR studies (5,6, 32). More-
over, we make use of HRP complexes prepared by reconstitut-
ing the apoprotein with the two modified hemins (5,6, 33),
deuterohemin and mesohemin, with \( R = H \) and ethyl,
respectively, in Fig. 1.

MATERIALS AND METHODS

Sample Preparation—HRP, type VI, was purchased from Sigma as
a lyophilized salt-free powder; the protein is predominantly isozyme
C. Purification was carried out as described previously (5,6). The
mesohemin and the deuterated hemins are the same ones utilized
previously (5,6, 16, 18, 32), except \([meso-^2H_4]mesohemin\), which was
prepared as described elsewhere (34). The positions of deuterium
of the heme are indicated by the prefix to the position with use of the
standard numbering scheme depicted in Fig. 1. Apo-HRP was pre-
pared by the method of Younami (35) and was reconstituted with
deuterated hemins and purified by the method of Di Nello (36). Solutions
for \(^2\)H NMR studies were 1-3 mm in protein in deuterium-
depleted \( H_2O \) (5 \times 10\(^{-3}\) natural abundance). For \(^1\)H NMR studies the
protein was dissolved in 99.8\% \( H_2O \). The solution pH, adjusted using
0.2 M HCl or 0.2 M NaOH, was measured with a Beckman model
3500 pH meter equipped with an Ingold microcombination electrode.
Excess solid KCN was added to the protein in solution to generate
cyanide-ligated ferric low-spin species of HRP. Compound 1 of
mesohemin-HRP and \([meso-^2H_4]mesohemin\) were generated by
adding 2 equivalents of \( H_2O \) to the corresponding resting-state
protein (5).

NMR Methods—Deuterium NMR spectra were recorded on a
 Nicolet NT-500 FT NMR spectrometer operating at 76.77 MHz in
the quadrature mode. The spectra were collected by using double
precision on 8192 data points over a 7-kHz band width; the 90\(^\circ\) pulse
was 25 \( \mu \)s. The number of transients/spectrum varied from 5 \times 10\(^3\)
to 150 \times 10\(^3\) depending on the protein concentration and the desired
signal to noise ratio. Peak shifts were referenced to the residual water
signal, which in turn was calibrated against internal 2,2-dimethyl-
silapentane-5-sulfonate (DSS). Chemical shifts are reported in parts/
million, with downfield shifts taken as positive. Spectra simulations
were done using the Nicolet curve analysis deconvolution routine,
NTCCAP, available on the Nicolet 1280 data system. The line-
broadening introduced by the apodization of the free induction decay
was subtracted from all line width measurements.

RESULTS

Pyrrrole Hydrogen Resonances—The 76.77-MHz \(^2\)H NMR
spectra of \([2,4-\(^2\)H_4]deutero hemin-HRP\) and its cyanide-
ligated complex in \( H_2O \) at 50 °C are illustrated in Fig. 2, A
and B, respectively. Below in \( A ' \) and \( B ' \) are the net computer
simulations of the respective experimental spectra, with the
components used in the simulation, shown in\( A ' \) and \( B ' \); the
line width for both 2-H and 4-H are determined as 1.28 \( \pm \)
0.12 kHz. For HRP-CN (Fig. 2B), the peaks a and b at -12
and -25 ppm are identified as the 2-H and 4-H by previous
\(^1\)H NMR studies (37). The peaks a and b at 40 and 50 ppm
similarly arise from 2-H and 4-H in HRP (Fig. 2A). The
resulting line width for 2-H and 4-H (1.31 \( \pm \) 0.31 kHz)
and shifts are listed in Table 1. The broad peak c centered near 5
ppm in both HRP and HRP-CN arises from residual -50% deuterium
that is introduced at the meso positions (16). The
narrow peaks, \( s \) and \( n \), arise from residual solvents, \( ^1\)H and
natural abundance \( ^2\)H of mobile methyls, respectively.

Meso-Hydrogen Resonances—The 360-MHz \(^2\)H NMR
spectra of mesohemin-HRP and mesohemin-HRP-I at 25 °C in
\( H_2O \) are given in A and B of Fig. 3. The shift patterns,
particularly for the four prominent methyls, a-d, are very
close to those for the analogous HRP and HRP-I containing
protoporhem or deuterohem iron, as reported previously (5,6).
The advantage of monitoring mesohemin-HRP-I by NMR is
that this compound I exhibits a significantly longer half-life
(>2 h) than the native enzymes (30 min) under comparable
conditions.

The 76.77-MHz \(^2\)H NMR traces of \([meso-^2H_4]HRP\) and its
cyanide-ligated complex in \( H_2O \) at 55 °C are illustrated in Fig.
4, A and B, respectively. In each case, it is clear that there
exists a very broad resonance, a, in the labeled complex not
present for unlabeled hemin. For high-spin \([meso-^2H_4]HRP\),
this peak is centered at -2 ppm, and for low-spin \([meso-^2H_4]HRP-
PCN\), it is centered at ~6 ppm. In the resting state
complex, the broad line a was simulated by a single Lorentzian

\( R \) vinyl, \( R \) hydrogen, and \( R \) ethyl.

![Fig. 1. Structure and numbering system for hemin](image-url)
$^2$H NMR of Horseradish Peroxidase

TABLE I

$^2$H NMR quadrupolar line widths of $^2$H-labeled hemins reconstituted in HRP complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^2$H Shift</th>
<th>$^2$H Line width</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2,4-$^2$H$_2$]Deuterohemin- &amp; 50</td>
<td>45.0</td>
<td>1.28 ± 0.15 kHz</td>
</tr>
<tr>
<td>HRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2,4-$^2$H$_2$]Deuterohemin- &amp; 50</td>
<td>42.3</td>
<td>1.28 ± 0.15 kHz</td>
</tr>
<tr>
<td>HRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[meso-$^2$H$_4$]HRP</td>
<td>55</td>
<td>-11.8</td>
</tr>
<tr>
<td>[meso-$^2$H$_4$]HRPCN</td>
<td>55</td>
<td>(3, 5, 7, 8)*</td>
</tr>
<tr>
<td>[meso-$^2$H$_4$]mesohemin-</td>
<td>55</td>
<td>-12 (composite)</td>
</tr>
<tr>
<td>HRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2,4-$^2$H$_2$]HRP</td>
<td>50</td>
<td>66.4</td>
</tr>
<tr>
<td>[2,4-$^2$H$_2$]HRPCN</td>
<td>50</td>
<td>5.6</td>
</tr>
<tr>
<td>[1,5-$^2$H$_2$]HRPCN</td>
<td>55</td>
<td>2.8</td>
</tr>
<tr>
<td>[1,3-$^2$H$_2$]HRP</td>
<td>55</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Taken from $^1$H NMR data obtained by nuclear Overhauser effects; Ref. 37.

FIG. 2. 76.77-MHz $^2$H NMR spectra of [2,4-$^2$H$_2$]-deutero- hemin-HRP (A) and its cyanide-ligated complex (B) in deuterium-depleted H$_2$O at 50 °C, pH 7.0. Computer simulation of the experimental spectra using the NTCCAP (see "Materials and Methods") curve-fitting program is shown in A' and B' respectively. The individual components used in the computer simulation are given in the bottom traces A" and B". Previously known (37) shifts for the 2,4-H resting-state HRP and its cyanide complex and the position of the residual HOD signal (peak s) along with the expected signal (peak c) for the partial deuteration at the meso-H positions were used in the simulation of the experimental spectra. The signal due to the natural abundance mobile methyl groups (peak n) is also taken into account in simulation.

FIG. 3. Hyperfine shifted portions of 360-MHz $^1$H NMR spectra of mesohemin-HRP (A) and mesohemin-HRP compound I (B) in 99.8% $^2$H$_2$O, pH 7.0, and 25 °C. The compound I complex was generated by adding 2 equivalents of H$_2$O$_2$ to the solution of mesohemin-HRP. The peaks due to the four methyl groups of mesohemin (see Fig. 1) are labeled as a-d in both spectra.

peak, as well as the solvent (s) and residual $^2$H-methyl (n) line, as shown in A' and A". In [meso-$^2$H$_4$]HRPCN, the positions of the four nonequivalent meso-H signals are known from $^1$H nuclear Overhauser effect studies (37), so that the simulated spectrum in Fig. 4B' was generated by optimizing the equal line widths of four equally intense peaks centered at 3, 5, 7, and 8 ppm, as well as the solvent (s) and the natural abundance methyl (n) signals, as shown in Fig. 4B". The resulting line widths are given in Table 1.

The location of the meso-$^2$H signals in compound I was pursued only with [meso-$^2$H$_4$]mesohemin-HRP-I because of the significantly improved stability of this derivative with respect to regeneration of the resting state. The 76.77-MHz $^2$H NMR trace of resting state high-spin [meso-$^2$H$_4$]mesohemin-HRP is illustrated in A of Fig. 5; the broad peak, a, centered at -12 ppm must originate from the exclusively meso-deuterated positions (for mesohemin, deuteration of the meso positions does not introduce deuteration at any other position of the porphyrin (34), in contrast to the case with native hemin). When the sample is converted to [meso-$^2$H$_4$]mesohemin-HRP-I by adding 2 equivalents of H$_2$O$_2$, the trace shown in Fig. 5B results. The intensity at -12 ppm is gone, but there is now evidence for a broad peak, a', centered at 15–20 ppm downfield from DSS. As this sample is allowed to partially decay back to the resting state, the trace in Fig. 5C results, for which some area is lost in the region 15–20 ppm, with concomitant regeneration of some of the known meso-
Table I. The fits indicate essentially unchanged 4-H, line width (0.75 kHz) upon formation of cyanide complex (see text).

Vinyl Proton Resonances—The 76.77-MHz 1H NMR traces of [2,4-(vinyl-2H2)2]HRP and its cyanide-ligated complex are displayed in A and B of Fig. 6. The vinyl-1H shift for individual vinyls in both cases are known (5, 32, 37), and the pair of 1H peaks are found at the appropriate positions; that of the 2-vinyl-H, in HRPCN overlaps the residual solvent 1H peak (s) as well as the natural abundance mobile methyl groups, a, are also taken into account in the simulation. Note that the broad signal moves from the right to left side of the solvent 1H peak upon formation of cyanide complex (see text).

Methyl Resonances—The 76.77-MHz 1H NMR spectra of [1,3-(C2H3)2]HRP and [1,5-(C2H3)2]HRPCN in H2O at 50 °C are illustrated in A and B of Fig. 7, with the complete simulation and the component peak given in A', A'', B', B'', respectively. The known (5, 6) unequal degree of deuteration of the 1-C2H3 (65%) and 5-C2H3 (95%) accounts for the intensity difference of the two methyls in Fig. 4B and allows unambiguous assignment of a and b to the 5-CH3 and 1-CH3, respectively. The pair of peaks a and b in Fig. 4A for HRP appear at the known position for 1-CH3 and 3-CH3 (37). For each methyl in both complexes, the simulation yields line widths ~90-95 Hz. The methyl line width for the 3-CH3 peak in [1,3-(C2H3)2]HRP was determined in the temperature range 5–50 °C, and a plot of log line width versus reciprocal temperature leads to a straight line (not shown) whose slope yields the activation energy, $E_a \sim 4.2 \text{ kcal/mol}$, for the correlation time $\tau_c = \tau_{c,exp}(-E_a/RT)$, for the quadrupolar relaxation.

Discussion

The 1H NMR spectra for each labeled heme incorporated into the various HRP resting state derivatives allowed clear detection of its resonance, although the line widths often do not allow resolution of the nonequivalent component peaks (i.e. the four meso-1H in HRP or HRPCN (Figs. 3 and 4)). In particular, the broad meso-1H envelope is observed in HRP and mesohemin-HP (Figs. 4 and 5), in contrast to the...
\[ ^2\text{H} \text{NMR of Horseradish Peroxidase} \]

Fig. 6. 76.77-MHz \(^2\text{H}\) NMR spectra of high-spin, resting state \([2,4-(\text{vinyl}-^2\text{H}_2)_2]\text{HRP}\) \((A)\) and its cyanide-ligated low-spin complex \((B)\) in deuterium-depleted \(\text{H}_2\text{O}\) at 50 °C, pH 7.0. The computer simulation of the experimental spectra are shown in \(A'\) and \(B'\), respectively. The individual components of the simulated spectra are given in \(A''\) and \(B''\). The peaks \(a\) and \(b\) are assigned to 2- and 4-vinyl-\(^2\text{H}_2\), respectively, while the peaks \(s\) and \(n\) represents the residual solvent signal and the natural abundance mobile methyl groups in the protein, respectively.

Previous unsuccessful attempts \((30)\) can be derived from \(^2\text{H}\) NMR even in such large enzymes as HRP \((42 \text{kDa})\), which yield line width for individual peaks up to 1.4 kHz for meso-\(^2\text{H}\) and pyrrole-\(^2\text{H}\) signals. The vinyl-\(^2\text{H}_2\) peaks are somewhat narrower, \(\sim 0.8\) kHz, and hence easier to detect.

In contrast, the methyl groups yield extremely narrow lines, which for low-spin HRP-CN \((-80\) Hz) are essentially the same as the \(^1\text{H}\) line widths \((5, 6, 32)\). The observed \(^2\text{H}_2\) line widths are very similar in low-spin HRP-CN and high-spin HRP, 80–90 Hz, in spite of the fact that the \(^1\text{H}\)-methyl line widths are \(\sim 5\) times broader in the latter complex \((5, 32)\). The dominant paramagnetic dipolar relaxation for \(^1\text{H}\) in HRP is decreased by a factor of 42 to \(<10\) Hz for \(^2\text{H}\), and this minor contribution, when compared to quadrupolar relaxation, accounts for the observation of similar \(^2\text{H}_2\) line widths in HRP and HRP-CN. Hence the major advantage of \(^2\text{H}\) versus \(^1\text{H}\) detection of methyl peaks in highly paramagnetic enzymes is clearly demonstrated.

HRP Spin State—The pyrrole 2-H and 4-H signals for resting state deuterohemin-HRP are clearly resolved in the low-field region of the spectrum where the same signals resonate in both high-spin ferric model compounds \((14, 16)\) and met-aquomyoglobin \((18)\). Primarily high-spin and intermediate-spin ferric hemes exhibit pyrrole-H contact shifts far downfield and near DSS, respectively \((14, 16, 24)\). Hence the ferric heme contact shift pattern for pyrrole protons and methyl groups provide direct evidence that the dominant spin state for resting state is \(S = 5/2\).

The previous failure to detect the pyrrole-H proton signals in the \(^1\text{H}\) NMR spectrum of deuterohemin-HRP due to area loss upon deuterating the 2,4-position is due to the fact that these signals were broad compared to the methyl signals and would have resonated under or very near one of these narrower signals \((5)\). The virtue of direct \(^2\text{H}\) detection as opposed to area loss in \(^1\text{H}\) NMR upon label incorporation is again clearly demonstrated.

Ligation State of HRP—The \(^2\text{H}\) NMR trace for high-spin, native meso-\(^2\text{H}\)-labeled HRP clearly gives rise to an intense broad peak centered at \(-2\) ppm \((\text{Fig. 4A})\) that must originate from the meso-\(^2\text{H}_2\), inasmuch as the peak is completely absent in unlabeled HRP. With a diamagnetic position for meso-\(^2\text{H}\) of \(-10\) ppm, the net contact shift, therefore, is \(-12\) ppm upfield. Direct evidence for the meso-\(^2\text{H}\) assignment is obtained from a comparison of meso-\(^2\text{H}\) shifts in resting state HRP reconstituted with modified hemin. Studies on five-coordinate, high-spin model compounds has demonstrated that the meso-\(^2\text{H}\) shift is upfield, with the magnitude of the
shift depending on the nature of the 2,4-substituent (14, 16). Electron-donating ethyl groups produced an upfield bias relative to the vinyl groups of native heme. The $^2H$ trace for $[\text{meso-}^2H]_4$mesohemin-HRP (Figs. 4 and 5A) clearly demonstrates the presence of a broad resonance centered at $-12$ ppm that can be unambiguously attributed to the $\text{meso-}^2H$s. Moreover, the broad resonance centered near 0 ppm for $[2,4-^2H]_4$deuterohemin-HRP (Fig. 2A) must also originate from the known residual meso-deuteration of this modified hemin (18).

Hence all three resting state complexes exhibit clearly upfield, albeit small, contact shifts characteristic of five-coordinate model compounds possessing a high-spin ferric ion (14, 16). Our NMR interpretation, therefore, strongly favors a five-coordinate ferric ion, as also supported by resonance Raman spectroscopy (20). Previous NMR relaxation measurements have also been presented to support the absence of a water molecule, but these interpretations relied on implicit assumptions about the mobility of either the whole water molecule or its protons (21, 22). The present NMR data is interpreted solely on the basis of the occupation of the sixth site, and is independent of any kinetic properties of this molecule.

**Compound I Radical Ground State**—Compound I of native HRP is insufficiently stable with respect to decay to yield a useful $^2H$ NMR spectrum. The significantly greater stability of compound I of mesohemin-HRP, probably due to the stabilization by the electron-donating ethyl groups, allows the clear observation that the $-12$ ppm $\text{meso-}^2H$ signal of the resting state is essentially absent, but that there is now intensity in an extremely broad resonance in the downfield region 15–20 ppm (Fig. 5B). As this sample is allowed to partially decay to the resting state (Fig. 5C) the low-field broad peak loses intensity, and that at $-12$ ppm partially recovers. Hence we attribute the broad 15–20 ppm low-field peak to the composite of the $\text{meso-}^2H$ in mesohemin-HRP-I.

The strong similarity of the apparent methyl-$^2H$ NMR contact shifts in resting state mesohemin-HRP (Fig. 3A) and mesohemin-HRP-I with the previously published spectra of the analogous peaks of native HRP and HRP-I (5, 6) argues that the electronic structures are the same for two hemins in both the resting state and in compound I.

The relatively small downfield contact shift for the $\text{meso-H}$s relative to the methyls in mesohemin-HRP-I does not support the presence of significant $\pi$ spin density at the meso positions of compound I (25–27). Based on the similarity of the compound I heme methyl shifts in HRP-I and mesohemin-HRP-I, this argues that similarly small $\text{meso-H}$ contact shifts are likely in HRP-I. These data therefore favor the $\alpha_\beta$ over the $\omega_\alpha$ description of the orbital ground state for the porphyrin radical species (25–27). This result is unexpected, since the $\omega_\alpha$ ground state would place the orbital hole nearest the proposed substrate binding site (38, 39).

**Heme Substituent Mobility**—The observed 1.3-kHz line widths for pyrrole-$^2H$s in $[2,4-^2H_2]_4$deuterohemin-HRP and its cyanide adduct, together with the $\sim 190$ kHz quadrupolar coupling constant for a proton attached to an sp$^2$ carbon (28, 40), yield a correlation time, $\tau_\rho$, of 22 ns at 55 °C via Equation 1. The simulation of the $[\text{meso-}^2H]_4$HRP-I spectrum using the known four $\text{meso-}^2H$ shifts (37) similarly and independently leads to a $\sim 1.4$ kHz $^2H$ line width (Table I, Fig. 4B), and hence a similar value for $\tau$. An estimate of the overall tumbling time of HRP based on its molecular weight yield is 20 ns. Hence the correlation time for the heme is that for the reorientation of the whole protein, and therefore the heme, not unexpectedly, is immobile within the protein matrix. Similar conclusions had been reached via $^2H$ NMR on analogous myoglobin complexes (40). Thus the pyrrole-$^2H$ NMR line widths allow the direct determination of the effective molecular weight of a heme protein, which in oligomeric proteins, could aid in determination of the degree of association.

The heme methyl groups exhibit very narrow line widths which are essentially the same in low-spin and high-spin states, confirming the dominance of quadrupolar effects (28, 29, 40) and the negligible influence of paramagnetism on relaxation properties. Using the methyl quadrupolar coupling constant of $\sim 170$ kHz (28) and the reorientation time of 22 ns at 55 °C determined above, an immobile line width of $\sim 0.98$ kHz is predicted for Equation 1. With free methyl rotation, Equation 2 predicts a line width $\sim 100$ Hz, which is consistent with the observed line width. Thus methyls exhibit completely motionally averaged line widths, and hence the methyl-$^2H$ line width can be used directly to estimate the protein tumbling time via Equation 2 with potentially greater accuracy than Equation 1 with the immobile pyrrole-$^2H$ line widths because the methyl quadrupolar coupling constant is less sensitive (28) to detailed environment that that of an sp$^2$ hybridized carbon. Hence the methyl-$^2H$ NMR line width can serve as a convenient probe of overall molecular motion, and thereby provide a measure of the effective molecular weight of a heme protein in solution.

The plot of log of the methyl line width versus reciprocal temperature, therefore, yields the activation energy for overall tumbling of the whole complex, and hence allows the estimation of the pyrrole-$^2H$ line width at temperatures lower than 50 °C where the signals are insufficiently resolved to yield direct line widths by computer fit. It may be noted that the location and assignment of the 1-C$^2H_2$ and 5-C$^2H_2$ signals at 2.8 and 6.4 ppm, which are obscured by the intense diamagnetic envelope in the $^1H$ NMR spectrum (32), are as previously determined from indirect saturation transfer experiments (37).

The vinyl line widths are significantly smaller ($\sim 0.8$ kHz) than those of the pyrrole-$^2H$ (1.3 kHz). However, the quadrupolar coupling constant is not known, and while such sp$^2$ hybridized C-H values are near 190 kHz, values range to 160 kHz (28). Thus the line width does not provide unequivocal evidence either for or against internal mobility on the nanosecond time scale. The likelihood that this line width reflects significant mobility has been suggested by detecting a rotationally locked vinyl in HRP-I (6). Other observations similarly support the premise that vinyls are much more constrained in HRP than in myoglobin (41).

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**REFERENCES**

\textbf{\textit{\textsuperscript{2}H NMR of Horseradish Peroxidase}}