Deuterium Nuclear Magnetic Resonance of Deuterium-labeled Diacetyldeuterohemin Incorporated into Sperm Whale Myoglobin*

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The heme derivative 2,4-diacetyldeuterohemin deuterated in the methyl groups of the acetyl moieties was reconstituted with sperm whale apomyoglobin and the two labeled methyl groups in the protein environment were observed by deuterium nuclear magnetic resonance spectroscopy. The results were compared to the free heme form as the dimethyl ester in chloroform and in a pyridine-water mixture, as well as in the zinc complex form. Under most conditions the two methyl resonances overlap each other to a large degree. Resonance width at half-height is of the order of 25 Hz for the protein and approximately one-third as much for the free heme at 16° and is little affected by conversion to paramagnetic derivatives. Chemical shifts for the oxy- and carbonmonoxymyoglobins are very similar. In cyanoferri-myoglobin a positive pseudo-contact contribution of 3.04 ppm was computed to explain a relative upfield shift offset in part by a small negative contact shift contribution. The cyanoferri-myglobin resonance was sensitive to the presence of phosphate buffer as well as to cyclopropane. The aquoferrimyoglobin form shows distinct resonances for the two methyl groups, with the downfield resonance considerably broadened. The expected effects of temperature on chemical shift were observed, the paramagnetic derivatives showing an effect and carbonmonoxymyoglobin showing none. The relaxation behavior was gauged from the line widths and from measurements of spin-lattice relaxation time, \( T_1 \). The effective rotational correlation time is of the order of 50 ps for the liganded myoglobin forms. The temperature dependence of the line widths may imply an increased rotational freedom with increasing temperature. The broadening observed in the aquoferrimyoglobin case is indicative of restricted internal rotational motion of one of the methyl groups. The method is suitable for probing the more mobile structures in proteins and retains its value in the neighborhood of paramagnetic centers.

The technique of simplification of \( ^1H \) NMR spectra of large molecules such as proteins by the introduction of deuterium is well known and widely practiced (2, 3). The converse application of \( ^2H \) NMR to aqueous solutions of proteins bearing specific sites of enrichment with deuterium has only just begun (4). The natural abundance of deuterium is sufficiently low that specific enrichment can lead to a simple spectrum in which resonances are readily assigned. This characteristic will be even more favorable than for \( ^1H \) in many applications (5, 6). Provided that rotational motion is sufficiently rapid, as found for example in a methyl group in a protein (1, 7, 8), the line width of the \( ^2H \) resonance can be narrow enough to allow ready observation by standard Fourier transform NMR techniques (4).

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Relaxation processes of the \( ^2H \) nucleus with \( I = 1 \) are dominated by the quadrupolar mechanism (9–11). Line broadening by paramagnetic centers, being proportional to the square of the magnetogyric ratio, \( \gamma \), is less marked for \( ^2H \) than for \( ^1H \) (9). The spin-spin relaxation time, \( T_2 \), is inversely proportional to line width, neglecting factors mentioned below. An estimate given by Diehl (9, 12) is that the ratio of the \( T_2 \) expected for \( ^2H \) to the \( T_2 \) expected for \( ^1H \), equivalent to \( (\gamma_H/\gamma_D)^2 \), equals approximately 42.5. Here the subscripts \( H \) and \( D \) refer to the proton and deuteron, respectively. Hence the effect of paramagnetic substances on the width of the \( ^2H \) resonances will be approximately 2% as pronounced as on the width of the \( ^1H \) resonances (9, 12). The predicted gain in resolution with \( ^2H \) was qualitatively confirmed in a comparison of deuterated and protonated complexes of acetylacetone with paramagnetic transition metal ions (13). The \( ^2H \) line width was reported to be smaller than the corresponding proton line width by a factor between 9 and 40 (12). This lower sensitivity of \( ^2H \) to paramagnetic substances is of potential value in the study of metalloproteins.

We report here \( ^2H \) NMR studies of derivatives of sperm...
whale myoglobin formed from the apoprotein reconstituted with 2,4-diacetyldeuterohematin deuterated in the methyl groups of the acetyl moiecties (14). The acetyl groups presumably occupy the space in the reconstituted protein structure (15) that is occupied by the vinyl groups in the native protein, toward the interior of the heme pocket and in van der Waals contact with several nonpolar amino acid side chains (16, 17). Proton NMR studies of low spin myoglobin have yielded considerable information about resonances of methyl groups on the heme and about other functional groups near the heme (18, 19), and less detailed identification of vinyl group resonances (20).

The deuterated methyl groups in the myoglobin can be observed by 1H NMR spectroscopy (4), and the observations yield information concerning the electronic configuration of the heme and the mobility of the label within the protein framework. The derivatives studied are the oxy- and carbonmonoxyferromyoglobin and the cyanoferrimyoglobin, and, to a lesser degree, the high spin aquoferrimyoglobin derivatives. In the present study, measurements of spin-lattice relaxation time, $T_1$, support the conclusion based on resonance line width that the methyl groups undergo rapid internal rotational motions. The results also bear out the expected insensitivity of the 1H resonances to paramagnetic line broadening.

**EXPERIMENTAL PROCEDURE**

$D_{24}$-2,4-Diacetyldeuterohematin. 2,4-Diacetyldeuterohematin was prepared according to Fischer and Zeile (21). The compound was esterified with concomitant removal of the iron in a mixture of dry acetic acid, dry acetic anhydride, 1/5 (v/v) of concentrated ($14 N$) sulfuric acid, and $D_24$-diacetyldeuteroporphyrin-OH (22). After purification by alumina chromatography, the iron was reintroduced by the method of Alben et al. (23), yielding the pure $D_{24}$-2,4-diacetyldeuterohematin-OH. Selective deuteration of the acetyl group of this compound was obtained at room temperature by treatment with CH$_3$OD containing $7\%$ D$_2$SO$_4$ (w/v) for 24 hours in the absence of light, as described previously (14). The ester function was removed according to Fischer et al. (24) by hydrolysis in deuterated solvents. A single component was observed by paper chromatography (25, 26).

$D_{24}$-2,4-Diacetyldeuteroporphyrin-OH. $D_{24}$-2,4-diacetyldeuterohematin-OH was dissolved in CH$_3$COOD and treated with a freshly prepared, saturated solution of ferrous sulfate in concentrated DCl under nitrogen (27). $D_{24}$-2,4-diacetyldeuteroporphrin-OH was extracted with chloroform, concentrated, and chromatographed on alumina. It was crystallized from chloroform/methanol. The melting point was 234-236°C (28). The ultraviolet spectrum corresponded to that already reported (29).

$D_{24}$-Zn-2,4-Diacetyldeuteroporphyrin-OH. 100 mg of $D_{24}$-diacetyldeuteroporphyrin-OH was dissolved in 90 ml of CH$_3$Cl, and added to 60 ml of hot, dry methanol. A saturated solution of 300 mg of zinc sulfate in methanol was added and the reaction mixture was refluxed for 2 hours in the dark, during which time a change in color from red to purple was observed. The conversion was monitored by the disappearance of the prominent absorbance maxima at 518, 552, 588, and 640 nm (29), after which the solvent was evaporated, a small amount of water was added, and the product extracted with chloroform. The extracts were dried over sodium sulfate, concentrated, and chromatographed on alumina. The eluate was concentrated and the product crystallized from chloroform/isooctane, 1/4. Absorbance maxima in chloroform were observed at 592, 555, 512 (shoulder), and 430 nm. The melting point was 284-287°C. The zinc compound probably bears H$_2$O as a ligand (30). The metal may be removed easily by the procedure described above for the removal of the iron from the hemin.

$D_{24}$-4-Diacetyldeuterohematin-Ferrimyoglobin. Sperm whale apomyoglobin (31) was treated at 4°C with a 1.2 molar ratio of the deuterohematin which had been taken up in 1 ml of 0.1 N NaOH and immediately diluted 10-fold with water. After the pH of the solution had been adjusted to 10.5, it was dialyzed first against cold distilled water and then against 0.1 M phosphate buffer at pH 6.2. The reconstituted myoglobin was chromatographed on CM-50 Sephadex with pH 6.2 phosphate buffer, ionic strength 0.1 M. The purity of the product was checked by cellulose acetate electrophoresis. It was lyophilized after deionization on a column of Reex 1-300 (Fischer).

$D_{24}$-4-Diacetyldeuterohematin-Oxymyoglobin. Oxymyoglobin was prepared by an adaptation by Shire (32) of the procedure of Dixon and McIntosh (33). A $10^4$ fold excess of sodium dithionite was dissolved in 2 ml of 0.1 M Tris-HCl buffer, pH 8.6, and applied to a Sephadex G-25 column (Fine, Pharmacia, 25 x 2 cm) equilibrated with the Tris buffer, pH 8.6. The ferricyoglobin sample was then applied and the protein eluted through the dithionite and off the column with the same buffer. The minimized exposure to dithionite and its oxidation products seems responsible for the good stability of the oxymyoglobin (32). The eluate was concentrated by Amicon ultrafiltration to a concentration of 5 to 6 mM for measurements.

$D_{24}$-2,4-Diacetyldeuterohematin-Carbonmonoxymyoglobin. The oxymyoglobin eluate was stirred gently while a slow stream of CO was passed over the surface. The protein solution was concentrated by ultrafiltration to 5 to 6 mM for NMR measurements.

$D_{24}$-2,4-Diacetyldeuterohematin-Cyanoferrimyoglobin. A $10^4$ fold excess of KCN was added to a solution of the ferrimyoglobin, 5 to 6 mM, in phosphate buffer, pH 6.7. The cyclopropane derivative of this material was prepared by passing cyclopropane over the surface of the protein solution with mild agitation (34).

Sample Preparation for NMR. The protein solutions were prepared as described, 5 to 6 mM. The sample of $D_{24}$-2,4-diacetyldeuterohemin-OMe (4 mg) was dissolved in a mixture of pyridine and water, 0.75 ml and 0.25, respectively, containing 4 mg of KCN. The zinc derivative was prepared at a concentration of 4 mg in 1 ml of either chloroform or 3/1 pyridine/water, by volume. The concentration of $D_{24}$-2,4-diacetyldeuteroporphyrin-OMe and of $D_{24}$-4-diacetyldeuterohematin-OMe was 1 mg in 1 ml of chloroform.

**1H NMR Measurements.** The Varian HR 220 spectrometer was operated at 33.77 MHz in the pulsed Fourier transform mode. Unless otherwise stated the ambient probe temperature was 15-17°C. External D$_2$-Me$_2$Si was used as a reference throughout the experiments. For measurements on the protein samples 32,768 accumulations were taken with a recycle time of 0.211 s. The small molecules were measured with 4,096 accumulations at a recycle time of 1.08 s. For the measurements at other temperatures the probe temperature was checked before and after the accumulation with variation of not more than 1°C. Measurements of $T_1$, spin-lattice relaxation time, were made by the inversion recovery method (35, 36).

Other methods and procedures, such as pH determinations and absorbance measurements, followed previous practice (8). All chemicals were reagent grade and the water was distilled and deionized.

**RESULTS**

General Characteristics of Protein Spectra. The 1H NMR spectrum of each sample shows a prominent resonance for HOB about 4.2 to 4.6 ppm downfield of D$_{19}$Me$_2$Si in no case interfering with the observation of the resonances of the two deuterated methyl groups. The two methyl resonances overlap each other considerably under many conditions but are distinguishable in certain protein samples. In the following results the spectra are presented in terms of chemical shifts in parts per million referred to D$_{19}$Me$_2$Si. Smaller changes in resonance position may be presented in Hz ($\times 33.77$), as are all values for line width, $\Delta$$\delta$, denoting the width of a resonance at half-height.

Four conditions of the myoglobin are considered. Two diamagnetic states are represented by the O$_2$ and CO derivatives. Two paramagnetic states are presented by the cyanoferrimyoglobin and aquoferrimyoglobin. Emphasis is placed in the present study on the geometry is to be expected in that the iron atom can be taken as located in diamagnetic states are represented by the 0, and CO derivatives (33). To assign the two methyl resonances overlie each other to a great extent in the spectra, the two methyl resonances overlie each other considerably under many conditions but are distinguishable in certain protein samples. In the following results the spectra are presented in terms of chemical shifts in parts per million referred to D$_{19}$Me$_2$Si. Smaller changes in resonance position may be presented in Hz ($\times 33.77$), as are all values for line width, $\Delta$$\delta$, denoting the width of a resonance at half-height.

Four conditions of the myoglobin are considered. Two diamagnetic states are represented by the O$_2$ and CO derivatives. Two paramagnetic states are presented by the cyanoferrimyoglobin and aquoferrimyoglobin. Emphasis is placed in this paper on the first three states in which broadly similar geometry is to be expected in that the iron atom can be taken to be nearly within the plane of its porphyrin ligands (37).

Fig. 1 shows the spectra of cyanoferrimyoglobin (A), carbonmonoxymyoglobin (B), and oxymyoglobin (C). In each case the two methyl resonances overlaid each other to a great extent in the spectra.

1. The abbreviation used is: Me$_2$Si, tetramethylsilane.
Fig. 1 (left). $^1$H NMR spectra measured by pulsed Fourier transform spectroscopy at 33.77 MHz at 15–17°C. The protein is myoglobin prepared from sperm whale apomyoglobin and D$_2$-2,4-diacetyl-deuterohemin. A, cyanoferrimyoglobin, 5 to 6 mM, with 10-fold excess of KCN in phosphate buffer, ionic strength 0.1 M, pH 6.7. B, carbonmonoxymyoglobin, 5 to 6 mM, 0.1 M Tris-HCl buffer, pH 8.6. C, oxymyoglobin, 5 to 6 mM, 0.1 M Tris-HCl buffer, pH 9.0. External D$_2$-Me$_2$Si was used as a reference, and its separately recorded spectrum is overlaid by computer in each case, with chemical shifts expressed in parts per million. Spectra are delivered from 32,768 accumulations with a recycle time of 0.211 s.

Fig. 2 (right). Temperature dependence of (A) the line width and (B) chemical shift in Hz plotted against the reciprocal of the absolute temperature. The concentrations of the protein solutions are given in the legend to Fig. 1, and conditions of measurements were identical except for the alterations in probe temperature. ○, carbonmonoxymyoglobin; Δ, cyanoferrimyoglobin; •, D$_2$-2,4-diacetyldeuterohemin-OMe in pyridine-water containing excess KCN, 4096 accumulations with 1 s recycle time. See text for details.

extent and have similar line widths. The resonances of the cyanoferrimyoglobin lie approximately 2.4 ppm to higher field than those of the other two forms. A separation of perhaps 0.2 ppm between the overlapping resonances may be estimated for the carbonmonoxymyoglobin in Fig. 1B. There are slight indications of downfield shoulders in the other two spectra. The ferro forms appeared to be comparably stable to the natural myoglobin. Variations in pH of approximately 1 unit above and below pH 8 were explored without evidence of spectral changes. A larger pH variation was avoided because of the danger of autooxidation during the 2 hours of signal accumulation. In the case of cyanoferrimyoglobin no significant changes in spectrum were evident over the pH range between 5.5 and 10.

The observed chemical shift values for the different forms of the myoglobin derivative are collected in Table I. The chemical shift of the resonance observed for the cyanoferrimyoglobin was sensitive to the presence of phosphate and moved approximately 0.4 ppm downfield when the phosphate buffer containing KCN was replaced with 0.05 M KCN alone. This substantial change may reflect phosphate binding that is in keeping with crystallographic observations (17). Phosphate is also known to affect the on and off kinetics of cyanide binding (38) and to stabilize the protein under certain conditions (39). Another adduct defined by crystallographic analysis (40) and known to form in solution (34) is that with cyclopropane. As shown in Table I the equilibration of the cyanoferrimyoglobin with cyclopropane results in a downfield shift nearly one-half as great as that caused by the phosphate.

Free Hemin Forms—Table I includes chemical shift values for the overlaid methyl resonances of the free D$_2$-2,4-diacetyldeuteroporphyrin-OMe, differing from the protein inclusion by the presence of the methyl esters and observed in organic solvents. The chemical shift position in CHCl$_3$ of the resonance is -3.05 ppm. The hemin derivative is prepared as the chloride but may undergo some exchange for hydroxyl ion (23).

The cyanide derivative in pyridine-water shows a resonance centered at -1.06 ppm. The zinc derivative serves as a diamagnetic analog (41, 42) and shows a resonance centered at -1.06 ppm. The zinc derivative serves as a diamagnetic analog (41, 42) and shows a resonance centered at -1.06 ppm.
Various chemical shift components, defined in the text, are listed. See equation 1. The negative sign indicates positions in parts per million downfield of external D$_2$-MeSi.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Chemical shift contribution</th>
<th>Line width Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>D$_2$-2,4-diacetyldeuterohemin-OMe Zn</td>
<td>CHCl$_3$</td>
<td>$-2.60$</td>
<td>$9$</td>
</tr>
<tr>
<td>D$_2$-2,4-diacetyldeuterohemin-OMe Zn</td>
<td>Pyridine-H$_2$O + KCN</td>
<td>$-2.42$</td>
<td>$8$</td>
</tr>
<tr>
<td>Oxymyoglobin</td>
<td>Tris, pH 8.6</td>
<td>$-3.05$</td>
<td>$23$</td>
</tr>
<tr>
<td>CO-myoglobin</td>
<td>Tris, pH 8.6</td>
<td>$-3.20$</td>
<td>$28$</td>
</tr>
<tr>
<td>D$_2$-2,4-diacetyldeuterohemin-OMe</td>
<td>Pyridine-H$_2$O + KCN</td>
<td>$-1.06$</td>
<td>$-2.60^a$ $+3.04$ $-1.50$</td>
</tr>
<tr>
<td>CN-ferrimyoglobin</td>
<td>H$_2$O + KCN, pH 6.7</td>
<td>$-1.20$</td>
<td>$-2.42^a$ $+3.04$ $-1.68$</td>
</tr>
<tr>
<td>CN-ferrimyoglobin</td>
<td>Phosphate buffer, pH 6.7 + 10-fold excess KCN</td>
<td>$-0.8$</td>
<td>$-3.10^a$ $+3.04$ $-1.14$</td>
</tr>
<tr>
<td>CN-ferrimyoglobin + cyclopropane</td>
<td>Phosphate buffer, pH 6.7 + 10-fold excess KCN + saturated cyclopropane</td>
<td>$-0.96$</td>
<td>$-3.10^a$ $+3.04$ $-0.90$</td>
</tr>
</tbody>
</table>

Diamagnetic analogs:
- $^a$ Zinc compound as diamagnetic analog (in CHCl$_3$).
- $^* $ Zinc compound in pyridine/H$_2$O/KCN as diamagnetic analog.
- $^c$ With $-3.10$ ppm an average value for the diamagnetic protein analog.

The chemical shift of the methyl resonances of D$_2$-2,4-diacetyldeuteroporphyrin-OMe in CHCl$_3$ was $-2.74$ ppm.

**Temperature Dependence of Line Widths and of Chemical Shifts**—Estimates of line widths at $16^\circ$ are listed in Table I. Apart from the usual systematic and instrumental contributions to observed line widths, chemical shift nonequivalence between the two methyl groups may be involved. The variation of line width with temperature is illustrated in Fig. 2A where the ordinate expresses its value in Hz plotted against reciprocal of temperature (K) as abscissa. In each case the line width narrows with increasing temperature, that for D$_2$-2,4-diacetyldeuterohemin-OMe cyanide reaching a limiting value. The two forms of the myoglobin have similar line width over the conditions measured. The protein preparations show a distinctly greater line width than the free hemin form or its diamagnetic analog (Table I).

The corresponding observations on temperature dependence of chemical shifts are presented in Fig. 2B. The carbonmonoxymyoglobin shows no effect of temperature on chemical shift. The paramagnetic cyanoferrimyoglobin does show a shift to lower field with increasing temperature, as does the hemin cyanide.

**Aquoferrimyoglobin Form**—The spectrum of the aquoferrimyoglobin form at pH 6.7 is shown in Fig. 3. The two methyl group resonances are now separated, with a broad component centered at $-8.44$ ppm and a sharp component at $-7.07$ ppm. This high spin form, $S = 5/2$, is characterized by an equilibrium between high and low spin states that is estimated at 12% high spin at the temperature of the measurements, $17^\circ$ (43, 44). The iron is also believed to be displaced from the plane of the heme toward the proximal histidine residue, affecting the geometry around the heme (17). The separation of the two methyl resonances contrasts with the observations of the liganded forms in Fig. 1, as does their considerable downfield displacement. The chemical shift of D$_2$-2,4-diacetyldeuterohemin-OMe in CHCl$_3$ was $-3.05$.

**Spin-Lattice Relaxation Time, $T_1$**—The inversion recovery technique was used for determination of $T_1$ in the solutions represented in Fig. 1. Values for the cyanoferric, carbon-
monoxo-, and oxymyoglobin forms were 36, 34, and 43 ms, respectively. The standard deviations (7, 8) were 3.5, 2.5, and 3.4 ms, respectively. Since each spectrum analyzed represented the overlap of the resonances of both methyl groups the $T_1$ values are averages.

**Discussion**

**Diamagnetic and Paramagnetic Contributions to Chemical Shifts**—The observed chemical shifts may be correlated to a first approximation by using the diamagnetic species for reference in considering the paramagnetic species. The paramagnetic contributions for the low spin iron porphyrin, $S = 1/2$, system in pyridine-water containing KCN have been thoroughly investigated for $^1H$ NMR (42), and other solvents have been considered (45). The axial ligands of the diacetyldeuterohemin-OME in pyridine-water with an excess of KCN have not been positively identified, but it will be assumed that one is pyridine and the other cyanide (27).

That part of the chemical shift contribution by the paramagnetic electron occurring through $\sigma$ or $\pi$ bonds is called the contact shift, $\Delta\alpha_{con}$. The space through space coupling by electron-nuclear dipolar interaction produces an additional pseudocontact shift, $\Delta\alpha_{pseud}$ (42, 45). The observed chemical shift, $\Delta\alpha_{obs}$, will also experience contributions from ring currents, $\Delta\alpha_{rc}$, and be controlled by the chemical factors peculiar to the structure of the diamagnetic compound considered in the absence of ring current effects, $\Delta\alpha_{diam}$. The combination of these terms is summarized in Equation 1:

$$\Delta\alpha_{obs} = \Delta\alpha_{diam} + \Delta\alpha_{pseud} + \Delta\alpha_{rc} + \Delta\alpha_{con}$$

The sum of the first two terms is estimated in what follows from $\Delta\alpha_{con}$ for appropriate diamagnetic species. Estimates of the two paramagnetic term contributions can be made as follows. Assuming a single Kramers doublet, $\Delta\alpha_{pseud}$, in parts per million, can be computed for the heme or heme protein case where the three g-factor components of the electron spin are anisotropic as (42, 46-48):

$$\Delta\alpha_{pseud} = \frac{4a}{3} \left[ \frac{\Delta \mu_B^2}{\gamma_p^2 S(S+1)} \right] \left[ \cos^2 \alpha - \sin^2 \alpha \right]$$

In this equation $\Omega$ is the polar angle between the z axis and the radius through the center of the electron spin to the given nucleus, and $\Psi$ is the polar angle between the projection of $\Omega$ in the $xy$ plane and the $x$ axis, $\delta$ is the Bohr magneton, $S$ is the electron spin and $T$ is the absolute temperature. $K$ is a constant and expresses the reduction of the unpaired spin in the iron orbitals where it has an anisotropic g-factor. $K$ is taken as 0.75, making the assumption that only the spins in the iron atomic orbitals contribute to $\Delta\alpha_{con}$ (49).

The computations according to Equation 2 were made for the $^1H$ case with the same assumptions as were made by Shulman et al. (42), who present a fuller discussion. The g-factor values for low spin hemins at low temperature were taken, based on the pyridine-water-solvent containing excess KCN: these are 3.4 for $g_x$ perpendicular to the heme plane, and 1.84 and 0.74 for $g_y$ and $g_z$ which express the anisotropy of the unpaired electron in the plane (42). It is assumed that $g_x$ and $g_y$ interchange rapidly enough to appear as weighted averages, $(g_x)^2 - (g_y)^2 = (g_x^2 + g_y^2)/2$. It is assumed further that the deuterated methyl groups are in the plane of the porphyrin, an assumption supported by the x-ray structure of the nickel derivative of 2,4-diacetyldeuterohemin-OME, from which also a value of $r$ of 7.4 A was taken (49). The value so computed for $\Delta\alpha_{pseud}$ is $+3.04$ ppm, a contribution in an upfield direction.

With $\Delta\alpha_{pseud}$ computed, $\Delta\alpha_{con}$ known, and $(\Delta\alpha_{diam} + \Delta\alpha_{rc})$ estimated from diamagnetic cases, $\Delta\alpha_{con}$ can be estimated by difference from Equation 1. Table I collects the results and estimates of chemical shift contributions for the various compounds. Listed first are the diamagnetic compounds which are used to arrive at estimates of $(\Delta\alpha_{diam} + \Delta\alpha_{rc})$ to apply to the paramagnetic cases. The Zn (II) derivative yielded values of $\Delta\alpha_{con}$ of 2.60 and 2.42 ppm downfield of Me$_3$Si in chloroform and in the pyridine-water-KCN mixture, respectively. The oxy- and carbonmonoxo- forms of the myoglobin yield an average of 3.10 ppm downfield for the diamagnetic protein analog. These three values are taken as alternative estimates in Table I of $(\Delta\alpha_{diam} + \Delta\alpha_{rc})$ entered for the paramagnetic cases in the lower part of the table. The value of $\Delta\alpha_{con}$ of +3.04 ppm (upfield shift) is also included, and the values of $\Delta\alpha_{con}$ are shown by difference. The compounds represented are the free hemin derivative in pyridine-water with cyanide, and the cyanoferriyoglobin with and without phosphate and with cyclopropane in addition to phosphate.

The values of $\Delta\alpha_{con}$ estimated in Table I are not large. The contact contribution is downfield as expected (42). The small value of $\Delta\alpha_{con}$ implies that the bond system leading through the acetyl group shows little $\pi$ character. The various assumptions about the magnitude of $(\Delta\alpha_{diam} + \Delta\alpha_{rc})$ will be uncertain to some extent because of the details of the environments of the heme probably vary in every case. An example of this is offered by the cyanoferriyoglobin under the three different conditions. Phosphate may well bind in the region of ligand entry to the iron site (38, 39).

**Temperature Dependence of Chemical Shifts**—The temperature dependence of the chemical shifts shown in Fig. 2B follows the expected pattern. The diamagnetic carbonmonoxo-yoglobin resonance band shows no temperature dependence, whereas the paramagnetic cases do so (Ref. 42, cf. Equation 2), shifting to lower field with increasing temperature so as to be closer to the position of the diamagnetic analog. An opposite trend seen with the protoporphyrin-OLE analog was ascribed to conformational changes induced by the temperature change (50).

**Line Width**—As shown in Figs. 1 and 3, and recorded in Table I, the line widths of the methyl resonances near 15' in the myoglobin derivatives are all near 25 Hz and are broader by a factor of 3 or 4 than for the free hemin compounds. The line width of $^1H$ resonances of the diamagnetic compounds is determined mainly by the quadrupolar mechanism (9). The following relationships are valid (51).

$$\Delta\nu = \frac{1}{2} \frac{1}{T_2} \left( \frac{1}{T_1} + \frac{1}{T_{1p}} \right)$$

which reduces to $1/2\Delta\nu = 1/\nu$ for $T_1 = T_{1p}$, extreme narrowing, and $g = 0$, to

$$\Delta\nu = \frac{1}{2} \frac{1}{T_{1p}} \frac{1}{T_{1p}}$$

Here $(e\gamma Q/h)$ is the quadrupolar coupling constant, $\tau_p$ is the correlation time which involves the overall motion of the protein expressible in the rotational correlation time, $\tau_R$, and one or more correlation times for the internal rotation of the quadrupolar nucleus around the pertinent axes, $\omega_F$ is the resonance frequency, and $g$ is the asymmetry parameter which may be neglected in most cases (51). Quadrupolar coupling constants of $^1H$ in methyl groups are often approximately 170 kHz (52-54). $T_2$ and $T_R$ are, respectively, the spin-spin and spin-lattice relaxation times of the $^1H$ nucleus. Assuming a Lorent-
zian shape for the resonance, the observed \( \Delta A \), the line width at half-height expressed in Hz, will equal \( 1/\pi T_i^* \), where instrumental and other factors cause \( T_i^* \) to be somewhat shorter than \( T_{1\theta} \) in Equations 3 and 4. In the present cases resonance overlaps may contribute to \( \Delta \delta \). For the carbonmonoxy- and oxy-myoglobin cases \( T_i^* \) was estimated as 12 ms, and \( T_1 \) was reported above as 36 ms.

The similarity of the line widths observed for the diamagnetic and paramagnetic species (Table I) confirms that paramagnetic effects are not large (9, 12), and that both dipole-dipole and scalar coupling terms in the Solomon-Bloembergen (55, 56) expression are small. For the cyanoferrimyoglobin case \( T_i^* \) was estimated as 12 ms, and \( T_1 \) was reported above as 36 ms.

As has been pointed out for the cyanoferrimyoglobin derivative (4), the line width is mainly determined by the internal rotation of the acetyl group around its axes. This is also the case for the oxy- and carbonmonoxy derivatives. Assuming relative rigidity of the bulky porphyrin skeleton within the protein matrix, the internal rotation will involve the two bond axes in the acetyl group, the contributions of which are not distinguishable by the present observations. The dominant contribution of the internal rotation to \( T_1 \) is illustrated by computing the line width based solely on the over-all tumbling of the protein. In that case \( T_1 \) equals \( \tau_0 \) which from \( ^{13} \)C NMR relaxation measurements has been shown to be about 22 ns (7, 8). This leads through Equation 3 to computed line widths for the resonances in question of approximately 1000 Hz (4). Internal rotation accounts for much of the 40-fold narrowing of the protein. In that case \( \tau_0 \) equals \( \tau_{1\theta} \) which from \( ^{13} \)C NMR relaxation measurements has been shown to be about 22 ns (7, 8). This leads through Equation 3 to computed line widths for the resonances in question of approximately 1000 Hz (4). Internal rotation accounts for much of the 40-fold narrowing observed (Table I). The effective correlation time, \( \tau_{1\theta} \), by Equation 4 utilizing \( T_{1\theta} \) values is of the order of 50 ps for each of the protein preparations. This value is in keeping with estimates for the alanine methyl groups in sperm whale myoglobin based on \( ^{13} \)C relaxation measurements (7, 8) and is consistent with the behavior of methyl groups in small peptides (57).

The present case in which two contributions to internal rotational motion are present does not readily reduce to the more manageable form with a single internal rotational motion exemplified by the alanine methyl groups mentioned above (7, 8). Approximate models for the latter case applied to \( ^{1} \)H NMR relaxation have been dealt with by Zeidler (58), Glase et al. (54), and by Wilbur and Jonas (59). The observed values of \( T_1 \) appear to be at least an order of magnitude too large when compared with such computations, undoubtedly the consequence of neglecting the presence of more than one mode of internal motion (60). Sperm whale myoglobin preparations enriched with \( ^{13} \)C in the methyl group of the 2 methionine residues (1) have yielded \( T_1 \) values at two spectrometer field strengths that could not be fitted to the simple case either.\(^2\)

Presumably in the methionine case also there are important internal motions other than that of the methyl group about its axis. The potential value of the systematic preparation of various enriched forms for analyzing internal motions in a protein has been outlined (6).

Temperature Dependence of Line Widths—As shown in Fig. 2A the line widths decrease as the temperature is increased. The free heme shows a limit of narrowing in the temperature range covered by the protein cases. The variation of line width for the small molecule may reflect a change in aggregation (61).


Its immediate cause is probably a partial hindrance of rotation of the acetyl group around its axis, most probably around the carbonyl porphyrin axis. Similar restricted rotation is found for the 2,4-substituents of mesoheme and protohemin-0-acyl forms according to the chemical shift behavior (62). For the protein cases, variations in internal freedom of rotation probably account for the observed dependence, since any changes directly in \( \tau_0 \) are probably without effect. Otsuka (63), in explaining the temperature dependence of the spin state equilibrium of hemoproteins, proposes a cooperative lowering of van der Waals contacts between the porphyrin and the protein moieties. The differential broadening of one methyl resonance in the high spin aquoferrimyoglobin form seen in Fig. 3 may represent a differential restraint on rotation if paramagnetic or other effects are not responsible. Restriction of heme methyl group rotation has been invoked by Morishima and Iizuka (64). Variations in packing density within myoglobin are recognized (65) and can be expected to be altered in passing from the liganded ferromyoglobin or low spin ferrimyoglobin to the aquoferrimyoglobin structure.

Conclusion—The results presented here show that the \( ^{1} \)H NMR technique is sensitive to changes around the heme, including those promoted by phosphate and cyclopropane (Table I). The expectation that the technique is applicable to the paramagnetic heme forms is borne out. The technique applies to structural components in proteins that undergo internal rotational motions considerably more rapid than those associated with over all tumbling. The probe appears suited to the assessment of partial restrictions on rotational freedom within the protein (6, 8, 63–65). The technique can exploit the well developed experience with deuterium labeling established primarily for use in conjunction with proton NMR (2, 3, 6, 17, 66, 67). It is equally applicable to the study of peptides.\(^3\)

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