

NMR STUDY OF DYNAMIC PROPERTIES OF HIGH-SPIN FERRIC PORPHYRINS

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ABSTRACT

Proton n.m.r. relaxation times have been used to characterize some dynamic properties of paramagnetic model haem complexes which may be relevant to the investigation of the electronic structure and function of haemo-proteins. T_1 relaxation of the porphyrin methyl protons in high-spin ferric complexes is shown to arise from dipolar coupling, where the correlation time is the electron spin-lattice relaxation time, τ_s . τ_s , in turn, is determined by modulation of the zero field levels by the tumbling of the complex in solution. The experimental ratio of T_1/T_2 is in agreement with the value predicted by the calculated correlation time.

Proton line broadening for certain porphyrin protons by a T_2 process is shown to reflect the motion of the iron atoms relative to the haem plane. The non-equivalent sides of the high-spin ferric porphyrins, which are due to an out-of-plane displacement of the iron, are averaged by a mechanism whereby the iron moves from side to side through the porphyrin 'hole'. This rapid 'inversion' suggests that the iron atom possesses a high degree of mobility relative to the porphyrin plane.

INTRODUCTION

Since the initial observation¹ of isotropically shifted proton resonances for the haem moiety in oxidized ferricytochrome *c*, n.m.r. in paramagnetic macromolecules has developed² into a powerful new tool for investigating structure and structure-function relationships in such metallo-proteins. The advantage of studying such proteins is that the hyperfine fields³ generated in the vicinity of the metal ion induce line broadening and large chemical shifts outside the normal range of diamagnetic shifts which characterize the bulk of the macromolecule. These shifts³ and the line broadening⁴ can serve as sensitive probes for the local environment near the active site, since the electron-nucleus interactions are relatively short-ranged.

One of the classes of paramagnetic metallo-proteins which has received the most attention is the class of haemo-proteins⁵, the haemoglobins, myoglobins and cytochromes. Numerous n.m.r. studies^{1,2,5} of these proteins have indicated that the observed paramagnetic shifts and/or linewidths are highly characteristic of the electronic structure of the metal ion and the tertiary structure of the protein. Unfortunately, the sheer size and complexity of such macromolecules present formidable problems in interpreting changes in the n.m.r. spectra in terms of changes in the electronic and/or tertiary structure near the active site.

As an aid towards partial understanding of the relationship between structure and the n.m.r. parameters, extensive use has been made of simple model porphyrin complexes⁶⁻¹² which exhibit some of the salient physical, spectroscopic and/or chemical properties of the active sites. Under favourable circumstances, the chemical or structural information derived from such model compounds may be directly applicable towards understanding the molecular basis for certain biological functions of the proteins.

Previously, we have directed our attention to the electronic structure of model compounds as manifested in their paramagnetic shifts^{6,7}. We will concern ourselves here with n.m.r. relaxation studies of two dynamic properties of such model compounds which bear on the ability to observe n.m.r. spectra of high-spin, HS, ferric haemo-proteins^{10,12,13}, and which are relevant to characterizing¹¹ the mobility of the iron atom relative to the haem plane.

It is well known that the ability to observe well-resolved n.m.r. spectra in haemo-proteins, as well as other paramagnetic complexes, depends critically on the oxidation and spin states of the metal ion⁴. For the haemo-proteins⁵, most work has focused on the low-spin, LS, ferric species, which exhibit very narrow lines. Much less attention has been directed towards the HS forms^{7,9,12,13}, which typically yield much more poorly resolved spectra. In order to understand the nuclear relaxation in HS ferric species, we have investigated¹⁰ the proton linewidths (T_2 s), and in some cases T_1 s, of a series of model compounds of the synthetic porphyrins, tetra-*p*-tolylporphyrin, *p*-CH₃-TPP, with general formula *p*-CH₃-TPPFeX, as illustrated in Figure 1, where X is a halide or pseudo-halide.

For the case of the reversible binding of oxygen to haemoglobin or myoglobin, it has been known for some time that the addition of the sixth ligand (O₂) to the active site consisting of an HS ferrous porphyrin with an axial histidyl imidazole causes a spin state change from HS ($S = 2$) to LS ($S = 0$). The systematic characterization of the stereochemistry¹⁴ and magnetic properties of a variety of model iron porphyrin complexes has revealed that

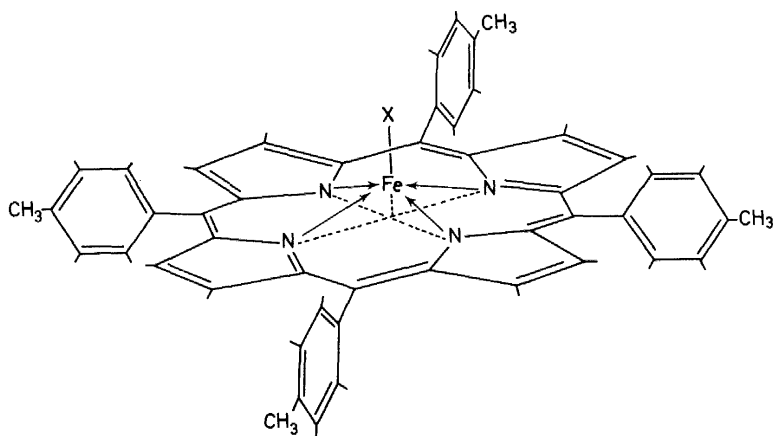


Figure 1. Structure of tetra-*p*-tolylporphyrin iron(III) chloride, *p*-CH₃-TPPFeCl. Note the displacement of the iron in the direction of the halogen atom X.

the HS forms suffer a displacement of the iron atom out of the porphyrin plane, reflecting the inability of the tetrapyrrole hole to accommodate the large HS ions. This accounts for the dominance of five-coordination in HS iron porphyrins, as exemplified in *Figure 1*. On addition of a sixth ligand, the spin multiplicity of the ground state decreases, yielding diamagnetic ferrous and spin-doublet ferric species whose reduced ionic radii are easily accommodated within the porphyrin plane. This pattern of HS, out-of-plane, and LS, in-plane, iron is also observed in haem proteins¹⁴⁻¹⁶, with displacements of $\sim 0.5 \text{ \AA}$ for ferric ions. X-ray data for ferrous haem proteins has not yet yielded this displacement distance, though it has been estimated^{3, 16} to be $\sim 0.8 \text{ \AA}$.

This conversion of iron(II) from an out-of-plane, HS, to an in-plane, diamagnetic, six-coordinate species upon oxygenation is typified by the tetra-haem protein, haemoglobin¹⁷. A remarkable property of this protein is that it exhibits cooperativity among its sub-units, even though the haems are separated by some $\sim 30 \text{ \AA}$. The biochemical trigger which precipitates this cooperativity has been proposed¹⁸ to originate from the sizeable rearrangement imposed on the protein chain of the globin molecule on the reduction of the distance between the F-8 histidine and the porphyrin π plane by some 0.8 \AA upon oxygenation of one sub-unit. This process is diagrammatically presented in *Figure 2*. In fact, within the 'entatic' theory of the action of haem proteins, Williams has proposed¹⁹ that the protein enforces a strained configuration on the haem moiety such that there exists some form of 'mobility' of the iron atom relative to the porphyrin π plane. This 'entatic' state is thought to facilitate the $\text{HS} \rightleftharpoons \text{LS}$ interconversion required for efficient uptake

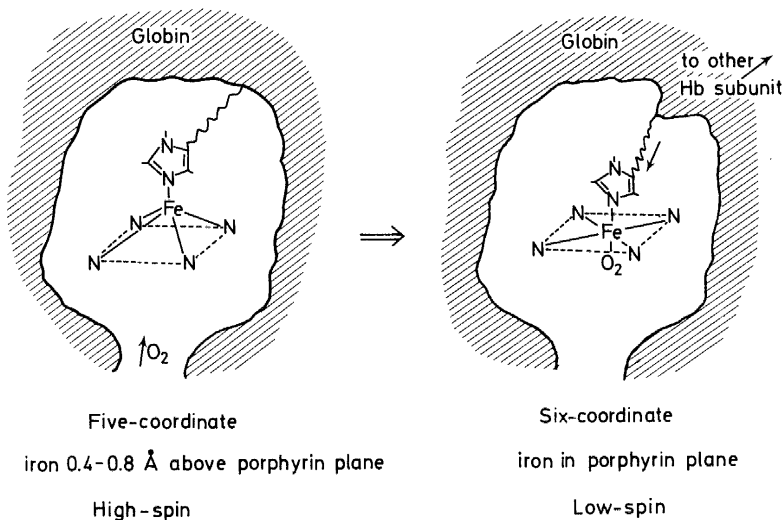


Figure 2. Pictorial representation of the rearrangement of the haem cavity which accompanies the conversion of high-spin, five-coordinate iron to low-spin, six-coordinate iron upon binding oxygen. Pulling on the globin chain attached to the histidine makes the haem cavity of the neighbouring sub-unit more accessible for the next oxygen. This globin rearrangement due to the motion of the iron relative to the haem plane is thought to trigger the cooperativity in haemoglobin.

and release of oxygen. To date, however, nothing quantitative is known about the magnitude of the stabilization energy resulting from the out-of-plane displacement of the iron atom, so that no realistic estimate can be made of the role played by the protein in lowering this barrier towards bringing the metal nearer the plane. The magnitude of this barrier would lead to insight into the degree of 'mobility' of the iron atom relative to the porphyrin plane.

We will show here that n.m.r. linewidths can, under certain favourable conditions, yield information¹¹ on the mobility of the HS iron relative to the porphyrin plane.

Nuclear relaxation by T_1 processes

In the absence of scalar hyperfine coupling, nuclear relaxation in paramagnetic complexes proceeds primarily via an intramolecular nuclear-electron dipolar interaction^{4,20}. The appropriate longitudinal (T_1) and transverse (T_2) relaxation times are given by

$$T_{1N}^{-1} = 2B \left[3\tau_c + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \right] \quad (1)$$

$$T_{2N}^{-1} = B \left[7\tau_c + \frac{13\tau_c}{1 + \omega_s^2\tau_c^2} \right] \quad (2)$$

where $B = \gamma_N^2 g^2 \beta^2 S(S+1)/15r^6$ (r = metal-nucleus distance). τ_c is the dipolar correlation time²¹ with

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} \quad (3)$$

where τ_r and τ_s are the correlation times for rotational motion of the complex, and the electron spin-lattice relaxation time, respectively. In a Lorentzian line, the linewidth, δ , is related to T_2 by

$$\pi\delta = T_{2N}^{-1} \quad (4)$$

In the limit of fast motion, where $\omega_s^2\tau_c^2 \ll 1$, we have

$$T_{1N}^{-1} = T_{2N}^{-1} = 20B\tau_c \quad (5)$$

In slow correlation times, with $\omega_s^2\tau_c^2 \gg 1$, we obtain

$$T_{1N}^{-1} = 6B\tau_c \quad (6)$$

$$T_{2N}^{-1} = 7B\tau_c$$

or

$$T_{1N}/T_{2N} = 7/6 = 1.16 \quad (7)$$

Both equation (5) and equation (6) predict a linear dependence on τ_c of the relaxation rates of a series of isostructural complexes with identical spin states and g values. Two possible limiting cases can be envisaged for the dependence of T_2 s or τ_c .

Case (a). If $\tau_s \gg \tau_r$, then $\tau_c = \tau_r$, and the relaxation rates should depend only on the rotational tumbling time of the complex, which may be assumed to be invariant in a series of isostructural compounds.

Case (b). If $\tau_s \ll \tau_r$, $\tau_c = \tau_s$, in which case the trend in relaxation times in a series of isostructural complexes would reflect relative τ_s 's.

It may be noted that with a knowledge of the structure (r) and the magnetic properties (g , S), equations (2) and (4) permit the determination of τ_c . The validity of this calculated τ_c may be established by independently measuring T_1 and demonstrating that either $T_1/T_2 = 1.0$ if $\tau_c^{-1} < \omega_s$ or $T_1/T_2 = 7/6$ if $\tau_c^{-1} > \omega_s$. Comparison of τ_c with reasonable available estimates of τ_r will determine whether τ_r or τ_s dominates τ_c .

Nuclear relaxation by T_2 processes

Such relaxation occurs in the commonly observed phenomenon of line broadening due to chemical exchange between two (or more) magnetically nonequivalent sites where the exchange rate, k , in frequency units is comparable to the difference in chemical shifts, in hertz, between the two sites²². With respect to elucidating the mobility of the iron relative to the haem plane in the five-coordinated, HS, ferric porphyrin shown in *Figure 1*, it should be noted that this out-of-plane displacement of the iron¹⁴ makes the two sides of the porphyrin plane non-equivalent. Hence, for the m -H of the *meso*-phenyl groups, which crystal structures as well as molecular models reveal to be sterically inhibited from free rotation¹⁴, two separate resonances may be expected. Such a pair of peaks has been observed^{7, 9, 11}, as illustrated in the proton n.m.r. trace in *Figure 3*.

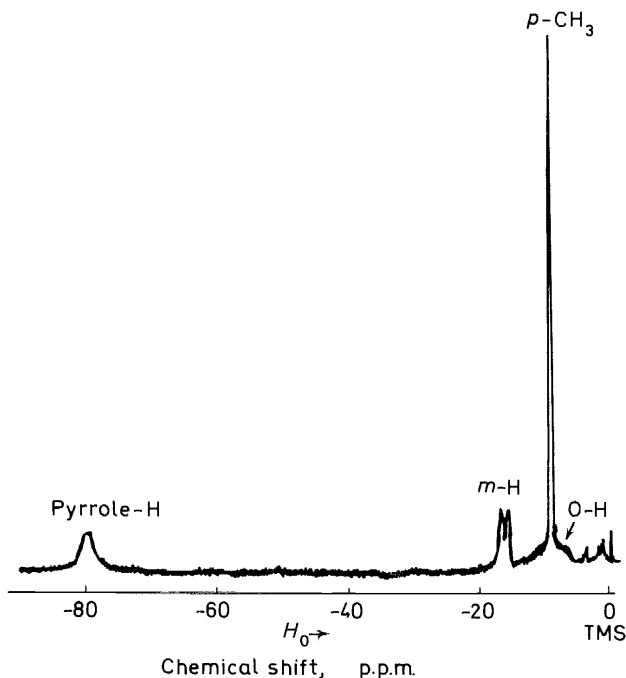


Figure 3. Proton n.m.r. trace of a 0.05 M CDCl_3 solution of $p\text{-CH}_3\text{-TPPFeCl}$ at 35°C : shifts are given in p.p.m. from TMS.

The dynamic averaging of these non-equivalent *m*-H environments can be effected by forcing the iron atom to pass from one side of the porphyrin, through the 'hole', to the other side. One simple method for inducing¹¹ this 'inversion' is by the addition of an excess halide ion which could interchange the two *m*-H environments via the mechanism depicted in *Figure 4*. If the rate of exchange can be made fast enough, standard analysis of the line broadening²² will yield the kinetic parameters of interest.

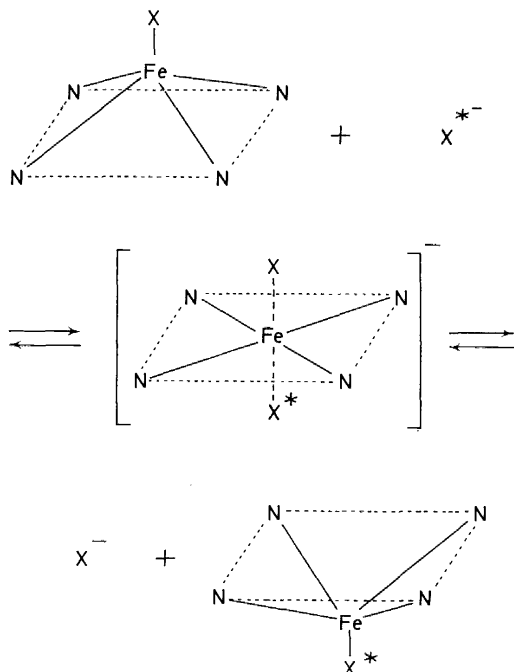


Figure 4. Mechanism for porphyrin 'inversion' via halogen exchange by which the non-equivalent *m*-H signals are dynamically averaged.

EXPERIMENTAL

Sample preparation—The following compounds, previously characterized¹⁰, were used: *p*-CH₃-TPPFeX with X = Cl, Br, I, N₃, and *p*-CH₃-TPPCrX, with X = Cl, I, N₃. Solutions were prepared approximately 0.02 M in chloroform-*d*. In the solutions where excess halide ion was required, this was added in the form of the appropriate tetra-*n*-butyl-ammonium halide salt, Bu₄N⁺X⁻.

NMR spectra—Proton linewidth (*T*₂) measurements for the methyl protons were made on a Jeol PS-100 pulse Fourier transform spectrometer interfaced with a Digilab NMR-3 128K word disc data system. Proton measurements were made at 23.5 kG, or 100.0 MHz. All shifts are in p.p.m., referenced against internal TMS. The linewidth, δ , defined as the full width of the peak at half-height, was obtained for delay times between successive pulses

which were $\geq 10T_1$ s. T_2 s were obtained by the relation $\pi\delta = T_2^{-1}$ after correcting the observed linewidth for the linewidth observed for the same position in an analogous diamagnetic nickel(II) porphyrin complex; hence, magnetic inhomogeneity effects and diamagnetic relaxation contributions have been eliminated in calculating the T_2 due to paramagnetic relaxation. Methyl proton T_1 measurements were made on a 0.01 M CDCl_3 solution of $p\text{-CH}_3\text{-TPPFeCl}$ using the usual $180^\circ\text{-}\tau\text{-}90^\circ$, or 'inversion-recovery', pulse sequence²³. The 90° pulse width was determined to be 20 μs . The carrier frequency was located 1.6 kHz downfield (at higher frequency) of the methyl resonance to insure the 90° pulse. Although this arrangement caused 'folding-over' of the pyrrole-H peak located 5.0 kHz below the carrier frequency, the folding-over occurred in a portion of the 8 kHz band width which was of no interest. τ values in the $180^\circ\text{-}\tau\text{-}90^\circ$ sequence were selected in the closest interval permitted by the Digilab pulse controller. T_1 values were obtained by the standard analysis²³ of the relation

$$\ln(I_\infty - I_\tau) = 2 \ln I_\infty - \tau/T_1 \quad (8)$$

where I_τ and I_∞ are the methyl peak intensities with delay times between the 180° and 90° pulses of τ and $\geq 10\tau$.

RESULTS

Table 1 summarizes the T_2 and T_1 data for the methyl resonances of $p\text{-CH}_3\text{-TPPFeX}$. Also included for comparison are the analogous data for the $p\text{-CH}_3\text{-TPPCrX}$ complexes¹⁰. T_1 and T_2 values are given in ms. The proton n.m.r. traces for the methyl region as a function of τ in the $180^\circ\text{-}\tau\text{-}90^\circ$ pulse sequence are illustrated in Figure 5 for $p\text{-CH}_3\text{-TPPFeCl}$. The complete

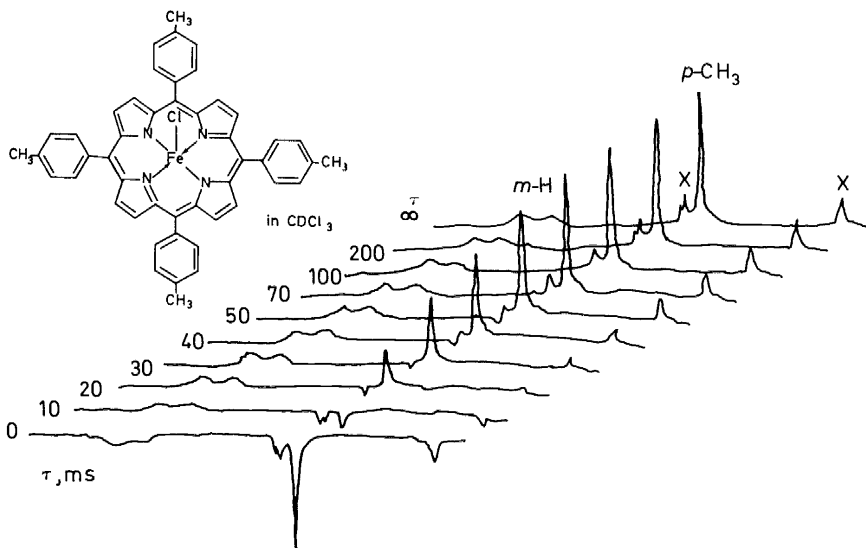


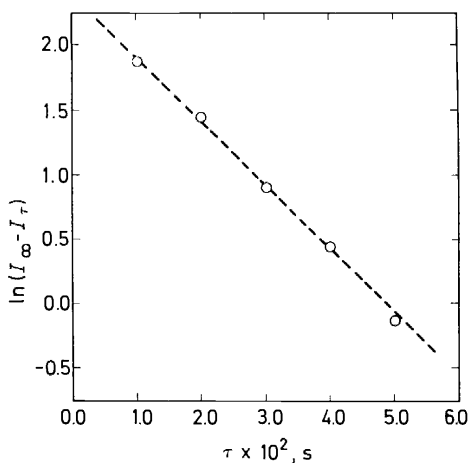
Figure 5. Proton n.m.r. traces for $p\text{-CH}_3\text{-TPPFeCl}$ in CDCl_3 in the methyl region (see Figure 3) as a function of τ obtained by the $180^\circ\text{-}\tau\text{-}90^\circ$ pulse sequence: τ is given in milliseconds.

Table 1. Methyl proton relaxation times in $p\text{-CH}_3\text{-TPPMX}^a$.

M \ X		I	Br	Cl	N ₃
M = Cr(III)	CH ₃ T ₂ s	4.5 ± 0.4	—	4.9 ± 0.4	4.5 ± 0.4
M = Fe(III)	CH ₃ T ₂ s	48 ± 5	26 ± 3	19 ± 2	17 ± 2
	CH ₃ T ₁ s	—	—	21 ± 1	—
	Rel. ZFS from T ₂	1.6	1.1	1.0	0.9
	Rel. ZFS ^b from IR	1.83	1.32	1.00	0.82

(a) T₁s and T₂s in milliseconds, at 30°, ~0.02M in CDCl₃.

(b) Data taken from Ref. 26.

Figure 6. Plot of $\ln(I_\infty - I_\tau)$ versus τ , obtained from Figure 5, for $p\text{-CH}_3\text{-TPPFeCl}$ in CDCl_3 ; the slope yields $T_1 = 21$ ms.

trace, reported earlier^{7,9}, is reproduced in Figure 3. The plot of $\ln(I_\infty - I_\tau)$ versus τ for $p\text{-CH}_3\text{-TPPFeCl}$ is shown in Figure 6.

The effect of $[\text{Cl}^-]/[p\text{-CH}_3\text{-TPPFeCl}]$, temperature and complex concentration on the $m\text{-H}$ linewidth for solutions of $p\text{-CH}_3\text{-TPPFeCl}$ containing excess Cl^- in the form of tetra-*n*-butyl ammonium chloride, $\text{Bu}_4\text{N}^+\text{Cl}^-$, are illustrated in Figure 7.

DISCUSSION

Methyl proton relaxation in $p\text{-CH}_3\text{-TPPMX}$ —The results in Table 1 indicate that the T_2 s for $\text{M} = \text{Cr(III)}$ are insensitive¹⁰ to X, while those for Fe(III) vary dramatically with X. Using equations (2) and (4), with $r = 9.5 \text{ \AA}$ for $p\text{-CH}_3$, $g = 2.0$, $s = \frac{3}{2}$ and the linewidth due to paramagnetic relaxation, we obtain $\tau_c = 4 \times 10^{-10}$ s for the Cr(III) compounds. Dielectric relaxation data on haemin²⁴ and estimates by the Debye equation¹⁰ agree on a value for $\tau_r = 3$ to 5×10^{-10} . Hence, for Cr(III) we have case (a), with $\tau_c = \tau_r$. The condition $\tau_s \gg \tau_r$ for Cr(III) complexes is consistent with their well-resolved

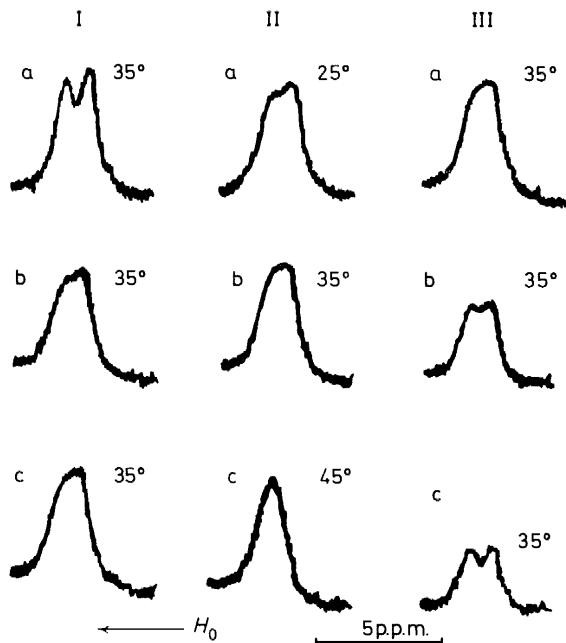


Figure 7. Proton n.m.r. trace for *m*-H in *p*-CH₃-TPPFeCl in CDCl₃: I. 0.04 M complex at 35° with (a) no Bu₄NCl, (b) 0.17 M Bu₄NCl, (c) 0.26 M Bu₄NCl; II. 0.04 M complex, 0.26 M Bu₄NCl at (a) 25°, (b) 35°, (c) 45°; III. [Bu₄NCl]/[complex] \cong 6.4, with [complex] at (a) 0.04 M, (b) 0.027 M, and (c) 0.02 M.

ambient temperature e.s.r. spectra. The insensitivity of T_2 to X is due to the fact that the extremely large size of the porphyrin complex dominates its motional characteristics in solution, with X acting only as a minor perturbation.

For *p*-CH₃-TPPFeCl, for example, the corrected linewidth gives $T_2 = 19$ ms, which with equation (2) yields $\tau_c = 2.5 \times 10^{-11}$ s. Since $\omega_s = 4.13 \times 10^{11}$ s⁻¹ at 23.5 kG, $\omega_s^2 \tau_c^2 \gg 1$, and we expect $T_1/T_2 = \frac{7}{6} = 1.16$. The 180°- τ -90° pulse sequence data for *p*-CH₃-TPPFeCl, illustrated in Figures 5 and 6, yield $T_1 = 21$ ms. The experimental ratio, $T_1/T_2 = 1.1$, is therefore consistent with the prediction for the calculated τ_c . However, T_1 and T_2 are in fact within experimental error of each other, so that not too much emphasis can be placed on the exact value of τ_c .

The calculated τ_c for *p*-CH₃TPPFeCl is much shorter than τ_r , which should be the same for Cr(III) and Fe(III) complexes, so that we conclude that we have case (b) for the Fe(III) species, with $\tau_c = \tau_s$. The variation in T_2 s for the Fe(III) complexes with X therefore reflects systematic changes in τ_s . Theoretical consideration²⁵ of electron-spin relaxation in complexes with $s \geq 1$ have indicated that τ_s is usually determined by the modulation of the zero field splitting, ZFS, by the motion of the complex in solution. Since the motion of the complexes can be assumed to be independent of X, we obtain the relation¹⁰

$$\tau_s^{-1} \propto D^2 \quad (9)$$

which, when combined with equation (6), yields

$$T_2 \propto D^2 \quad (10)$$

That the observed trend in T_2 s, $I > Br > Cl > N_3$, is semi-quantitatively consistent with this relaxation mechanism is evidenced by the great similarity of the relative values of the ZFS parameters obtained from the relative T_2 s, and the relative values of the ZFS parameter determined directly²⁶ by far infra-red e.s.r. techniques for the analogous deuterohaemins. These two sets of values for the relative ZFSs are also listed in *Table 1*, with the $X = Cl$ ZFS normalized to unity.

Since the ligand field strengths of X vary in the order $N_3 > Cl > Br > I$, it can be concluded that increasing axial field strength will broaden the n.m.r. signal of an HS ferric haem, while decreasing it will yield narrower lines. Although no reports on linewidth changes have appeared for ferric haemo-proteins, our study suggests that the observation of such changes could, in principle, be interpreted in terms of changes in the axial field of the haem group.

Axial mobility of HS iron—On addition of excess halide ion, in the form of $Bu_4N^+Cl^-$, to a solution of $p-CH_3-TPPFeCl$, it was observed¹¹ that the two $m-H$ peaks broadened and collapsed into a single peak, as illustrated in *Figure 7*. This figure depicts the effects of added chloride ion, temperature and concentration on the dynamic averaging of the two signals which establish a bimolecular mechanism for the process, as predicted by the model in *Figure 4*.

The elimination of an alternative mechanism for averaging the two $m-H$ environments, namely phenyl group rotation, was based on the observation¹¹ of a similar collapse of the diastereotopic α -methylene doublet for octaethylporphyrin iron(III) chloride⁹. For this porphyrin, it was shown that only 'inversion' can average the two peaks.

Analysis of the line broadening has not yet permitted the determination of the kinetic parameters owing to the extensive ion pairing of $Bu_4N^+Cl^-$. However, the rate of inversion, $k \sim 10^2-10^3 \text{ s}^{-1}$, indicates that the barrier to bringing the metal into the plane is not too large.

Further evidence for only a small difference between the in-plane and out-of-plane configurations for the HS ferric ion is derived from the proton spectrum of the analogous iodide complex, $p-CH_3-TPPFeI$. This compound exhibits some 'inversion' even in the absence of added I^- , exhibiting a broadened set of $m-H$ peaks for a 0.04 M $CDCl_3$ solution. These broadened lines sharpen significantly when either the concentration or temperature is lowered. At 0.08 M the linewidth indicates an inversion rate in the range 10^3-10^4 s^{-1} .

Preliminary evidence suggests that the mechanism for averaging the two $m-H$ peaks in the iodide complex differs from that for the chloride complex¹¹ (*Figure 4*). The likely mechanism for the iodide is due to dissociation of the complex:



where the signal averaging occurs primarily because of the rapid 'inversion' of the ionic species. Hence, this greatly increased rate of 'inversion' for the

dissociated species indicates that the HS iron is highly mobile with respect to its position above the porphyrin plane. This preliminary result suggests that attributing¹⁹ an important role to the globin molecule in lowering the barrier for bringing the iron near the porphyrin is perhaps unnecessary. Further n.m.r. work in progress should shed more light on this problem.

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