Proton Nuclear Magnetic Resonance Studies on Haemin Chloride† in Pyridine–Water Solution

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The effect of adding water to a pyridine (py) solution of protoporphyrin IX iron(III) chloride (haemin chloride) has been investigated by high-resolution (500 MHz) proton n.m.r. spectroscopy. When the amount of water is small (50% or less by weight), several iron(III) porphyrin species exist in solution in equilibrium; the solution contains six-co-ordinated high-spin [Fe^{III}(pp)(py)Cl], 'low-spin' [Fe^{III}(pp)$(py)_2$]Cl, and the aqua-haemin complex [Fe^{III}(pp)(py)(H_2O)]Cl (H_2pp = protoporphyrin IX). At higher concentrations of water, only [Fe(pp)(py)(H_2O)]Cl exists in the solution. The dependence of proton chemical shifts on pH was investigated and the results interpreted on the basis of a rapid exchange mechanism between the aqua and hydroxo complexes of the haemin. The aqua complex shows anomalous temperature dependence of the isotropic proton shifts for the ring methyl protons. These results, together with the temperature dependent magnetic moments in solution, indicate the existence of a spin equilibrium between the high-spin and low-spin states of the iron(III) ion in [Fe(pp)(py)(H_2O)]Cl in solution.

The haemoproteins are an important class of protein which show a large variety of biological functions.[1,2] The active site of the reduced haemoprotein contains a haem group which is a protoporphyrin iron(II) complex. In the oxidised form the haem group is haemin, which is an iron(III) protoporphyrin complex. The nature of axial ligands, the redox potentials, and the spin state of the iron in the active site are crucial in determining the structure–function relationship in haemoproteins.[3]

The study of ligand-exchange reactions with concomitant change in structure–function relationship has attracted considerable attention in recent years. Protoporphyrin IX‡ iron(III) chloride (henceforth called haemin chloride, Figure 1) in neat dry pyridine (py) shows the presence of an equilibrium mixture of high-spin ($S = \frac{5}{2}$) chloropyridineiron(III) protoporphyrinate, [Fe^{III}(pp)(py)Cl], and predominantly low-spin ($S = \frac{1}{2}$) [Fe^{III}(pp)$(py)_2$]Cl, in addition to the existence of a small amount of low-spin ($S = 0$) [Fe^{II}(pp)$(py)_2$], formed slowly over a period of time. This has been verified by optical spectroscopy,[4] n.m.r.,[4] and Mossbauer[5] studies. However, when organic solvents such as chloroform or methanol are added to the haemin chloride–pyridine solution formation of the iron(II) species is known to be inhibited.[6]

The structure and physical properties of iron(III) porphyrins in pyridine–water solutions have received considerable attention.[7–11] In particular, Degani and Fiat[10] reported a proton n.m.r. study on haemin chloride in pyridine–water and established that both pyridine and water were axially co-ordinated forming a six-co-ordinated aqua iron(III) protoporphyrin complex, [Fe(pp)(py)(H_2O)]Cl. They observed an anomalous temperature dependence of the isotropic proton shifts (i.p.s.) for the ring methyl protons, the i.p.s. showing sharp 'discontinuities' near room temperature. Degani and Fiat[10] had assumed that only the above aqua complex was present in the solution and ascribed the anomalies in the temperature dependence of the i.p.s. to a high-spin ⇌ low-spin thermal equilibrium in the complex. The study did not take into account the possibility of multiple equilibria of various haemin complexes existing in the solution and the dependence of these equilibria on the concentration of water present in the solution. Bartocci *et al.*[12] have recently reported u.v.–visible spectra of haemin chloride in pyridine–water at different pH and observed that the aqua species in the solution show an acid ⇌ base (aqua ⇌ hydroxo) equilibrium as the pH changes. Although such an equilibrium is known to exist in aqua metmyoglobin,[13,14] detailed n.m.r. investigation of the aqua ⇌ hydroxo equilibrium in simple model haem systems is lacking. More recently, Castro and Anderson[11] have studied by u.v.–visible spectroscopy [Fe(oep)Cl] (H_2oep = 2,3,7,8,12,13,17,18-octaethylporphyrin) in pyridine–water and observed the various species present in solution in equilibrium.

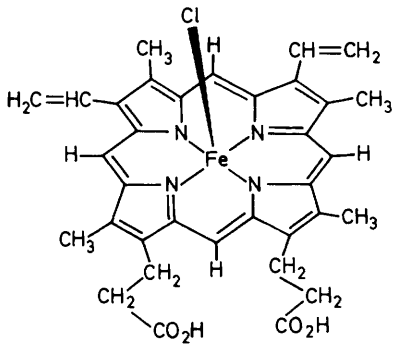

Figure 1. The molecular structure of protoporphyrin IX iron(III) chloride (haemin chloride)

A detailed accurate high resolution n.m.r. investigation of haemin chloride in pyridine–water as a function of temperature and pH appeared desirable. First, it is not clear from the studies of Degani and Fiat why the spin-equilibrium transition in the aqua haemin complex should be so sharp while such spin transitions in haem proteins and metalloporphyrins are generally of the 'continuous' type occurring over a wide temperature range.[13–15] Secondly, it is known that haemin chloride in pure pyridine stabilises several iron(III) and iron(II) porphyrin complexes with different spin states.[6] It would be interesting to examine the effect of the gradual addition of water on these complexes. Thirdly, an n.m.r. study as a function of pH and temperature at different concentrations of water would aid

† Chloro(3,8,13,17-tetramethyl-7,12-divinylporphyrin-2,18-dipropionato)iron(III).

‡ 3,8,13,17-Tetramethyl-7,12-divinylporphyrin-2,18-dipropionic acid (H_2pp).

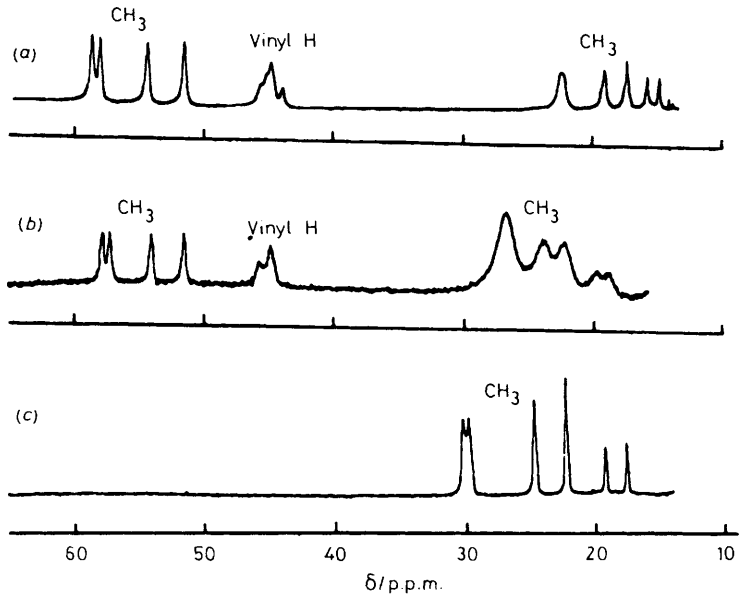

Figure 2. The ^1H n.m.r. resonances of the methyl protons in the high- and low-spin regions of haemin chloride in water–pyridine solutions at ratios (*a*) 0:1, (*b*) 0.1, and (*c*) 5:1 (v/v). Concentrations of the solutions are *ca.* 10 mol dm^{-3} although they are not identical in each run. The number of transients taken for the data acquisition are also not the same in each case

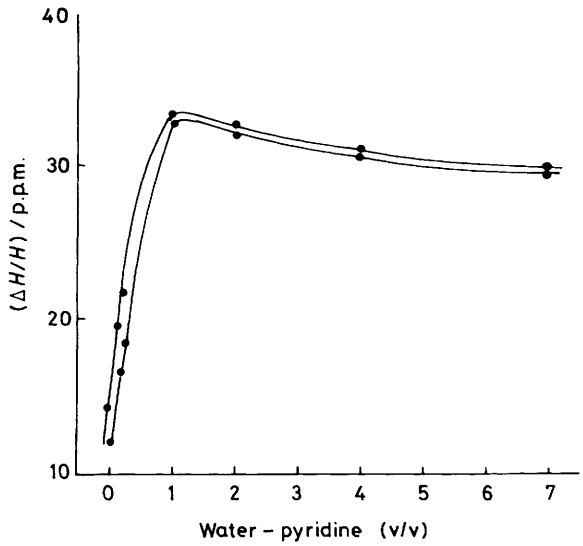

Figure 3. Variation in the low-spin two methyl proton i.p.s. (in the range 10—35 p.p.m.) with increasing amounts of water

our understanding of the electronic properties of the aqua and hydroxo species formed in the solution. These reasons together with the observation[10] that the aqua complex has structural similarity to the prosthetic group in aqua metmyoglobin prompted us to undertake a detailed proton n.m.r. and magnetic study in solution. In this paper we report the temperature dependence of magnetic moments and accurate ^1H n.m.r. measurements (at 500 MHz) on haemin chloride in $[^2H_5]$-pyridine–D_2O at different pH and water–pyridine ratios.

Experimental

All materials used were pure, their purity being checked by n.m.r., visible, and analytical measurements. Proton n.m.r. spectra were recorded on a Bruker AM 500 MHz Fourier-transform n.m.r. spectrometer. The temperatures were stable to within ±0.5 °C. The chemical shifts are reported with respect to sodium $[^2H_4]$3-trimethylsilylpropionate as internal standard. Downfield shifts are taken as positive.

Optical spectra were recorded on a Cary 17D u.v.–visible spectrometer and pH measurements performed with a digital pH meter. The pH was adjusted by dropwise addition of 0.1 mol dm^{-3} HCl or NaOH (DCl or NaOD for n.m.r. experiments). The pH was measured at 25 °C before and after each run and the reproducibility was ±0.1 unit. No isotopic correction was applied to the pH values however. Measurements were always made on freshly prepared solutions since aged solutions were found to give poorly reproducible results. Typical concentrations of the haemin solutions were 5—10 mmol dm^{-3} (see later comments on aggregation). Solution magnetic moments were determined using the Evans method.[16]

Results and Discussion

Part of the proton n.m.r. spectra of haemin chloride in pure dry pyridine and pyridine–water are shown in Figure 2. The general features and assignments of various resonances for iron(III) porphyrin complexes are well documented in the literature,[6,10,17,18] which we have followed here. We shall consider only the methyl proton resonances, since they can be easily identified, assigned, and are sensitive to structural and electronic changes.

In pure $[^2H_5]$pyridine two sets of characteristic methyl resonances are seen: one in the range 50—60 p.p.m. and the other in the range 15—25 p.p.m. The methyl resonances at 50—60 p.p.m. are characteristic of the six-co-ordinated high-spin monopyridine haemin chloride complex [Fe(pp)(py)Cl],[4,6b] where the pyridine and chloride are believed to be axially co-ordinated. The methyl resonances at 15—25 p.p.m. in pure pyridine have been assigned[4,6] to the 'low-spin' bis(pyridine) complex, $[Fe(pp)(py)_2]Cl$. Figure 2 also shows the effect of addition of water on the methyl resonances. As the water is progressively added the intensity of the high-spin resonances (50—60 p.p.m. region) decreases although their i.p.s. remain

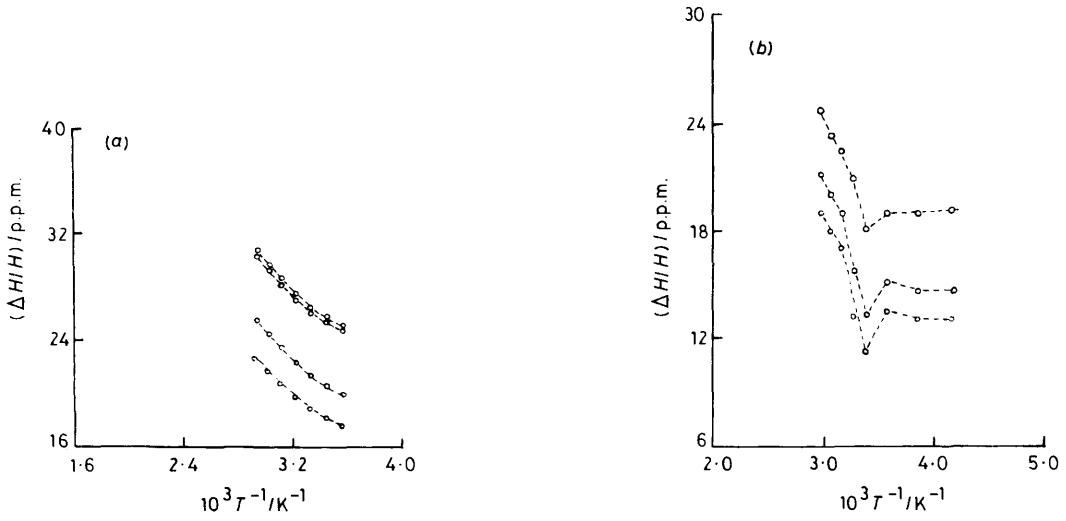

Figure 4. The temperature dependence of the low-spin methyl proton resonances at water–pyridine ratios (*a*) 5:1 and (*b*) 0.1:1 (v/v). The data in Figure 4(*a*) refer to pH *ca.* 7.6

nearly unchanged. When the water concentration became very high (water–pyridine ≥ 1:1 v/v), these resonances completely disappeared. The 'low-spin' resonances lying at 15—25 p.p.m. in pure pyridine also decreased in intensity but moved towards the lowfield side as the concentration of water increased. For a water–pyridine ratio of 1:1 or more, these resonances lie in the range 23—30 p.p.m. Figure 3 shows the variation in the methyl i.p.s. with increasing water concentration.

The above observations suggest that as the water is slowly added, the high-spin monopyridine and low-spin bis(pyridine) complexes are progressively converted into the aqua complex, [Fe(pp)(py)(H_2O)]Cl, so that at very high concentrations (water–pyridine ≥ 1:1 v/v) the solution contains only the aqua complex. At lower water concentrations, however, the solution contains all the three complexes in equilibrium: high-spin [Fe(pp)(py)Cl], low-spin [Fe(pp)($py)_2$]Cl, and the aqua complex [Fe(pp)(py)(H_2O)]Cl. It is interesting that Degani and Fiat[10] were unable to detect [Fe(pp)(py)Cl] presumably due to instrumental limitations, although their experiments were done at low water concentrations (*i.e.* 7—8% water, see Figure 1 of ref. 10). They had erroneously assumed, therefore, that only the aqua complex was present in the solution.

Temperature Dependence of N.M.R. Shifts.—Figure 4 shows the temperature dependence of the methyl protons resonances at two water–pyridine ratios. When the amount of water is small (*i.e.* water–pyridine 0.1:1 v/v), the isotropic shift varies in a complicated manner with temperature and shows sharp discontinuity [Figure 4(*b*)]. Degani and Fiat[10] observed an almost similar variation and ascribed the sharp changes in the shift to a phase transition between the high- and low-spin states of the aqua species. It is evident from the foregoing observations that at the concentration of water as in Figure 4(*b*) several iron(III) species exist in equilibrium, and hence the observed temperature dependence of the i.p.s. cannot be ascribed to the aqua complex alone. Figure 4(*a*) however shows the temperature dependence of the i.p.s. of the methyl protons in the presence of a large excess of water. The qualitative difference in the two situations is obvious. As we have discussed above, the solution in the case of Figure 4(*a*) contains only the aqua haemin species, and hence the temperature dependence of the i.p.s. for the aqua haemin complex [Fe(pp)(py)(H_2O)]Cl.

We now discuss the results of Figure 4(*a*) in some detail, especially in terms of the electronic structure of the aqua haemin complex. The four methyl and vinyl proton resonances show similar temperature dependence, deviating strikingly from Curie law. It is observed that the i.p.s. increase with increasing temperature, which is opposite to the Curie law. The variable temperature i.p.s. data give a clear indication that the iron(III) ion in the aqua complex does not possess a simple high-, low- or intermediate-spin ground state. The room temperature magnetic moment of the sample in solution is found to be 4.29 B.M. and the magnetic moment increases with increasing temperature (see later). By analogy with very similar results observed for other iron(III) porphyrins, especially [Fe(oep)-($py)_2$]X,[19–21] the most likely explanation is that the aqua complex is involved in a thermal high-spin ⇌ low-spin spin equilibrium with low-spin being the energetically favoured ground state.

The above suggestion can be checked by a theoretical calculation of the i.p.s. on a high-spin ⇌ low-spin thermal spin-equilibrium model. The calculation is based on a crystal-field model and similar to that recently described by us and others.[6b,19b,22–24] The i.p.s., ($\triangle H/H$), arising from the presence of unpaired electrons consists of two contributions, the contact and the dipolar terms [equation (1)] where the contact

$$(\Delta H/H)_{\text{i.p.s.}} = (\Delta H/H)_{\text{c.s.}} + (\Delta H/H)_{\text{d.s.}} \quad (1)$$

and dipolar terms, $(\triangle H/H)_{\text{c.s.}}$ and $(\triangle H/H)_{\text{d.s.}}$, can be written by equations (2) and (3); r is the magnitude of the radius vector

$$(\Delta H/H)_{\text{c.s.}} = h\sum A_i\langle S_{iz}\rangle/(g_N\beta_N H) \quad (2)$$

$$(\Delta H/H)_{\text{d.s.}} = (\tfrac{1}{3}N)(K_\perp - K_\parallel)(3\cos^2\theta - 1)/r^3 \quad (3)$$

between the metal ion and proton, θ is the corresponding angle between the radius vector normal to the porphyrin plane, A_i is the hyperfine coupling constant associated with the state i, and $K_\perp - K_\parallel$ is the anisotropy in the molar susceptibilities. As discussed earlier,[6b,19b] the $K_\perp - K_\parallel$ and $\sum A_i\langle S_{iz}\rangle$ terms are calculated using crystal-field parameters appropriate to the spin-equilibrium model. The geometric factors are obtained from the known structural data. Additionally the mixing within states by spin-orbit coupling (ζ) and the difference in the metal–

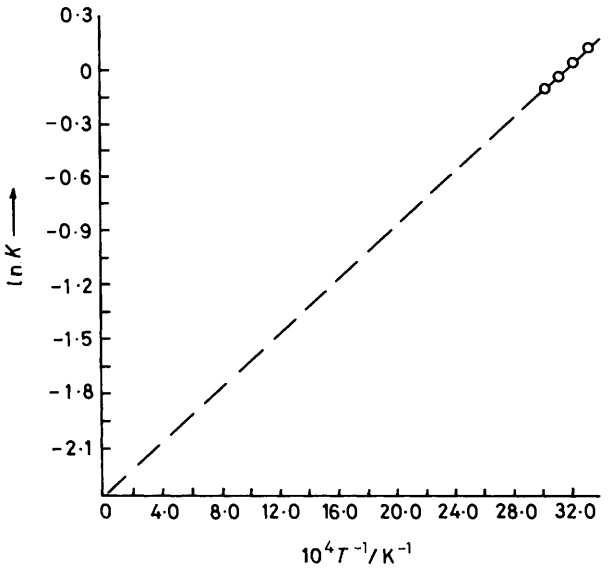

Figure 5. The temperature variation of the equilibrium constant (K) for the spin-transition $S = \frac{5}{2} \rightleftharpoons S = \frac{1}{2}$ of haemin chloride in a water–pyridine ratio of 5:1 (v/v)

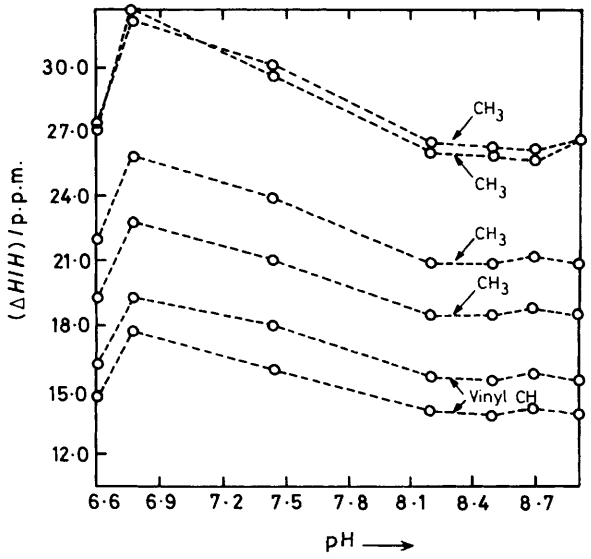

Figure 6. Variations of the i.p.s. of the low-spin methyl and vinyl proton resonances (range 10—35 p.p.m.) at different pH (water–pyridine *ca.* 5:1 v/v)

ligand bonding in the two spin states (through the vibrational partition function, η) were included. Assuming octahedral symmetry as a first approximation the calculation gave a good fit to the data of Figure 4(*a*) for a $^6A_1 - {}^2T_2$ energy separation of 525 cm^{-1}. In the calculation $\zeta = 400$ cm^{-1} and $\ln \eta = 2.0$ were assumed.[6b] Inclusion of axial symmetry in the crystal field gave results which generally agreed with the above conclusion (the dipolar term was found to be less significant). These simple calculations therefore support the view that the aqua haemin complex in pyridine–water exists in the crossover region between $S = \frac{5}{2}$ and $S = \frac{1}{2}$ with the latter state lying *ca.* 525 cm^{-1} below the former.

Temperature Dependence of the Magnetic Moment.—The measurements of the magnetic moment of the haemin chloride–pyridine–water solution at the water concentration of Figure 4(*a*), when only the aqua complex $[Fe(pp)(py)(H_2O)]Cl$ is present, gave the following values: T/K, μ/B.M.: 300, 4.29; 310, 4.36; 320, 4.43; 330, 4.49; and 340, 4.50. Within the validity of the Curie law the value of μ^2 should be independent of temperature for pure high-spin or low-spin ground-state complexes. The temperature dependence of μ^2 however displays gross deviation from the Curie law and confirms the above deduction that the aqua haemin complex exhibits a thermal spin equilibrium between high- and low-spin states.

The equilibrium constant, K, for the high-spin $\rightleftharpoons$ low-spin spin transition, and its temperature dependence can be calculated by using the relationship[15] (4), where $\mu_{h.s.}{}^2$ and $\mu_{l.s.}{}^2$ are taken as 35 and 4 $B.M.^2$ respectively (h.s. = high-spin, l.s. = low-spin).

$$K = \frac{\mu_{h.s.}{}^2 - \mu^2}{\mu^2 - \mu_{l.s.}{}^2} \quad (4a)$$

$$\ln K = -\Delta H^\circ/RT + \Delta S^\circ/R = -\Delta G^\circ/RT \quad (4b)$$

Figure 5 shows a plot of $\ln K$ *vs.* $1/T$ together with a least-squares fitted line (---) which gives the thermodynamic parameters ΔH° *ca.* −6.3 kJ mol^{-1} and ΔS° *ca.* −21 J K^{-1} mol^{-1}. A positive slope of the $\ln K$ *vs.* $1/T$ fit indicates[15] that in the aqua haemin complex the ground state is low-spin. As the temperature is raised the high-spin state is increasingly populated because of a favourable contribution of the entropy term to the free energy of formation of the high-spin state. This simple consideration indicates that the enthalpy of the reaction, ΔH°, can be correlated to the energy difference between the $^6A_1$ and $^2T_2$ states (−540 cm^{-1}) and the entropy change is related to the change in vibrational partition functions.

pH Variation Study.—Figure 6 illustrates the dependence of the i.p.s. of haemin chloride–pyridine–water solutions on the pH (range 6.6—8.9). As the pH of the solution increases the n.m.r. lines become considerably broadened so that for pH > 9.0 the quality of the spectra is quite poor. The isotropic shift is quite sensitive in general to small changes in the pH, and sharply moves downfield as the pH of the solution increases from 6.6 to *ca.* 7, beyond which the i.p.s. moves upfield as the pH increases further.

Visible spectral studies[12] in the pH range 8.5—11.7 established the presence of an acid–base equilibrium involving a species with pK_a *ca.* 10.5. This equilibrium has been assigned[12] to the process: $[Fe^{III}(pp)(py)(H_2O)]^+ \rightleftharpoons [Fe^{III}(pp)(py)(OH)] + H^+$. In the entire pH range, the propionic acid groups are completely deprotonated. We have recorded the u.v. spectrum of haemin chloride in pyridine–water at pH 8.5 and observed a band centred around 266 nm, which may be due to the presence of pyridinium salts. However at pH values lower than 7.4 the carboxylate groups would be protonated. The pK_a value of this second equilibrium is difficult to obtain due to the limited solubility of the complex.

Since the pK_a of the aqua–hydroxo equilibrium is 10.5[12] the aqua complex would be the sole species at pH *ca.* 7.6 and the hydroxo species will be the sole species at pH 13.5; at intermediate pH, the two species would exist in equilibrium. The observation of only one resonance for each proton suggests that the exchange between the aqua and hydroxo species is fast on the n.m.r. time-scale.

We have noted earlier that at pH *ca.* 13.5 only the hydroxo species are likely to exist in the solution. We measured the magnetic moments at room temperature in solution as a function of pH and observed rather surprisingly that the magnetic moment at pH *ca.* 13 was nearly zero (pH, μ/B.M.:

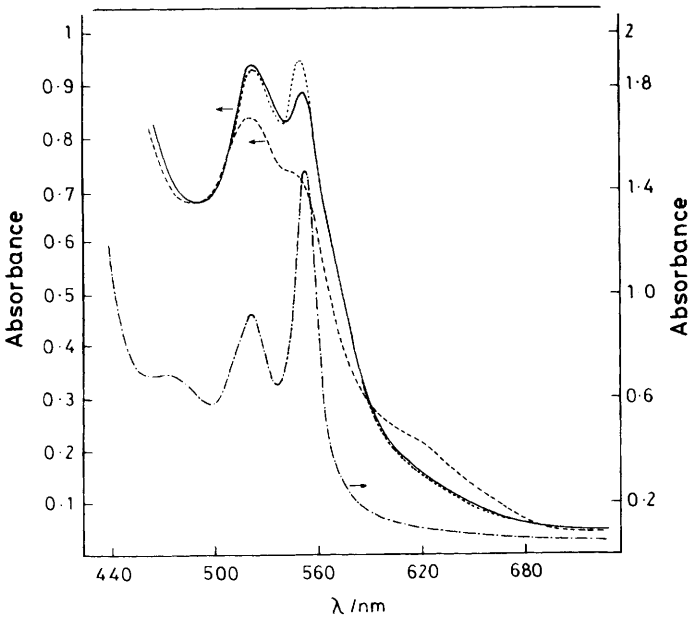

Figure 7. Visible spectra of haemin chloride in: dry pyridine (immediate) (———), dry pyridine (after several hours) (– – – –), pyridine with $NaClO_2$ (suspension) (— — —), and pyridine with $Na_2S_2O_4$ (suspension) (· · · –)

7.0, 4.3; 11.0, 2.14; 13, *ca.* 0). This could be due to hydroxide-induced dimerisation[12] or reduction.[25] The visible spectrum is rather inconclusive in resolving this ambiguity although it seems to favour the former possibility. The sharp change in the i.p.s. below pH 7.4 is presumably due to protonation of the propionic carboxylate groups. It is interesting that there is considerable change in the chemical shifts as a result of this protonation equilibrium. The temperature variation of the i.p.s. at pH 6.6 shows behaviour similar to Figure 4(*a*) suggesting that protonation of the carboxylate groups did not change the nature of the spin equilibrium in the aqua haemin complex.

Stability of Iron(II) *Species in Pyridine–Water.*—We have noted earlier that haemin chloride is autoreduced slowly to low-spin iron(II) species in a solution of pure dry pyridine.[4,6b] Even in the presence of air, the autoreduction appears to dominate over the possible oxidation by aerial oxygen. It has however been found that the presence of water in the solution significantly decreases the possibility of autoreduction of iron(III) porphyrins.[26] In the visible spectrum of haemin chloride in dry pyridine (Figure 7) the ratio of the intensity of the α band maximum (*ca.* 555 nm) to that at the minimum between the α and β bands (β band at *ca.* 525 nm) increases with time, indicating autoreduction of Fe^{III} to Fe^{II}. Reduction of haemin with $Na_2S_2O_4$ in dry pyridine (Figure 7) gives the pure Fe^{II} spectra with the α band being more intense than the β. On the other hand, haemin in dry pyridine in the presence of a small amount of an oxidising agent like $NaClO_2$ returns the Fe^{III} spectra having the more intense β band with a shoulder near the α band. The haemin chloride in 20% pyridine–water (pH *ca.* 7.0) gives spectra very similar to that in dry pyridine with an oxidising agent. Moreover we have found that this spectrum does not change even over a long period of time. Addition of $K_3[Fe(CN)_6]$ to the pyridine–water solution hardly causes any change. These observations indicate that in pyridine–water haemin chloride favours the Fe^{III} state. It has recently been reported[27] that pyridine does not reduce Fe^{III} porphyrins in benzene or dimethylformamide solutions; the same appears to be true for the solution of haemin chloride in water–pyridine.

Conclusions

Degani and Fiat[10] have drawn attention to the structural similarity that exists between aqua metmyoglobin and the aqua haemin complex. In the former the iron(III) ion is axially co-ordinated to water and imidazole of the histidine residue of the protein globin. The iron(III) ion in the latter is axially co-ordinated to water and pyridine. We attempted to stabilise an aqua imidazole iron(III) species, as a better model for metmyoglobin, by dissolving haemin chloride in *N*-methylimidazole (mim) and D_2O. The ^1H. n.m.r. spectrum of the solution gave the chemical shift of the ring methyl protons at 19.72, 19.50, 18.75, and 15.49 p.p.m. in mim–D_2O (1:1), indicating formation of a low-spin ($S = \frac{1}{2}$) bis(mim) complex. This was confirmed further by the temperature variation study, which conformed to the expected Curie law. Addition of excess of water did not change the n.m.r. spectrum indicating that the aqua complex is not formed even at high water–mim ratios. The relative ease of the formation and stability of the aqua pyridine complex in preference to the aqua mim complex seems to suggest as if the protein-bound imidazole behaves like a weak ligand and to some extent resembles the binding of pyridine. No doubt the situation is not so simple as the protein plays an important stereochemical role in modulating the nature and strength of the ligand field in the metalloproteins especially in deciding the role of the apoprotein-bound imidazole as an axial ligand.

The present study has highlighted that the aqua haemin complex $[Fe(pp)(py)(H_2O)]Cl$ is exclusively present in the haemin chloride–pyridine–water solution only when the concentration of water is high. At low water concentrations, the system is complicated and shows presence of the high-spin mono- and low-spin bis-pyridine iron(III) complexes in addition to $[Fe(pp)(py)(H_2O)]Cl$. The hydrophilicity (presence of water) of the medium surrounding the haem inhibits the iron(II) state and favours formation of the iron(III) state.

The aqua haemin complex shows a thermal spin equilibrium between the $S = \frac{5}{2}$ and $S = \frac{1}{2}$ states with S lying rather close to the ground $S = \frac{1}{2}$ state, and the spin equilibrium is very similar to that observed in many haemoproteins including ferrihaemoglobin hydroxide.[21,28] This is however in contrast to the high-spin behaviour of the metmyoglobin. On the other hand, dependence of the chemical shifts on pH indicates an aqua $\rightleftharpoons$ hydroxo equilibrium similar to that observed in aqua metmyoglobin. Thus, although the aqua haemin complex is structurally similar to the aqua metmyoglobin, the electronic properties are not entirely similar, which reinforces the importance of the modulation of the structure of haem by controlling the axial imidazole ligation.

Acknowledgements

The work reported here was carried out at the 500-MHz National N.M.R. Facility, Tata Institute of Fundamental Research. We are grateful to the staff for their co-operation.

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Received 30*th December* 1987; *Paper* 7/2271