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Characterization of pH Dependent Axial Ligation Changes of Monomer and Dimer Forms of Iron(III) Uroporphyrin I in Aqueous Solution

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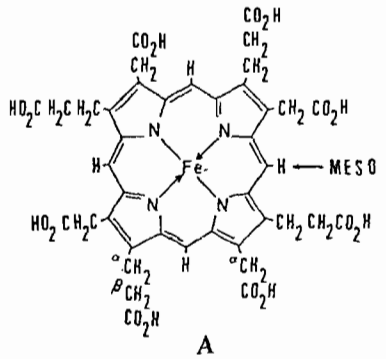
Abstract

It has been demonstrated that aqueous solutions of ferric uroporphyrin I chloride contain two species that are resolvable by proton NMR spectroscopy. These have been identified as monomeric and dimeric species. The proton NMR spectra of monomer and dimer forms of ferric uroporphyrin I chloride exhibit a dramatic pH dependence. This behavior is interpreted as a change in axial ligation from the five coordinate hydroxo complex at high pH to the six coordinate di-aquo complex at low pH for the ferric uroporphyrin monomer. Transition to the same low pH form is also achieved by the ferric uroporphyrin dimer, however the pH dependent behavior involves changes in coordination as well as in the extent of dimerization. The apparent pK for this process is 8.75.

Introduction**

Attention to ferric and ferrous porphyrins arises from their use as relatively simple models for understanding heme protein behavior [1, 2]. The vast majority of NMR work on porphyrins has been carried out in organic solvents due to problems of solubility and aggregation which complicates interpretation of NMR results in aqueous solution [3]. Nevertheless there are several important reasons for attempting to understand the aqueous solution properties of metalloporphyrins. These include the relevance of axial water and hydroxide ion ligation to the iron porphyrin coordination state in intact heme proteins [4], participation of aqueous porphyrins in redox mediated catalysis [5], implication of aqueous hemin in malaria chemotherapy [6], interpreting porphyrin aqueous solution dynamics [7, 8], structure [7, 9], and bonding [9].

One of the simplest porphyrins with respect to its aqueous solution behavior is Urohemin I (ferric uroporphyrin I chloride; FeURO; A). Its structure belies its extensive water solubility and several spectroscopic studies have revealed that its concentration dependent alkaline solution behavior is interpretable on the basis of a monomer-dimer equilibrium [7, 9, 10–14]. One of the advantages of Urohemin I is that at high pH the observed interconversion between the two solution species is slow on the NMR time scale. This has allowed characterization of both species, including the status of axial



A

ligation [7]. In this respect Urohemin I is unique, since most other ferric porphyrins that have been studied by NMR exhibit fast exchange kinetics for aggregation [1, 3, 9, 17, 18]. The results described herein show that in aqueous solution the pH dependence of the monomeric FeURO proton NMR spectrum is characteristic of a transition from a predominantly five coordinate complex at high pH to a monomeric six coordinate complex at low pH. Similarly, the FeURO dimer is transformed from a

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**Abbreviation, FeURO – Iron(III) uroporphyrin I chloride.

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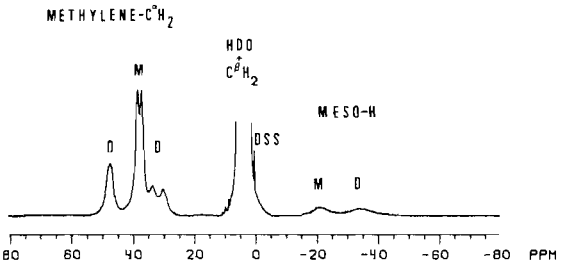

Fig. 1. Proton NMR spectrum of 9.0 mM Urohemin I at pH 12.8 in D_2O, 22 °C taken at 360 MHz. The resonance assignments are indicated as belonging to protons of the monomer (M) or dimer (D) forms. These assignments were made in ref. 7. Most of the residual water resonance centered at 4.6 ppm is omitted in this and subsequent figures for purposes of clarity.

five coordinate, face-to-face species [7], into the monomeric, six coordinate form at low pH.

Experimental

Materials and Methods

Iron(III) uroporphyrin I chloride was purchased from Porphyrin Products (Logan, Utah) and purified by column chromatography on Sephadex G-50-40 in 0.1 M KOH. As previously reported [15, 16], this procedure removes fluorescent contaminants which complicate Raman studies. Samples were further purified by precipitating the column eluent with ethanol, recrystallizing the porphyrin from aqueous ethanol (60% water–40% ethanol), vacuum drying then reprecipitating the FeURO from aqueous solution at pH 4–5. The samples so prepared were vacuum dried at room temperature over magnesium sulfate (Drierite). Heating was not used in order to prevent porphyrin decomposition and consequently some water of crystallization remains according to elemental analysis: found: C, 48.3%; H, 4.72%; N, 5.64%; calculated for FeURO·$5H_2O$ ($C_{40}H_{36}O_{16}N_4$-Fe·$5H_2O$): C, 48.6%; H, 4.26%; N, 5.44%.

Samples for NMR were dissolved in ^2H_2O (99.8%, Merck) at pH 12–14. No correction was made for this solvent and the meter readings are reported directly as pH′. Titrations were carried out with dilute KO^{2}H or ^{2}HCl (Merck) and pH′ was monitored before and after each spectrum employing a Beckman ϕ70 meter and combination pH electrode. The maximum pH′ variation in these unbuffered samples detected by this procedure was 0.10 pH units. Buffers could not be employed due to variable aggregation which is observed with increasing ionic strength in FeURO solutions [7, 9, 11].

Proton NMR spectra were recorded on NTC-200 and NTC-360 spectrometers operating at 200 and 360 MHz, respectively, in the quadrature detection

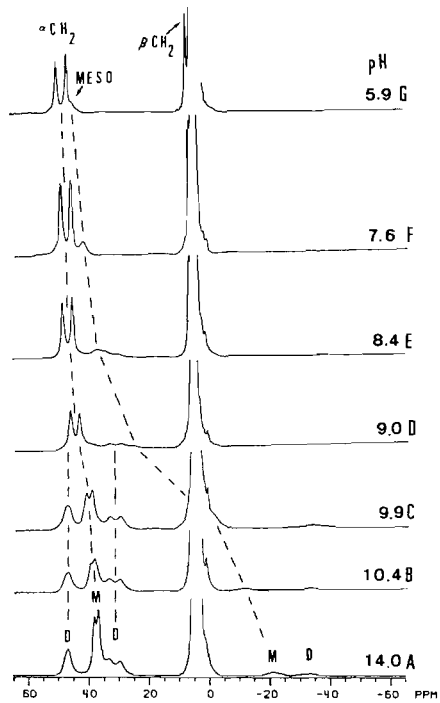

Fig. 2. Proton NMR spectrum of Urohemin I as a function of pH in D_2O at 22 °C, taken at 360 MHz. The dotted lines trace the behavior of individual resonances. This figure shows the dramatic titration behavior of the monomer meso resonance. At high pH this resonance appears upfield (A) and moves well downfield as the pH is lowered (B–G). Note also the pH dependent loss of intensity experience by the high pH dimer resonances (A–E).

mode. Active temperature regulation was employed with all spectra acquired at 23 ± 1 °C. Spectra were accumulated for solutions in 5 mm tubes with single pulse experiments employing the decoupler to partially saturate the residual water resonance and accumulating 2,000 to 10,000 transients. In general 8 K data points were acquired over spectral widths of 20 to 40 KHz. The observed proton resonances were internally referenced to water and are reported relative to external DSS.

Results and Discussion

FeURO exists in two detectable forms in aqueous solution that have been assigned as the monomer (M) and dimer (D) [7]. The assignments that were previously made are shown in Fig. 1. These forms have been characterized with regard to their temperature and concentration dependent interconversion at pH 12. At 24 °C the equilibrium constant for this process is 63 M^{-1} [7]. The assignments presented in Fig. 1 were made by comparison with other ferric porphy-

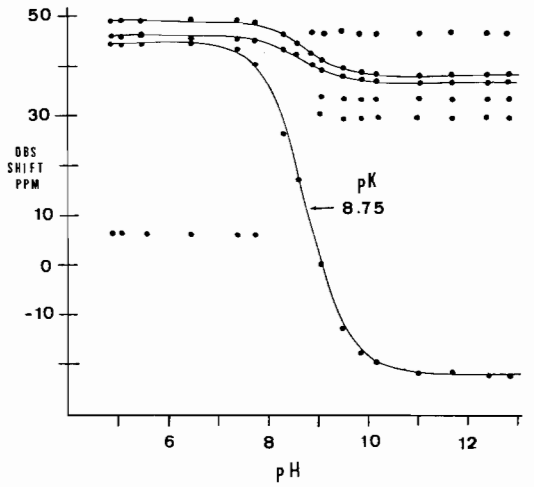

Fig. 3. Graph of observed proton hyperfine shifts against pH' for the monomer and dimer FeURO species. Lines drawn through the experimental data are nonlinear least squares fits to the Henderson-Hasselbach equation for a single proton ionization. The calculated pK for the meso proton resonance is shown on the figure. For the methylene shift the calculated pK's are 8.68 and 8.72 (±0.1) for the more downfield and upfield resonances of the pair, respectively.

rins and it was concluded that the dimer form is not a μ-oxo dimer, but a face-to-face pi dimer [7].

The observed hyperfine proton shifts shown in Figs. 1–3 and tabulated in Table I reflect the presence of iron centered paramagnetism in ferric porphyrins [1, 2]. These large shifts relative to diamagnetic proton NMR spectra are understood on the basis of a theory [1, 2, 20] that involves two mechanisms which may convey sizeable hyperfine fields to protons of the porphyrin periphery. The two mechanisms are the contact and pseudocontact, or dipolar, interactions. Therefore, the observed shifts presented in Figs. 1–3 are due to diamagnetic, dipolar, and contact contributions, as summarized in the following equations [1, 2, 20]

$$\left(\frac{\Delta H}{H}\right)_{obs} = \left(\frac{\Delta H}{H}\right)_{dia} + \left(\frac{\Delta H}{H}\right)_{dip} + \left(\frac{\Delta H}{H}\right)_{con}$$

where

$$\left(\frac{\Delta H}{H}\right)_{dip} = \frac{-\beta^2 S(S+1)}{9kT}\,[g_{\|}{}^2 - g_{\perp}{}^2]\,\langle(3\cos^2\theta - 1)/r^3\rangle$$

$$\left(\frac{\Delta H}{H}\right)_{con} = -A g \beta S(S+1)(3\gamma_N hkT)^{-1}$$

The form of these equations expressly assumes negligible contributions from the second order Zeeman interaction, strict Curie law behavior and effective axial symmetry. We have shown the first two assumptions to be valid for FeURO [7], at least over the temperature range of 0–70 °C. The structural symmetry of the porphyrin (A) argues for effective axial site symmetry in both five and six coordination geometries.

Figures 2, 3 and Table I demonstrate that the observed proton resonances are extremely pH depen-

TABLE I. Observed Hyperfine Shifts for the High pH Forms and Low pH Form of FeURO in Comparison to Other Representative Ferric Porphyrins with Determined Coordination Numbers. This Table is divided into Four Parts: (A) Experimentally observed Shifts for the High pH Forms showing the Upfield (Negative) Meso Shifts; (B) Five Coordinate Ferric porphyrins that also demonstrate Upfield Meso Shifts; (C) Experimentally observed Shifts for the Low pH Form demonstrating a Downfield (Positive) Meso Shift; (D) A Six coordinate Ferric Porphyrin showing Similar Downfield Meso Shifts. A More Extensive Collection of Porphyrins and Their Ligation Dependent Meso Shifts is given in Ref. 7, Table I.

Compound[6]	Solvent	pH'	Temp.[c]	Observed Shift (PPM)[a]				
				Pyrrole Substituents				Meso[d]
				$CH_2{}^{\alpha}$	$\Delta\nu_{1/2}$[e]	$CH_2{}^{\beta}$	$CH_3{}^{\beta}$	
A								
FeUROCl (Monomer)	D_2O	13.0	22	+38.1, 36.8,	350			−21.8
FeUROCl (Dimer)	D_2O	13.0	22	+46.7, 33.6, 29.7	400			−33.2
B								
OEPFeCl	$CDCl_3$		29	+43.1, 39.3			+6.6	−55
MPDMEFeCl	$CDCl_3$		25	+36, 35				−45

(continued overleaf)

TABLE I. *(continued)*

Compound[b]	Solvent	pH'	Temp.[c]	Observed Shift (PPM)[a]				
				Pyrrole Substituents				Meso[d]
				CH_2^{α}	$\Delta\nu_{1/2}$[e]	CH_2^{β}	CH_3^{β}	
C FeUROCl	D_2O	5.6	22	+49.6, 46.2	180	+6.9		+44.8
D $MPF(DMSO)_2^+$		DMSO-d^6	25	+46			+5.7	+40.39

[a]Observed shifts for FeURO referenced internally to the residual water resonance and reported relative to external DSS; (+) indicates downfield from DSS; (−) indicates upfield from DSS. Shifts for porphyrins in $CDCl_3$ or DMSO are reported relative to internal TMS and are taken from refs. 1 and 7, and references therein. Some difference in the observed shifts reported here and those in ref. 7, Table I, occur due to the different temperatures at which they were obtained and the well known Curie Law behavior of resonances in paramagnetic molecules. [b]Abbreviations: OEP = octaethylporphyrin; MPDME = mesoporphyrin dimethyl ester; MP = mesoporphyrin; DMSO = dimethylsulfoxide; URO = uroporphyrin I; D_2O = 99.8% deuterium oxide; $CDCl_3$ = deuterochloroform. [c]Degrees °C. [d]±1.5 ppm due to its large linewidth (≥1000 Hz). [e]Linewidth in Hz estimated at half peak maximum height for the CH_2^{α} resonances.

dent over the range of pH from 14 to 5 for both the monomer and dimer forms of FeURO. The general effect for the monomer is that the upfield resonance of the meso protons is shifted far downfield with decreasing pH. The pK observed for this process is 8.75 (±0.1) as shown in Fig. 3. The resonances of the methylene protons are observed moderately downfield and are shifted even further downfield at low pH. They exhibit narrower linewidths as a result of the low pH. For the dimer, chemical shift dependence is not observed. The dimer resonances lose intensity with decreasing pH until all of the dimer converts into the single low pH form (Fig. 2 B–F).

The behavior of the meso resonance gives information about the pH dependent solution dynamics because it has previously been demonstrated that the ferric porphyrin meso protons exhibit axial ligation dependent shifts [1, 7, 22]. This effect is summarized in Table I where it is shown that upfield (negative shifts) meso resonances are characteristic of five coordination in high spin ferric porphyrins, that is, one axial ligand in addition to the four equatorial pyrrole nitrogens from the porphyrin ligand. Table IB shows that monomer ferric porphyrins in a noncoordinating solvent yield sizeable upfield meso shifts. By the same reasoning presented elsewhere [7], the upfield meso protons' resonances of the high pH FeUROCl monomer and dimer are characteristic of five coordination (Table IA).

Downfield resonances for meso protons are observed for six coordinate high spin ferric porphyrins as demonstrated in ref. 7 and Table ID, leading to the assignment of six coordination for the low pH form of FeUROCl (Table IC). Protons of pyrrole

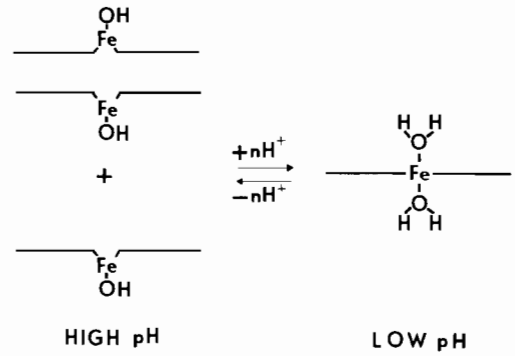

Fig. 4. Conceptualization of the species that predominate in high (A) and low (B) pH solutions of Urohemin I. This figure is not meant to imply any particular mechanism for the transition between high and low pH forms, but serves only to indicate how the state of axial ligation differs between forms. The porphyrin planes are indicated as heavy lines without peripheral substituents. A face-to-face, $\pi-\pi$ type dimer has been suggested as the best candidate to account for previous NMR analyses [7].

substituents are less sensitive to the state of axial ligation in high spin ferric porphyrins, although average shifts of α-CH_2 protons demonstrate a slight downfield bias (0.6–6 ppm) in six coordinated high spin ferric porphyrins compared to five coordinate forms (Table IB, D).

Given these facts and making the reasonable assumption that the observed shifts in the high spin FeURO forms have as their origin the same mechanism for electron spin delocalization as the synthetic ferric high spin porphyrins [1, 20], it is possible to conclude that the pH dependence of the hyperfine proton NMR spectrum for the FeURO

monomer is a result of changes in axial ligation. Data supporting the argument for similar mechanisms of electron spin delocalization in synthetic and natural porphyrins has previously been put forth [7]. The process that is envisioned is shown in Fig. 4a. At high pH (pH 11–14) five coordinate hydroxo complexes are believed to exist for both the monomer and dimer. The particular forms that are likely structures are shown in Fig. 4 and are characterized by an upfield meso proton resonance.
These are converted into a six coordinate, probably a bis aquo, complex at low pH. The low pH complex is assigned as a monomer for several reasons. (i) It is difficult to envision a symmetric dimer in which each of the pair of porphyrins is six coordinate due to the axial steric ligation which would interfere with close approach of the two porphyrin planes. Moreover, the observation of a single set of methylene resonances, as well as the single resonance for the meso protons, dictates that single, equal magnetic environments for these protons exist. A symmetric species is demanded by the data. (ii) The monomer (high pH) resonances titrate smoothly into the low pH form, whereas the dimer resonances do not shift but simply lose intensity, indicating transformation into the only low pH form. The total integrated resonance intensity at pH 14 is conserved at pH 7.0 within the error limits of graphical integration (±10%). This indicates that the high pH dimer form is, indeed, converted into the single low pH form. (iii) Raman and spectrophotometric investigations of other axially ligated metallouroporphyrins, such as the $Sn(OH)_2^-$ and VO derivatives, indicate that strongly bound axial ligands do interfere with $\pi-\pi$ aggregation [10, 11, 16, 24]. For most metallouroporphyrins $\pi-\pi$ aggregation normally occurs at acid pH as a result of reduced electrostatic repulsion caused by protonation, hence neutralization, of the peripheral carboxylates and lack of complete axial ligation [10, 11, 23, 24]. Aggregation is apparently blocked for urohemin by the six coordinate structure detected by NMR for the low pH species. This argues for the presence of two axially coordinated water molecules in the low pH form of urohemin. By analogy, the low spin, dicyanourohemin complex, which possesses two axially coordinated cyanide ligands, also does not form $\pi-\pi$ dimers, even at high pH and upon addition of 5 M salt [9, 14].

The identity of the axial ligands in each form is deemed reasonable in view of the nature of ligation in other aqueous ferric porphyrins [25–27]. Moreover, the NMR results presented here support the idea of a significant pH dependent change in axial ligand field strength in the following way. It has previously been demonstrated by proton [1, 28] and carbon [29] NMR that changes in the nature of axial ligation induce linewidth and shift changes that correlate with the zero field splitting parameter in ferric porphyrin complexes. This is valid for complexes in which the electron spin lattice relaxation time ($T_{1e} \approx 10^{-11}$ sec) is less than the rotational correlation time (τ_r). The change in linewidth, caused by lowering the pH, for pyrrole α-CH_2 substituents and the meso protons resonance is shown in Table I. It should also be pointed out in support of this argument that the pyrrole methyl shifts of ferric protoporphyrin **IX** in native metmyoglobin exhibit similar pH dependent shifts to those observed for the pyrrole methylene shifts of FeURO. Met-myoglobin undergoes a pH dependent axial ligation change from aquo to hydroxo forms with a pK of 9.1 [30–32] similar to our observations for ferric urohemin **I**. When water occupies the available sixth coordination position in the protein, the pyrrole methyl protons' resonances are narrower and lie farther downfield than when a hydroxide ion is the sixth ligand. Shift changes are between 28 and 57 ppm for the individual methyl resonances [30–32].

For the dimer form, the pH dependence indicates not only an axial ligation change, but conversion of the dimer to the same limiting low pH form as the monomer. Figure 2B–F shows that throughout the pH titration peaks characteristic of the dimer at high pH are converted into the common low pH form.

Acknowledgements

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