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Speciation of Ferriprotoporphyrin IX in Aqueous and Mixed Aqueous Solution Is Controlled by Solvent Identity, pH, and Salt Concentration

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The speciation of ferriprotoporphyrin IX (Fe(III)PPIX) in aqueous and mixed aqueous−organic solvents has been investigated by UV−vis, ^{1}H NMR, magnetic, and diffusion measurements. Fe(III)PPIX has been found to form monomers, $\pi-\pi$ dimers, μ-oxo dimers, and π-stacked aggregates of μ-oxo dimers depending on concentration, pH, the presence of salts, temperature, and solvent identity. This highlights the complexity of the behavior of Fe(III)PPIX in solution. However, the presence or absence of the μ-oxo dimer is clearly dependent on solvent, with a series of aprotic solvents (5.64 M DMSO, acetone, DMF, THF, 2,6-lutidine) all promoting μ-oxo dimer formation at pH 10. By contrast, protic solvents (methanol, ethanol, propanol, ethylene glycol, diethylene glycol, and formamide) at the same concentration and under the same conditions give rise only to the $\pi-\pi$ dimer variously mixed with monomer depending on solvent polarity. The $\pi-\pi$ dimer has previously been shown to be present in purely aqueous solution. In the presence of 4.25 M NaCl in aqueous solution, on the other hand, both UV−vis spectra and diffusion measurements suggest the presence of large π-stacked aggregates of μ-oxo dimers at pH 10. In aqueous DMSO at least, the temperature dependence of the dimerization constant shows that the process of μ-oxo dimer formation is endothermic and hence entirely entropy driven. This strongly suggests that formation of the μ-oxo dimer is driven by desolvation, with solvents that can act as both hydrogen bond donors and acceptors to the axial water/hydroxide ligand of Fe(III)-PPIX preventing formation of this dimer species, while those that cannot act as hydrogen bond donors facilitate it. The findings permit prediction of the Fe(III)PPIX species present in different mixed solvent systems and in the case of aqueous DMSO at any given pH, concentration, and temperature.

Introduction

As long ago as 1947, Shack and Clarke recognized that heme exhibits complex speciation behavior in aqueous solution.[1] Studies conducted on ferriprotoporphyrin IX (Fe(III)-PPIX) in the 1960s and 1970s led to contradictory conclusions. In 1969, Brown, Jones, and Lantzke isolated solid material from a solution of hemin (Cl-Fe(III)PPIX) by addition of solid NaOH to a basic solution and by addition of NaOH solution to hemin dissolved in DMSO. Infrared characterization demonstrated the presence of a Fe−O−Fe bond.[2] The following year, Brown, Dean, and Jones conducted a spectrophotometric study on aqueous solutions of Fe(III)PPIX and concluded that the concentration dependence of the visible spectrum was consistent with dimerization, but not higher aggregation.[3] In combination with the previous study, they proposed that the dimer is a μ-oxo dimer. On the other hand, an ultracentrifugation study in aqueous solution containing 1.2 M NaCl conducted by Blauer and Zvilichovsky provided evidence for large aggregates of Fe(III)PPIX μ-oxo dimer.[4] Subsequently, in 1975, O'Keeffe et al. conducted a study on a series of Fe(III)-porphyrins, including Fe(III)PPIX. They deliberately synthesized and isolated μ-oxo dimers, which they characterized by magnetic susceptibility measurements, ^{1}H NMR, and UV−vis spectroscopy. In their hands, the μ-oxo dimer of Fe(III)PPIX did not form spontaneously in aqueous solution but had to be induced by introduction of 10% pyridine into a solution made up in 0.1 M NaOH.[5] Despite these contradictions, over time it came to be widely accepted that the species present in aqueous solution is in fact the μ-oxo dimer.

Recently, there has been a surge in interest in the behavior of Fe(III)PPIX in aqueous solution, mainly owing to its possible role in a number of pathogenic conditions. Inappropriate release of heme (Fe(II)PPIX) from proteins followed by oxidation has been implicated in atherogenesis,

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cancer, hemolysis, and inflammation.[6−9] In addition, there is considerable interest the heme-uptake mechanisms of pathogenic Gram-positive bacteria such as *Staphylococci*, *Streptococci*, and others including Gram-negative bacteria which utilize host heme to satisfy their iron requirements.[10−14] Finally, blood-eating organisms such as the pathogenic helminth *Schistosoma mansoni* and the protozoan malaria parasite *Plasmodium* digest large quantities of host hemoglobin. In the process, heme is released and oxidized to Fe(III)-PPIX. This toxic byproduct is detoxified by incorporation into a crystalline product, hemozoin, in a biomineralization-like process.[15−17] This process is inhibited by a number of antimalarial drugs and is thus believed to be an important drug target.[18,19]

In a recent study, we showed that H_2O/HO-Fe(III)PPIX does not in fact spontaneously form a μ-oxo dimer in solution.[20] It does however, dimerize, and we proposed that the dimer is a $\pi-\pi$ dimer of two five-coordinate iron porphyrins with the H_2O/HO axial ligands directed outward. This model is very similar to the crystal structure reported by Cheng et al. for the perchlorate salt of cationic H_2O−Fe(III)-octaethylporphyrin.[21] This porphyrin exhibits an intermediate spin state as a result of weak antiferromagnetic coupling of the two Fe(III) centers ($\mu \approx 4.8$ at 300 K). The magnetic moment of the aqueous dimer of H_2O/HO-Fe(III)PPIX ($\mu = 4.21$)[22] is similar and much larger than the expected value of $\mu = 1.1$ for the μ-oxo dimer. Both the dependence of the UV−vis spectrum on concentration and diffusion measurements suggested that aggregates larger than dimers are not formed. On the other hand, when 10% pyridine is included in a solution of Fe(III)PPIX in 0.1 M NaOH, a visible spectrum typical of the μ-oxo dimer is formed and the magnetic moment decreases to 1.04, very close to that expected for this species at room temperature ($\mu = 1.1$). Diffusion measurements again demonstrated that large aggregates are not present. Subsequent to this study, Casabianca et al. have conclusively shown that the μ-oxo dimer dominates in 40% aqueous DMSO at concentrations used for NMR studies (4 mM) and especially under mildly alkaline conditions.[23] They have also shown that certain detergent micelles promote μ-oxo dimer formation.[24] Both of these observations are in accord with unpublished observations in our laboratory.

In view of the above, we have undertaken a comprehensive investigation of the role of solvents in mixed aqueous solutions and of high salt concentrations on Fe(III)PPIX speciation in solution. The findings have allowed us to propose a comprehensive hypothesis for the factors controlling Fe(III)-PPIX dimerization and higher aggregation, permitting prediction of the species likely to dominate in a given solvent mixture.

Materials and Methods

Materials and Instrumentation. Bovine hematin (HO-Fe(III)-PPIX) and bovine hemin (Cl-Fe(III)PPIX) were purchased from Sigma-Aldrich. All other reagents and solvents were of analytical or equivalent grade and were obtained from commercial suppliers and used without further purification. Glass-distilled water was used for all experiments. UV−vis spectra were recorded using a Varian Cary 100 UV−vis spectrophotometer with 1 or 0.1 cm path length quartz cuvettes. Temperature regulation was maintained to within 0.2 °C by means of a thermostated water bath. ^{1}H NMR spectroscopy was performed on a Varian Unity 400-MHz spectrometer. Deuterated solvents were used for all NMR measurements.

Washing of Glassware and Cuvettes. All volumetric flasks, vials, and NMR tubes were cleaned by washing with concentrated NaOH, followed by extensive rinsing with water. The equipment was then washed with 1 M HNO_3 and once again extensively washed with water. Glassware and cuvettes that were used for long periods of time or used for solutions of Fe(III)PPIX at high pH were soaked in 1 M NaOH for 1 h and then extensively washed with water. This equipment was subsequently soaked in 1 M HNO_3 overnight, washed with water, and then soaked in boiling water to rinse off excess acid. Cuvettes were air-dried, and all other equipment was dried in an oven at 393 K overnight. All Fe(III)PPIX solutions were dispensed using a Hamilton syringe, which was washed with dilute NaOH followed by thorough washing with distilled water.

UV−vis Spectra. A 1.00×10^{-2} M Fe(III)PPIX stock solution was prepared by dissolving hematin in 0.1 M NaOH. The solution was allowed to dissolve completely. A 40% (v/v) DMSO/aqueous mixture (5.64 M DMSO) was prepared and buffered with either 0.020 M *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) at pH 7 or 0.020 M *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) at pH 10. For all subsequent comparative studies in mixed aqueous solvents, a concentration of 5.46 M was used as the basis for comparison. Working solutions of Fe(III)PPIX (3.00×10^{-5} M) were prepared in the buffered mixed aqueous solvents from the stock Fe(III)PPIX solution.

High salt concentrations were prepared by dissolving NaCl (4.25 M) in distilled water buffered with CHES (0.020 M) at a pH of 10 in a volumetric flask. Working solutions of Fe(III)PPIX

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(3.00×10^{-5} M) were prepared in the salt solution from the stock Fe(III)PPIX solution. All UV−vis spectra were recorded in the range 300−800 nm at 299 K.

^{1}H NMR and Magnetic Susceptibility. For studies in aqueous solution, a 1×10^{-2} M Fe(III)PPIX solution was prepared by dissolving hematin (approximately 0.006 g, accurately weighed) in 0.1 M NaOD in D_2O. For studies in mixed aqueous solvents, a 1×10^{-2} M Fe(III)PPIX solution was prepared by dissolving hematin in 0.400 mL 0.1 M NaOD in D_2O. The hematin was allowed to dissolve completely, and then the solution was diluted into an aqueous/organic mixture with a final concentration of 5.64 M organic solvent in D_2O and a total volume of 2 mL. ^{1}H NMR spectra were recorded in the chemical shift range of −20 to 80 ppm. For magnetic susceptibility measurements, organic/aqueous mixtures with 2.82 M organic solvent (20% v/v in the case of DMSO) were used. Under these conditions, the solvent is predominantly water and the diamagnetic susceptibility of the solvent could be approximated by the value for water. A reference solution consisting of the mixed aqueous system without Fe(III)PPIX was dispensed into the outer tube of a coaxial NMR tube while the same solution containing Fe(III)PPIX was dispensed into the inner tube. In the case of methanol, the concentration of Fe(III)PPIX used was 1.0×10^{-2} M, while for the DMSO-, acetone-, and THF-containing solutions it was 2.0×10^{-2} M. The water peak (from H_2O present in the D_2O) was recorded at 303 K and the difference in chemical shift (Δf) between the reference and the sample was measured (in Hz).

The mass magnetic susceptibility of dissolved Fe(III)PPIX was determined by the Evans method according to eq 1[25]

$$\chi_g = \frac{-3\Delta f}{4\pi f m} + \chi_0 \tag{1}$$

where f is the operating frequency of the spectrometer (399.952 MHz), m is the concentration of Fe(III)PPIX in g/mL, and χ_0 is the mass magnetic susceptibility of the solvent (in this case water, -7.22×10^{-7} g^{-1}). The molar magnetic susceptibility χ_m is the product of χ_g and the molecular weight of the complex. The magnetic moment was calculated according to eq 2

$$\mu = 2.84\sqrt{\chi_m T} \tag{2}$$

where T is the temperature of the sample in the spectrometer.

Determination of the Dimerization Constant. Effects of pH on the spectrum of Fe(III)PPIX in aqueous DMSO and methanol were investigated. A stock solution of Fe(III)PPIX (1×10^{-2} M) was prepared by dissolving hematin in 0.1 M NaOH. A working solution (3×10^{-5} M) was prepared in aqueous/organic mixtures (at a constant concentration of 5.64 M) and buffered with CHES at pH 10. The pH of the solution was sequentially adjusted by small additions of 70% $HClO_4$. The pH was measured after each adjustment and the spectrum of the solution recorded at constant temperature. Titrations in aqueous DMSO solutions were conducted at six different temperatures to determine the effects of temperature on the dimerization process.

Formation of a μ-oxo dimer of Fe(III)PPIX was analyzed in accordance with the equilibrium given by eq 3:

$$2H_2O-\text{Fe(III)PPIX(aq)} \overset{K_D}{\rightleftharpoons} O(\text{Fe(III)PPIX})_2(aq) + 2H^+(aq) + H_2O(l) \tag{3}$$

Changes in the protonation state of the heme propionates are ignored as the experiment is performed in a pH range well above the pK_a of these groups, which are deprotonated under all conditions used in this study. The dependence of Soret band absorbance (A) on pH is given by eq 4

$$A = \frac{A_M}{[M]_T}[M] + \frac{2A_D}{[M]_T}\left(\frac{[M]_T - [M]}{2}\right) \tag{4}$$

where A_M is the absorbance of pure monomer, A_D is the absorbance of pure dimer, $[M]_T$ is the total concentration of Fe(III)PPIX, and [M] is the concentration of monomeric H_2O−Fe(III)PPIX given by eq 5

$$[M] = \frac{-[H^+] + \sqrt{[H^+]^2 + 8K_D[M]_T[H^+]^2}}{4K_D} \tag{5}$$

where K_D is the pH-independent dimerization constant. Absorbance data obtained at 400 nm were fitted to eq 5 to obtain K_D. The conditional dimerization constant at any fixed pH, $K_{D,obs}$ is then given by eq 6:

$$K_{D,obs} = \frac{K_D}{[H^+]^2} \tag{6}$$

Diffusion Experiments. Diffusion measurements were carried out in aqueous solution containing 4.25 M NaCl according to a method described by Linder et al.[26] A stock solution of Fe(III)PPIX (1×10^{-2} M) was prepared in 0.1 M NaOH. A working solution (3×10^{-4} M) was then prepared in buffered salt solution (0.02 M CHES, pH 10, 4.25 M NaCl). The Fe(III)PPIX solution was dispensed into the lower chamber of a diffusion apparatus and allowed to diffuse into a blank solution lacking Fe(III)PPIX, but otherwise of the same composition in the upper chamber. After 1 h at room temperature, samples from the upper chamber were collected and the spectra recorded. The temperature was recorded during each experiment. The experiment was repeated eight times and the diffusion coefficient calculated according to eq 7

$$D = \left(\frac{Ch}{C_0}\right)^2 \frac{\pi}{t} \tag{7}$$

where C is the final concentration of Fe(III)PPIX in the upper chamber, C_0 is the initial concentration of Fe(III)PPIX in the lower chamber which remains effectively constant, h is the height of the upper chamber, and t is diffusion time. The relative hydrodynamic radius of the species was approximated using the Stokes−Einstein relationship, eq 8, assuming spherical particles

$$D = \frac{k_B T}{6\pi\eta a} \tag{8}$$

where k_B is the Boltzmann constant, T is the absolute temperature, η the viscosity of the solution, and a is the hydrodynamic radius of the particle.

Results

Spectroscopic and Magnetic Evidence Shows That Fe(III)PPIX Forms a μ-Oxo Dimer in 40% (v/v) Aqueous DMSO, but Not in Aqueous Methanol. The UV−vis spectrum of Fe(III)PPIX in 40% (v/v) aqueous DMSO (5.64 M) at pH 10 closely resembles that of the μ-oxo dimer reported by O'Keeffe et al. (Figure 1a).[5] The spectrum has characteristic peaks in the charge-transfer

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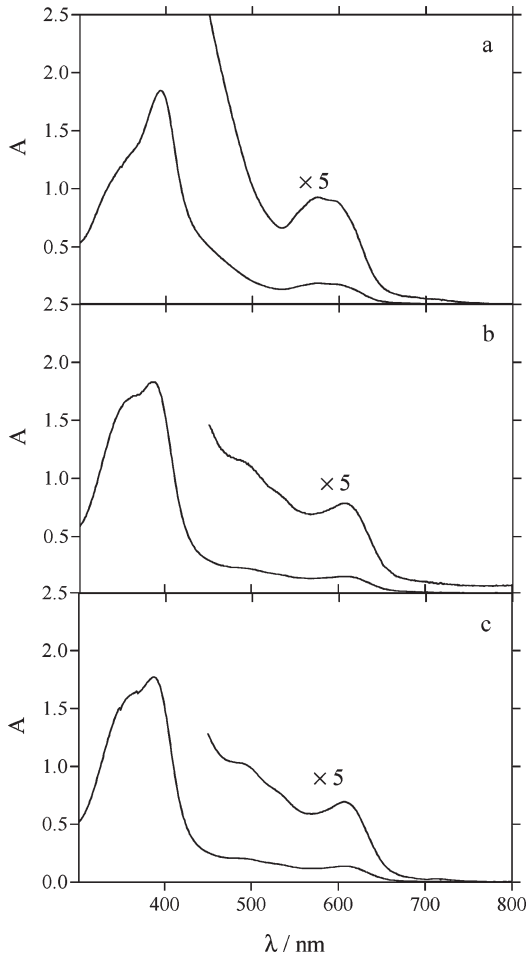

Figure 1. UV−vis spectra of 3×10^{-3} M Fe(III)PPIX in (a) 5.64 M aqueous DMSO, pH 10, (b) aqueous solution, pH 10, and (c) 5.64 M aqueous methanol, pH 10. All solutions were buffered with 0.02 M CHES. The spectrum in (a) exhibits a peak at 575 nm with distinct shoulder at 600 nm typical of the μ-oxo dimer, while those in (b) and (c) have a peak at about 605 nm.

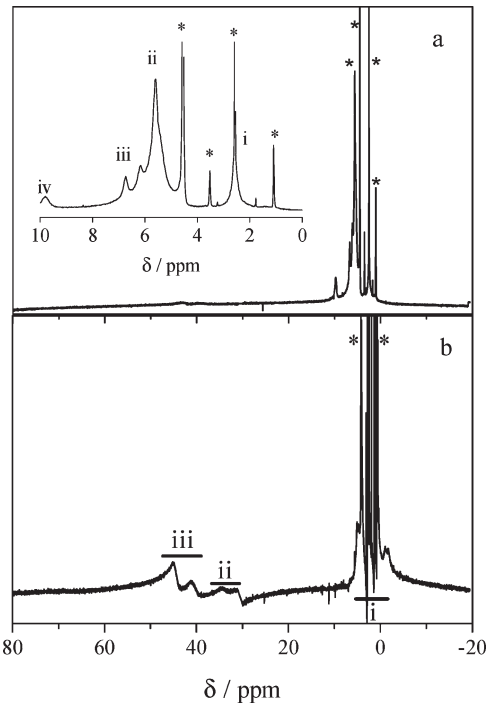

Figure 2. ^{1}H NMR spectra of 1×10^{-2} M solutions of Fe(III)PPIX in (a) 5.64 M DMSO-d^6 and (b) 5.64 M methanol-d^4. Both were prepared in 0.1 M NaOD dissolved in D_2O. The inset in (a) is an expansion of the 0−10 ppm region of the spectrum. On the basis of assignments by O'Keeffe et al. for a series of Fe(III)porphyrin μ-oxo dimers, the peaks in (a) can be assigned as (i) propionyl β-CH_2, (ii) methyl groups, and (iii) propionyl α-CH_2.[5] The peak marked (iv) is absent from O'Keeffe et al.'s spectra and likely corresponds to the vinyl α-CH protons, a group not present in the compounds investigated by those authors. The broadened *meso* peaks are expected to underlie the region where (ii) and (iii) occur. Assignment of peaks in (b) is the same as that reported by de Villiers et al. for the aqueous species[20] and follows Budd et al., who assigned the spectrum of the dimethyl ester of Cl−Fe(III)PPIX in chloroform.[42] The peaks are assigned as (i) *cis*- and *trans*-vinyl β-CH_2 and propionyl β-CH_2, (ii) vinyl and propionyl α-CH_2, and (iii) methyl groups. Again, *meso* peaks are expected to be obscured and are likely to be extremely broadened. Asterisks indicate solvent peaks.

region which differ markedly from the species present in aqueous solution (Figure 1b) which has previously been shown to be a $\pi-\pi$ dimer by de Villiers et al.[20] This observation is in accord with a recent report by Casabianca et al. showing μ-oxo dimer formation in aqueous DMSO[23] and is supported by the ^{1}H NMR spectrum of Fe(III)PPIX in DMSO-d_6/D_2O (containing 0.1 M NaOD) which falls within the range 0−10 ppm (Figure 2a), closely resembling the NMR spectra previously reported for μ-oxo dimers of Fe(III)porphyrins.[5] Even at lower concentrations of DMSO (20% v/v or 2.82 M in the presence of 0.1 M NaOD), the magnetic moment (μ) per Fe(III) center determined by the Evans method (Figure S1, Supporting Information) of 1.59 is comparable to that of Casabianca et al. (μ = 2.4) for hemin dissolved in 40% (v/v) aqueous DMSO.[23] These values are very different from the value of 4.21 for the $\pi-\pi$ dimer spontaneously formed in aqueous solution obtained by de Villiers et al.[20] Collectively, these observations confirm the findings of Casabianca et al. in aqueous DMSO,[23] as well as the utility of the UV−vis spectrum as a fingerprint for identifying the presence of the μ-oxo dimer.

In a solution of aqueous methanol at pH 10, with the same concentration of organic solvent as in the aqueous DMSO system (5.64 M), the UV−vis spectrum is not that of the μ-oxo dimer (Figure 1c). Rather, it is identical to that of the $\pi-\pi$ dimer observed in aqueous solution (Figure 1b). The NMR spectrum in the same concentration of methanol-d_4/D_2O (0.1 M NaOD) is similarly characteristic of the $\pi-\pi$ dimer (Figure 2b). Unlike the μ-oxo dimer, the spectrum gives rise to very large paramagnetic broadening, with peaks that lie in the range of −10 to +60 ppm, indicating the absence of strong antiferromagnetic coupling. Magnetic susceptibility measurements (Figure S2, Supporting Information) at lower concentration of methanol (2.82 M) yields a magnetic moment of 4.73 per iron center, a value similar to that observed in aqueous solution, and much larger than the value of 1.59 observed at the same concentration of DMSO.

Finally, a spectrophotometric pH titration of Fe(III)-PPIX in aqueous methanol (5.64 M) results in a series of spectra which do not exhibit isosbestic points and which when monitored at 610 nm exhibit an absorbance versus pH curve consistent with two deprotonation steps (Figure 3a). This is behavior typical of the $\pi-\pi$ dimer in aqueous solution as reported by de Villiers et al. which is characterized by two deprotonation steps.[20]

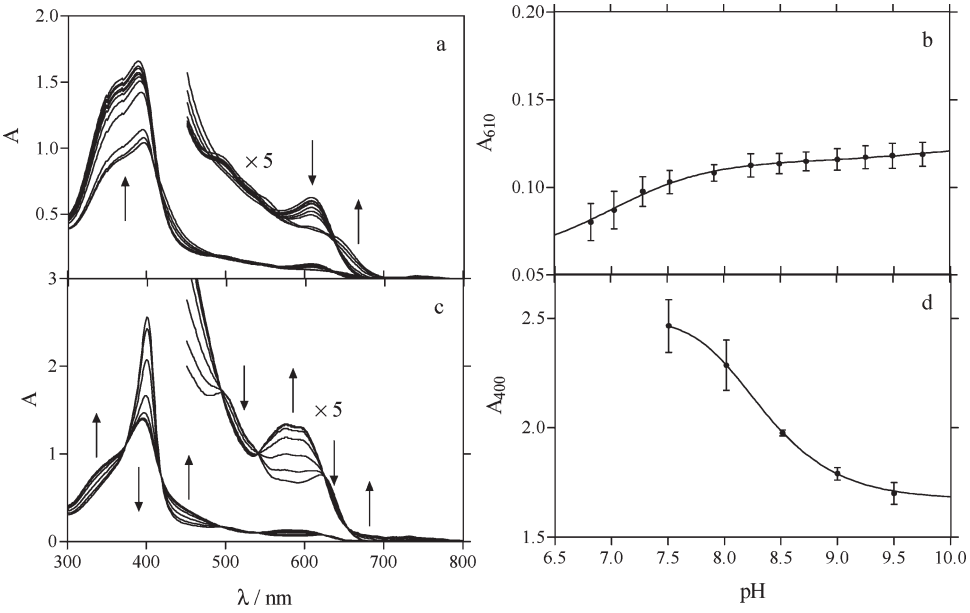

Figure 3. Dependence of UV−vis spectrum of Fe(III)PPIX on pH. (a) Changes in the UV−vis spectrum of Fe(III)PPIX in 5.64 M aqueous methanol as a function of pH. Note the absence of isosbestic points. (b) Dependence of A_{610} on pH fitted to a model including two deprotonation steps for Fe(III)PPIX in 5.64 M aqueous methanol. (c) Changes in the UV−vis spectrum of Fe(III)PPIX in 5.64 M aqueous DMSO as a function of pH exhibiting six isosbestic points. (d) Dependence of the Soret band (A_{400}) on pH in 5.64 M aqueous DMSO fitted to eq 5. In all cases, concentration of Fe(III)PPIX was 3×10^{-5} M, $T = 298$ K. Direction of change in spectra with increasing pH is indicated by arrows.

Conversion between the μ-Oxo and $\pi-\pi$ Dimers of Fe(III)PPIX in Mixed Aqueous Solvents Is Fully Reversible. The spectrum of a 3×10^{-4} M solution of the μ-oxo dimer of Fe(III)PPIX in aqueous DMSO (5.64 M) at pH 10 in a 0.1 cm cuvette is shown in Figure 4a. When this solution was diluted 10-fold into the same solvent mixture, a spectrum recorded using a 1 cm cuvette was almost identical, with only a small increase in intensity of the Soret band, consistent with a slight rise in the fraction of monomer species at lower concentration (Figure 4a). However, when the solution was diluted into aqueous methanol (5.64 M, pH 10), the spectrum observed was that of the $\pi-\pi$ dimer (Figure 4a). Conversely, the spectrum of 3×10^{-4} M Fe(III)PPIX in aqueous methanol (5.64, pH 10) recorded in the 0.1 cm cuvette was that of the $\pi-\pi$ dimer (Figure 4b). Ten-fold dilution into the same aqueous methanol solvent caused no appreciable change (Figure 4b), while dilution into aqueous DMSO (5.64 M, pH 10) resulted in conversion to the μ-oxo dimer (Figure 4b). These spectra unequivocally demonstrate that the conversion between μ-oxo dimer and $\pi-\pi$ dimer is fully reversible in mixed aqueous systems. Furthermore, the process is a thermodynamic, rather than kinetic phenomenon and depends only on the identity of the organic solvent.

Ten-fold dilution from the 3×10^{-4} M Fe(III)PPIX solution in aqueous DMSO into aqueous acetone (5.64 M, pH 10) resulted in a spectrum identical to that seen in aqueous DMSO at the same concentration (Figure 4a), suggesting that Fe(III)PPIX forms a μ-oxo dimer in aqueous acetone as well. Indeed, when a similar dilution of the aqueous methanol solution was made into the aqueous acetone mixture, the spectrum of the μ-oxo dimer was also obtained (Figure 4b). Formation of the μ-oxo dimer in basic aqueous acetone solution (acetone-d_6/D_2O, 2.82 and 0.1 M NaOD) was confirmed from the magnetic susceptibility of 1.52 determined by the Evans method (Figure S3, Supporting Information).

Thermodynamics of Fe(III)PPIX μ-Oxo Dimer Formation in Aqueous DMSO. The dependence of the UV−vis spectrum of 3×10^{-5} M Fe(III)PPIX on pH in aqueous DMSO (5.64 M) at 298 K is shown in Figure 3c. The spectrum at pH 7.49 has the sharp Soret band characteristic of the monomer. The presence of isosbestic points shows that only two species are present as a function of pH, the monomer and the μ-oxo dimer.

A fit of the Soret absorbance (A_{400}) versus pH data for the aqueous DMSO mixture to eq 4 describing μ-oxo dimer formation (Figure 3d) provides a value for the pH independent dimerization constant (K_D) of $5.0 \pm 0.9 \times 10^{-13}$ M at 298 K. The corresponding conditional dimerization constants at pH 7.4 and 10 ($K_{D,obs}$) can be readily calculated using eq 6. These values are respectively 317 M^{-1} and 5.0×10^{7} M^{-1}. The value of K_D permits the percentage of μ-oxo dimer species at any pH and total Fe(III)PPIX concentration to be predicted in this solvent system at 298 K (Figure 5a). This shows that at pH 7.4 and a total Fe(III)PPIX concentration of 2×10^{-6} M, conditions which have been used for measuring association constants of antimalarial quinolines with Fe(III)PPIX,[27−30] there is essentially no dimer present. On the other hand, at a concentration of 4×10^{-3} M, conditions used in the recent NMR study by Casbianca et al.,[23] the μ-oxo dimer exceeds 50% at the same pH.

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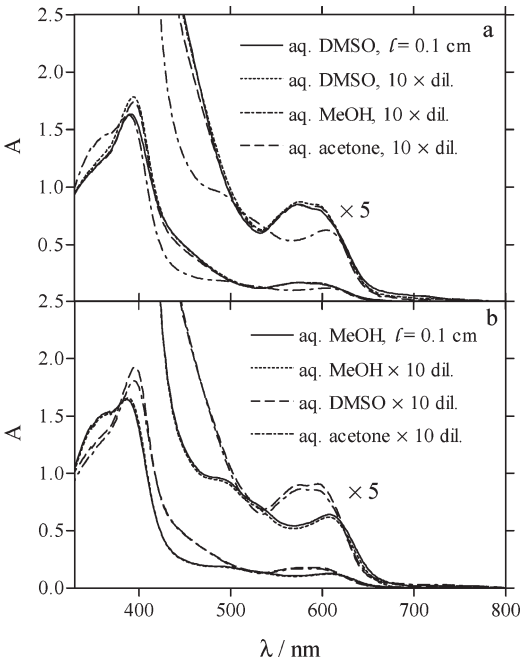

Figure 4. Reversibility of changes in UV−vis spectra of Fe(III)PPIX as a function of solvent composition. (a) The spectrum of 3×10^{-4} M Fe(III)-PPIX in 5.64 M aqueous DMSO, pH 10 recorded in a 0.1 cm cuvette and 10-fold dilutions of this solution into the same solvent and 5.64 M methanol at the same pH recorded in a 1 cm cuvette. (b) The spectrum of 3×10^{-4} M Fe(III)PPIX in 5.64 M aqueous methanol, pH 10 recorded in a 0.1 cm cuvette and 10-fold dilutions of this solution into the same solvent and 5.64 M DMSO. Ten-fold dilutions into 5.64 M acetone at the same pH recorded in a 1 cm cuvette are also shown in each case. Solutions buffered with 0.02 M CHES.

The value of K_D is strongly temperature dependent. A van't Hoff plot of log $K_{D,obs}$ versus $1/T$ calculated for pH 10 (Figure 5b) exhibits a negative slope, demonstrating that dimerization is endothermic. This means that the process is entirely entropy driven. Under these conditions, the value of ΔH is 61 ± 6 kJmol^{-1} and ΔS is 359 ± 19 JK^{-1}mol^{-1} (corresponding to a $T\Delta S$ value of 107 ± 6 kJ mol^{-1} at 298 K).

The Effects of Other Water-Miscible Organic Solvents on Fe(III)PPIX in Mixed Aqueous Solutions. As seen above, aqueous DMSO and acetone both result in μ-oxo dimer formation at pH 10, while aqueous methanol and pure aqueous medium give rise to a $\pi-\pi$ dimer. To better understand the role of the solvent, the UV−vis spectrum of Fe(III)PPIX was recorded in nine other solvents (5.64 M, pH 10). Using the spectrum as a fingerprint (Figure S4, Supporting Information), the dominant dimer species identified is shown in Table 1. The considerably sharper Soret band seen in the case of diethyleneglycol, 2-propanol and to a lesser extent ethanol suggests a significant proportion of monomer species is present. The spectrum in *tert*-butyl alcohol appears to have characteristics of both the μ-oxo dimer and monomer (or possibly $\pi-\pi$ dimer). In the case of tetrahydrofuran, the Soret band appears somewhat different to the other solvents. However, the charge transfer region of the spectrum indicates the presence of the μ-oxo dimer. This was confirmed by a magnetic susceptibility measurement using the Evans method (Figure S5, Supporting Information). The magnetic moment (2.00) is indicative of the μ-oxo dimer being the dominant species.

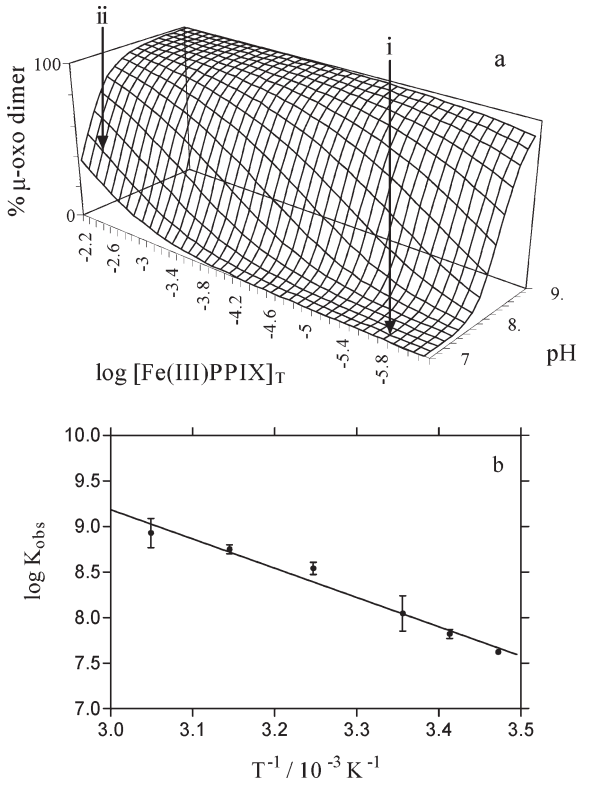

Figure 5. Effects of concentration, pH, and temperature on Fe(III)-PPIX dimerization in 5.64 M aqueous DMSO. (a) The percentage of Fe(III)PPIX present as the μ-oxo dimer as a function of pH and concentration (shown as a log value). Point (i) represents 2×10^{-6} M total Fe(III)PPIX at pH 7.5, conditions routinely used for measuring interactions with antimalarial drugs.[27−30] Here, Fe(III)PPIX is essentially entirely monomeric. Point (ii) represents 4×10^{-3} M Fe(III)PPIX at the same pH which is extensively converted to the μ-oxo dimer. This concentration was used in the NMR study of Casabianca et al.[23] The graph shown is for a temperature of 298 K. (b) The dependence of the conditional dimerization constant (log $K_{D,obs}$) at pH 10 on reciprocal temperature.

High Concentrations of NaCl Give Rise to Aggregated μ-Oxo Dimers of Fe(III)PPIX in Aqueous Solution. Evidence of μ-oxo dimer formation in 4.25 M NaCl is also observed in the UV−vis spectrum of Fe(III)PPIX in aqueous solution (Figure 6). However, the spectrum is different to those observed in mixed aqueous solutions, in that there is a combination of a broad Soret band suggestive of the $\pi-\pi$ dimer and a charge transfer region characteristic of the μ-oxo dimer. This spectrum suggests that the μ-oxo dimer formed under these conditions π-stacks to form large aggregates. In order to confirm this interpretation, the diffusion coefficient was measured in the presence of 4.25 M NaCl. This is compared to the diffusion coefficients previously reported for other Fe(III)PPIX species by de Villiers et al. (Table 2).[20] It is immediately apparent that the diffusion coefficient is considerably lower than those of monomeric and dimeric species. Assuming the presence of spherical particles, the Stokes−Einstein relationship (eq 8) can be used to calculate the apparent hydrodynamic radii of these particles. This demonstrates that the diffusing Fe(III)PPIX particles in 4.25 M NaCl are very much larger than those in other solvent systems. This result is consistent with an early report by Blauer and Zvilichovsky using ultracentrifugation that Fe(III)PPIX forms aggregates of 45−50 molecules in the presence of 1.2 M NaCl.[4]

Table 1. Observed Fe(III)PPIX Species Identified from UV−vis Spectra and the Dielectric Constants and ET_{30} Values of the Pure Organic Solvents Used in Mixed Aqueous Solutions, Each Containing 5.64 M Organic Solvent

solvent	Fe(III)PPIX species	ε_r	ET_{30}/kcal mol^{-1}[h]	protic/aprotic
water	$\pi-\pi$ dimer[a]	81.7[b]	63.1	protic
ethylene glycol	$\pi-\pi$ dimer	37[c]	56.3	protic
formamide	$\pi-\pi$ dimer	109[c]	55.8	protic
methanol	$\pi-\pi$ dimer	32.63[c]	55.4	protic
diethylene glycol	$\pi-\pi$ dimer/monomer	30.95[d]	53.8	protic
ethanol	$\pi-\pi$ dimer/monomer	24.3[c]	51.9	protic
2-propanol	$\pi-\pi$ dimer/monomer	18.3[c]	48.4	protic
dimethyl sulfoxide	μ-oxo dimer	45[c]	45.1	aprotic
tert-butyl alcohol	μ-oxo dimer/monomer	20.0[e]	43.3	protic
dimethylformamide	μ-oxo dimer	36.7[f]	43.2	aprotic
acetone	μ-oxo dimer	20.7[c]	42.2	aprotic
pyridine	μ-oxo dimer[a]	12.3[c]	40.5	aprotic
tetrahydrofuran	μ-oxo dimer	7.3[b]	37.4	aprotic
2,6-lutidine	μ-oxo dimer	6.9[g]	36.9	aprotic

[a]de Villiers et al.[20] [b]Guttman.[32] [c]CRC Handbook of Chemistry and Physics.[36] [d]Cwilinksa et al.[37] [e]El-Subruiti and Ahmed.[38] [f]Guo et al.[39] [g]Bakshi.[40] [h]Reichardt.[33]

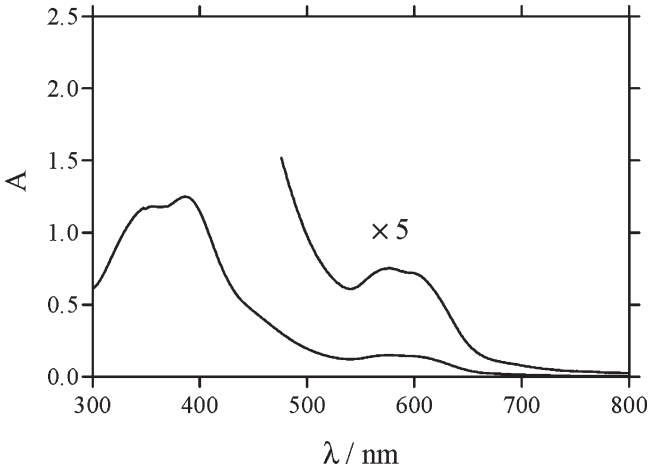

Figure 6. UV−vis spectrum of Fe(III)PPIX in the presence of 4.25 M NaCl at pH 10, 0.02 M CHES.

Discussion

The findings of this work emphasize the complexity of Fe(III)PPIX solutions. The species present are dependent on pH, Fe(III)PPIX concentration, electrolyte concentrations, and the identities of solvents present in mixed aqueous systems. These factors determine whether monomers, $\pi-\pi$ dimers, μ-oxo dimers or large aggregates are present. In addition, as we have shown previously,[20] findings can be further complicated by adsorption onto glassware and plasticware. For this reason, care needs to be exercised in the cleaning of glassware and cuvettes and plasticware must be avoided when attempting to conduct quantitative studies on Fe(III)PPIX in solution. We suspect that this behavior may be the main factor behind early reports that aqueous Fe(III)PPIX solutions are unstable and change slowly with time even when care is taken to avoid photodegradation and oxidation of the porphyrin ring.[31]

It has been known since the 1970s that 10% (v/v) pyridine can induce μ-oxo dimer formation in aqueous solution containing 0.1 M NaOH.[5] Recently, Casabianca et al. have shown that this dimer can also be formed in aqueous DMSO.[23] However, no systematic study of solvent effects appears to have previously been reported. The present work illustrates that a wide variety of solvents are capable of promoting μ-oxo dimer formation, while others do not. The dissociation and formation of the μ-oxo dimer also appears to be quite rapid. This is evident from the fact that a 10-fold dilution of 3×10^{-4} M μ-oxo dimer in aqueous DMSO (5.64 M) into aqueous methanol (5.64 M) results in conversion to the $\pi-\pi$ dimer as quickly as the spectrum can be obtained by manual mixing in a conventional spectrophotometer. Conversely, 10-fold dilution of 3×10^{-4} M $\pi-\pi$ dimer in aqueous methanol (5.64 M) into aqueous DMSO (5.64 M) results in conversion to the μ-oxo dimer by the time the spectrum is recorded. These observations also demonstrate that μ-oxo dimer formation is an equilibrium process depending on solvent conditions which determine which species is more stable. It is not the catalytic formation of a μ-oxo-bridged product which is inherently thermodynamically more stable regardless of the conditions in solution. It is also clear that there is no requirement that the solvent be an organic base. While pyridine is indeed both a Brønsted base and a Lewis base with a large donor number of 33.1, acetone which also induces μ-oxo dimer formation is not a Brønsted base and has a lower donor number (17.0) than either ethanol (19.2) or water (18),[32] neither of which induce formation of the μ-oxo dimer.

A closer look at solvent properties (Table 1) reveals that no correlation exists between the dimer species present and the dielectric constant of the solvent. For example, on the one hand water and formamide with high dielectric constants give $\pi-\pi$ dimers while acetone and pyridine with much lower dielectric constants induce μ-oxo dimer formation. On the other hand, DMSO and dimethyl formamide with higher dielectric constants than either methanol or ethanol give rise to μ-oxo dimers while the latter give $\pi-\pi$ dimers. By contrast, when an empirical microscopic measure of solvent polarity, the Dimroth−Reichard parameter or ET_{30} is used,[33] a clear correlation is observed (Table 1). Solvents with ET_{30} values $\geq$48.4 kcal mol^{-1} all give rise to $\pi-\pi$ dimers, while solvents with ET_{30} values $\leq$45 kcal mol^{-1} produce the

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Table 2. Diffusion Coefficients (D), Viscosity of the Solvent at 20 °C (η), Hydrodynamic Radii (a), and Apparent Hydrodynamic Volumes of Fe(III)PPIX Species Relative to Monomeric $(CN)_2$−Fe(III)PPIX (V/V_0) in Various Aqueous and Mixed Aqueous Solutions

species	$D/10^{-10}$ m^2	$\eta/10^{-3}$ kg m^{-1}s^{-1}	a/Å	V/V_0[a]
$(CN)_2$Fe(III)PPIX, pH 12	1.9 ± 0.2[b]	1.00[c]	11 ± 1	1
HO-Fe(III)PPIX, pH 11.9	1.2 ± 0.1[b]	1.00[c]	18 ± 3	4
$(Fe(III)PPIX)_2O$, 10% pyridine, 0.1 M NaOH	1.1 ± 0.1[b]	1.25[d]	16 ± 1	3
HO-Fe(III)PPIX, pH 10	1.6 ± 0.3	1.00[c]	13 ± 2	2
$(Fe(III)PPIX)_2O$, 4.25 M NaCl, pH 10	0.12 ± 0.03	1.64[c]	109 ± 27	973

[a]Mean volume relative to the bis-cyano species. [b]de Villiers et al.[20] [c]From CRC Handbook of Chemistry and Physics.[36] [d]From Dunstan et al.[41]

μ-oxo dimer. Even more strikingly, all of the solvents that give rise to $\pi-\pi$ dimers are protic while those that give the μ-oxo dimer are all aprotic except for *tert*-butyl alcohol.

The core of Fe(III)PPIX is often thought of as hydrophobic; however, in the case of five-coordinate H_2O/HO-Fe(III)PPIX it is in fact better considered to be amphipathic with the unligated face being hydrophobic and the face containing the axial H_2O/HO^- ligand being hydrophilic.[34] Formation of a $\pi-\pi$ complex entails interaction of the hydrophobic unligated faces of Fe(III)PPIX. This interaction, which is independent of the μ-oxo dimer formation process, is likely to be promoted by highly polar solvents. Thus, as water is replaced by solvents such as formamide and methanol the $\pi-\pi$ complex persists at least at low concentrations of the solvent. In fact, in the case of less polar solvents such as diethyleneglycol, ethanol and 2-propanol the increasing sharpness of the Soret band (Figure S4c−e, Supporting Information) strongly indicates significant monomerisation even at the low concentrations of solvent used in this study. Furthermore, it would appear that solvents which promote μ-oxo dimer formation (which are all even less polar as measured by their ET_{30} values) do not promote π-stacking, thus ensuring that the μ-oxo linked species are limited to dimers in these mixed aqueous solvents.

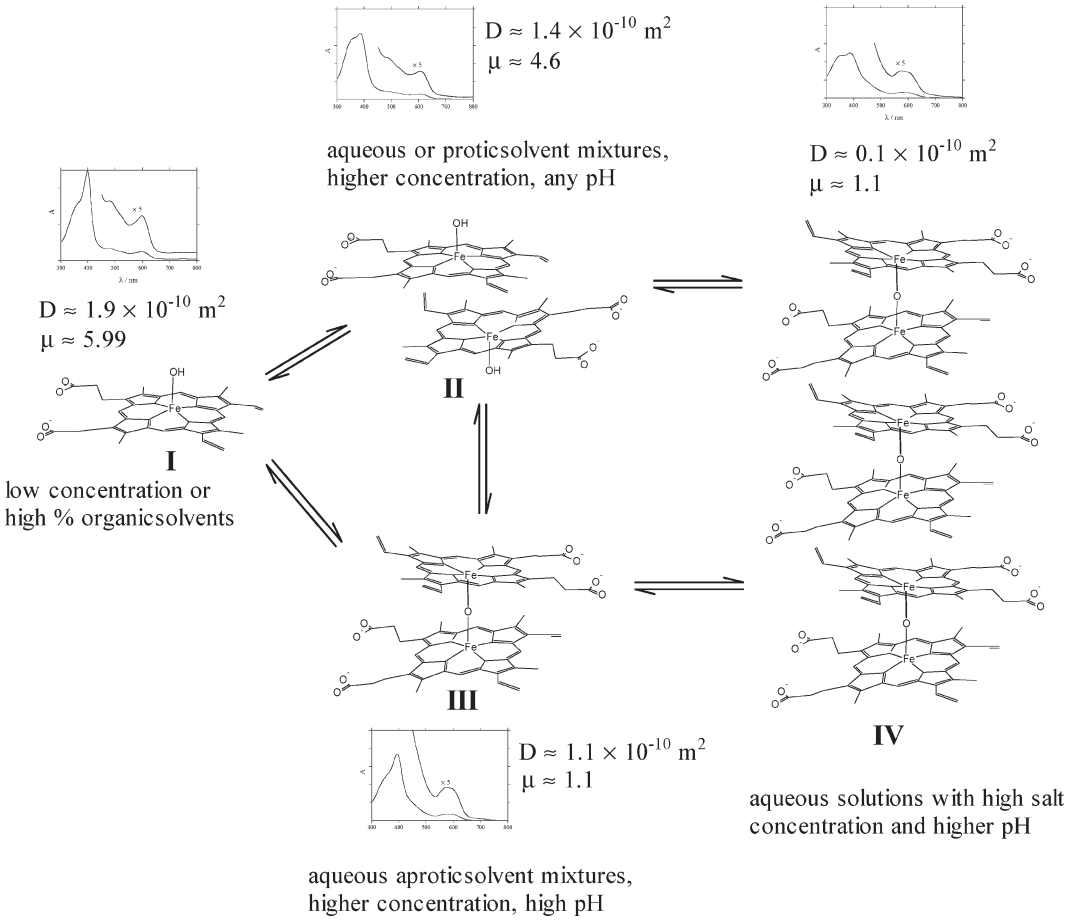
Investigation of the temperature dependence of the K_D value for μ-oxo dimer formation in aqueous DMSO (5.64 M) shows that this process is endothermic. In this mixed solvent system, the μ-oxo dimer will be somewhat more stable at 310 K (physiological temperature), however the increase in $K_{D,obs}$ from 317 M^{-1} to 822 M^{-1} at pH 7.4 will not have a major effect on speciation and the relevance of this in vivo is questionable, especially as this applies specifically to aqueous DMSO and is only relevant for mixed solvent systems with aprotic solvents. The endothermic nature of the process suggests that the stability of the μ-oxo dimer does not arise from any exceptional stability of the Fe−O−Fe bond. Rather, at high pH the formation of this dimer is entropy driven, strongly indicating that desolvation is responsible for its formation. This allows us to propose a hypothesis to explain the role of solvents in μ-oxo dimer formation. The axial H_2O/HO^- ligand can be expected to be strongly solvated by water molecules. This is supported by the crystal structure of aqua-Fe(III)octaethylporphyrin (H_2O−Fe(III)-OEP) reported by Cheng et al.[21] In this 5-coordinate cationic porphyrin, the unligated faces π-stack in the crystal, while the hydrophilic faces of adjacent molecules interact via a layer consisting of two ClO_4^- counterions and four included water molecules, with one ClO_4^- and two water molecules interacting with the axial H_2O ligand of each Fe(III)OEP. In H_2O−Fe(III)PPIX in pure aqueous solution, we can expect the axial water ligand to hydrogen bond to three solvent water molecules, two acting as hydrogen bond acceptors and one as a hydrogen bond donor, stabilizing this face and preventing further aggregation. As water begins to be replaced by a protic solvent such as methanol, this solvent will act as both hydrogen bond donor and acceptor, maintaining the stability of the hydrophilic face. On the other hand, when water is replaced by an aprotic solvent such as DMSO, it will only be able to replace water as a hydrogen bond acceptor. To maintain full solvation of the axial ligand, the solvation sphere will have to be enriched with water relative to the bulk. This will lower the entropy of this species, decreasing its stability and increasing ΔS upon desolvation. We hypothesize that it is largely this decrease in entropy which drives μ-oxo dimer formation in the presence of mixed solutions containing aprotic solvents. The one exception is the hindered protic solvent, *tert*-butyl alcohol which we deliberately chose to test this hypothesis. This solvent would be expected to solvate the axial H_2O/HO^- ligand less effectively than the other protic solvents for steric reasons and thus promotes μ-oxo dimer formation.

Finally, high concentrations of salts would be expected to disrupt solvation of the axial ligand by themselves strongly competing for the hydration sphere. This can be expected to lower the stability of the hydrophilic face of the porphyrin relative to the μ-oxo dimer. On the other hand, the solution remains highly polar and there is no weakening of the π-stacking interactions. The consequence is the formation of large π-stacked aggregates of μ-oxo dimers.

This complex set of equilibria is summarized in Scheme 1. It can be used to rationalize previous observations in the literature. For example, in water or water/alcohol mixtures the monomer (**I**) and $\pi-\pi$ dimer (**II**) are present. In mixed aqueous systems containing large proportions of an alcohol such as methanol which is not strongly coordinating, the monomer (**I**) dominates as we have shown previously.[20] In water at low or high pH the $\pi-\pi$ dimer (**II**) is overwhelmingly dominant. This species cannot aggregate beyond the dimer state and accounts for our previous observation and the original observation of Brown et al. that Fe(III)PPIX forms dimers, but not higher aggregates in aqueous solution.[3] In mixed aqueous systems containing a water-miscible aprotic solvent such as aqueous DMSO, pyridine, acetone, etc., the μ-oxo dimer (**III**) is formed. The long held view that the dimer present in aqueous solution is this μ-oxo dimer was based on the fact that Brown et al. had isolated the solid both by addition of solid NaOH (leading to high concentrations of Na^+) or by introduction of 1 M NaOH to a DMSO solution of hemin and demonstrated the presence of the Fe−O−Fe bond by infrared spectroscopy.[2] In light of the current study

(34) Under the pH conditions used in this study, the peripheral propionate groups are always negatively charged and unlikely to play a direct role in the trends observed.

Scheme 1. Representation of the Fe(III)PPIX Species Present in Aqueous and Mixed Aqueous Solution, Showing Characteristic UV−vis Spectra, Expected Diffusion Coefficients and Magnetic Moments (Conditions Favoring Each Particular Species Are Illustrated)



it is evident that species **IV** and **III** would indeed from under these conditions. In the absence of knowledge that aprotic solvents specifically promote μ-oxo dimer formation it was reasonable to suppose that **III** is the same species present in aqueous solution. Although these species appear to form only dimers in mixed aqueous systems, large aggregates of the μ-oxo dimers (**IV**) are formed in high salt concentrations. This accounts for the observations of Blauer and Zvilichovsky who correctly demonstrated the presence of aggregates in 1.2 M NaCl.[4] However, it cannot be ruled out that some of the studies suggesting large aggregates may also have been influenced by the strong tendency of Fe(III)PPIX to adsorb onto glass surfaces demonstrated by de Villiers et al.[20]

These findings for the first time permit predictions to be made about the species of Fe(III)PPIX likely to dominate under specific conditions in aqueous and mixed aqueous solution. In aqueous DMSO, concentration, pH, and temperature conditions can be chosen on the basis of these findings to selectively produce either predominantly the monomer or the μ-oxo dimer. Measurements of dimerization constants for other mixed aqueous systems should permit similar control over the species of Fe(III)PPIX present in other media.

Conclusions

This study shows that the aggregation state of Fe(III)PPIX in aqueous and mixed aqueous solution is largely controlled by solvation. This is likely to be a crucial factor in the behavior of hematin in other systems as well, such as detergents and lipids. A recent report by Casabianca et al. has shown that Fe(III)PPIX forms μ-oxo dimers within micelles of certain lipids such as TWEEN-20 and DTAB, but not SDS.[24] In unpublished studies in our laboratory, we have noted similar behavior for TWEEN-20 and SDS and have also found that TRITON X-100 also induces μ-oxo dimer formation. The situation in lipid droplets such as the lipid nanospheres in which hemozoin is now known to form within the malaria parasite is unknown.[35] It has also recently been shown that chloroquine can promote μ-oxo dimer formation. How this comes about is not clear, but the present study hints that chloroquine may act by perturbing the solvation of Fe(III)PPIX upon formation of a chloroquine-hematin complex. However, given the apparently fast interconversion between μ-oxo and $\pi-\pi$ dimers,

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the dominance of a particular species would not seem to rule out reactions occurring via either monomers or other intermediate species.

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Supporting Information Available: ^{1}H NMR spectra used for determination of magnetic moments using the Evans method and UV−vis spectra in various solvents. This material is available free of charge via the Internet at http://pubs.acs.org.