Aggregation of Fe(III)TPPS$_4$ on Biological Structures Is pH-Dependent, Suggesting Oxo-Bridging in the Aggregates

Victor E. Yushmanov

Instituto de Química de São Carlos, Universidade de São Paulo, São Carlos 13560-970, SP, Brazil

Received April 3, 1998

Interaction of the Fe(III) derivative of tetra(4-sulfonatophenyl)porphyrin (TPPS$_4$), and diamagnetic ZnTPPS$_4$ and metal-free TPPS$_4$, with the simplest models for membranes and protein reaction centers, aqueous (AM) and reversed (RM) ionic micelles, was studied by high-resolution ^{1}H NMR and proton magnetic relaxation measurements. AM were much more sensitive than RM to the bulky porphyrins, seemingly, because of the more restricted motion of surfactant chains in AM. TPPS$_4$ and its derivatives were incorporated into the AM of cationic cetyltrimethylammonium chloride (CTAC) or zwitterionic lysophosphatidylcholine (LPC) near the terminal part of their hydrocarbon chains, as evidenced by a strong upfield shift of the corresponding peaks. At a FeTPPS$_4$/CTAC molar ratio greater than 0.05 and a pH > 4, FeTPPS$_4$ partly formed nonparamagnetic aggregates, which dissociated into monomers at pH < 4. In CTAC RM, FeTPPS$_4$ was predominantly aggregated, the transition to the monomer form occurring upon acidification of the water RM interior to about pH −1. No similar pH dependencies were found for ZnTPPS$_4$ and TPPS$_4$. It is supposed that charged porphyrins may interact with cellular membranes. Characteristic pH dependence of the FeTPPS$_4$ aggregation in micelles suggests that aggregated units are bound through a μ-oxo-bridge. Similar mechanisms may be operative in other systems, such as porphyrin-protein.

Introduction

The importance of porphyrins and related compounds as therapeutic drugs and targeting agents has been widely recognized.[1−3] High affinity and phototoxicity of sulfonated *meso*-tetraphenylporphyrins to tumor cells make them promising compounds for photodynamic therapy (PDT) of cancer.[4] Radioactive derivatives of anionic tetra(4-sulfonatophenyl)porphyrin (TPPS$_4$) have been used as contrast agents in radiological imaging.[5,6] The paramagnetic Fe(III) and Mn(III) derivatives of TPPS$_4$ have recently attracted attention as prototypes for tumor-specific magnetic resonance imaging (MRI) contrast agents.[7,8]

The mechanisms of biological effects of porphyrins and metalloporphyrins may involve their penetration through membranes[9] and binding to proteins, e.g., to albumins in blood plasma.[10,11] Using ^{1}H NMR, electron spin resonance (ESR), and optical spectroscopic techniques, we have reported elsewhere the solution properties of TPPS$_4$ and dimerization and axial ligation of its Fe(III) and Mn(III) complexes,[12] and we have shown that the relaxation efficiency and blood transport of metalloporphyrins may be influenced greatly by their aggregation on proteins, particularly, bovine serum albumin (BSA)[13,14] and DNA.[15] The molecular nature of aggregates (either through the π-electron clouds interaction or μ-oxo-bridge), however, remained unclear. Similar aggregation of the diamagnetic free-base TPPS$_4$ upon binding to BSA has also been observed by optical spectroscopy,[16,17] suggesting that the same mechanism may underlie the aggregation of both free-base porphyrins and metalloporphyrins.

The porphyrin−protein model system, when used in physicochemical studies, has severe limitations. High molar concentrations of protein are inaccessible, and denaturation occurs beyond relatively narrow pH and temperature range. Moreover, the presence of numerous amino acids ionizable in the physiological pH range makes the protein properties extremely pH-dependent even within this range. For instance, BSA passes through its isoelectric point at mild acidification (pH 4.5−5.0), whereas in slightly basic media the tyrosine residues (which form part of the bilirubin binding sites) become negatively charged.[18] Thus, the interpretation of pH-dependent data may become ambiguous.

(1) Bonnett, R. *Chem. Soc. Rev.* **1995**, 19−33.
(2) Cannon, J. B. *J. Pharm. Res.* **1993**, *82*, 435−446.
(3) Ben-Hur, E.; Horowitz, B. *Photochem. Photobiol.* **1995**, *62*, 383−388.
(4) Berg, K.; Bommer, J. C.; Winkelman, J. W.; Moan, J. *Photochem. Photobiol.* **1990**, *52*, 775−781.
(5) Hambright, P.; Fawwaz, R.; Valk, P.; McRae, J.; Bearden, A. J. *Bioinorg. Chem.* **1975**, *5*, 87−92.
(6) Zanelli, G. D.; Kaelin, A. C. *Br. J. Radiol.* **1981**, *54*, 403−407.
(7) Patronas, N. J.; Cohen, J. S.; Knop, R. H.; Dwyer, A. J.; Colcher, D.; Lundy, J.; Mornex, F.; Hambright, P.; Sohn, M.; Myers, C. E. *Cancer Treat. Rep.* **1986**, *70*, 391−395.
(8) Fiel, R. J.; Button, T. M.; Gilani, S.; Mark, E. H.; Musser, D. A.; Henkelman, R. M.; Bronskill, M. J.; van Heteren, J. G. *Magn. Reson. Imaging* **1987**, *5*, 149−156.
(9) Ricchelli, F.; Gobbo, S.; Jori, G.; Moreno, G.; Vinzens, F.; Salet, C. *Photochem. Photobiol.* **1993**, *58*, 53−58.
(10) Lamola, A. A.; Asher, I.; Muller-Eberhard, U.; Poh-Fitzpatrick, M. *Biochem. J.* **1981**, *196*, 693−698.
(11) Datta-Gupta, N.; Malakar, D.; Dozier, J. *Res. Commun. Chem. Pathol. Pharmacol.* **1989**, *63*, 289−292.
(12) Yushmanov, V. E.; Imasato, H.; Tominaga, T. T.; Tabak, M. *J. Inorg. Biochem.* **1996**, *61*, 233−250.
(13) Yushmanov, V. E.; Tominaga, T. T.; Borissevitch, I. E.; Imasato, H.; Tabak, M. *Magn. Reson. Imaging* **1996**, *14*, 255−261.
(14) Tominaga, T. T.; Yushmanov, V. E.; Borissevitch, I. E.; Imasato, H.; Tabak, M. *J. Inorg. Biochem.* **1997**, *65*, 235−244.
(15) Gandini, S. C. M.; Yushmanov, V. E.; Perussi, J. R.; Tabak, M.; Borissevitch, I. E. *J. Inorg. Biochem.* **1999**, in press.
(16) Borissevitch, I. E.; Tominaga, T. T.; Imasato, H.; Tabak, M. *J. Lumin.* **1996**, *69*, 65−76.
(17) Borissevitch, I. E.; Tominaga, T. T.; Imasato, H.; Tabak, M. *Anal. Chim. Acta* **1997**, *343*, 281−286.
(18) Kragh-Hansen, U. *Pharmacol. Rev.* **1981**, *33*, 17−53.

10.1021/ic980377u CCC: $18.00
Published on Web 03/20/1999

Here, we address the nature of Fe(III)$TPPS_4$ aggregation upon binding to biological structures using the simplest models for membranes and protein reaction centers: aqueous (AM) and reversed (RM) ionic micelles[19−21] with stable characteristics in a wide pH, concentration, and temperature range exceeding the limits appropriate for biopolymers. It cannot be said a priori whether the micellar model represents well the real porphyrin binding sites on proteins; little is known about binding sites. Available reports mostly refer to the bilirubin binding sites at albumins.[18,22] Serum albumin is also known to be a heme-binding protein. However, some metalloporphyrins distribute differently among porphyrin binding sites on human serum albumin than does heme (Fe(III)protoporphyrin IX).[23] Also, binding sites of hemin and protoporphyrin derivatives on albumin differ from bilirubin binding sites.[18,22,23] Based on NMR and ESR data for the Fe(III)protoporphyrin IX, it has been suggested previously that the hemes in AM solutions seem to provide a possible model for the hemoproteins.[24]

Studies of the micellar systems are also expected to shed some light on the issue of interaction of ionic porphyrins with biological membranes and liposomes used for porphyrin drug delivery. It has been suggested that hydrophobic porphyrins can penetrate into the lipid regions of the membranes and distribute into protein-rich membrane domains,[9] whereas highly polar species were supposed to partition mainly in the aqueous compartments.[25] However, the interaction of water-soluble synthetic porphyrins with ionic micelles has been clearly shown.[26,27] Thus, studies of interaction of water-soluble porphyrin derivatives with biomimetic systems are relevant in view of the challenges faced in formulating these drugs into stable, effective, and safe-dosage forms.

Experimental Section

Sodium salts of $TPPS_4$, $ZnTPPS_4$, and $FeTPPS_4$, tetra(*N*-methyl-4-pyridyl)porphyrin (TMPyP) chloride (Midcentury), lysophosphatidylcholine (LPC, Sigma), *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate (HPS, Sigma), sodium dodecyl sulfate (SDS, BioRad), $CDCl_3$ (Carlo Erba and Aldrich), D_2O (Merck), sodium acetate and potassium phosphate (Mallinckrodt) were used as purchased. Cetyltrimethylammonium chloride (CTAC, Herga) was purified by acetone-methanol extraction.

Equilibration in the system porphyrin-AM at room temperature is slow and requires several days to reach full equilibrium, so the samples were prepared well in advance. In experiments with AM, all solutions were prepared in a 0.02 M acetate or phosphate buffer, the pH values were adjusted using a Corning-130 pH-meter with an Ingold glass Ag/AgCl semimicro combination electrode, and they were not corrected for the isotope effect in D_2O solutions. In the porphyrin-RM system, the equilibration time is much shorter compared with AM. In experiments with RM, no special precautions against water impurity in solvents and solutes, such as vacuum-drying and keeping over molecular sieves, were undertaken.

(19) Fendler, J. H.; Fendler, E. J. *Catalysis in Micellar and Macromolecular Systems*; Academic Press: New York, 1975.
(20) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley-Interscience: New York, 1982.
(21) Yushmanov, V. E.; Tabak, M. *J. Colloid Interface Sci.* **1997**, *191*, 384−390.
(22) Peters, T. Jr. *Adv. Protein Chem.* **1985**, *37*, 161−236.
(23) Breslow, E.; Chandra, R.; Kappas, A. *J. Biol. Chem.* **1986**, *261*, 3135−3141.
(24) Mazumdar, S.; Medhi, O. K.; Mitra, S. *Inorg. Chem.* **1988**, *27*, 2541−2543.
(25) Ricchelli, F.; Jori, G. *Photochem. Photobiol.* **1986**, *44*, 151−157.
(26) Kadish, K. M.; Maiya, G. B.; Araullo, C.; Guilard, R. *Inorg. Chem.* **1989**, *28*, 2725−2731.
(27) Kadish, K. M.; Maiya, G. B.; Araullo-McAdams, C. *J. Phys. Chem.* **1991**, *95*, 427−431.

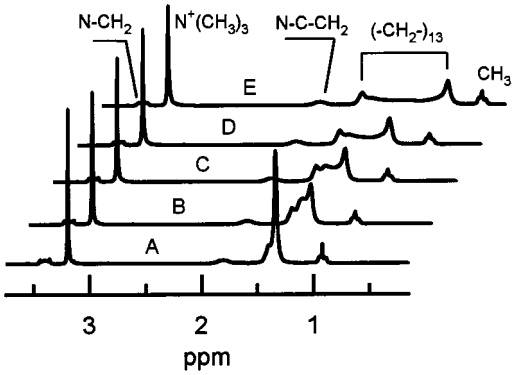

Figure 1. ^1H NMR spectra of 100 mM CTAC in D_2O at pH 5.0 in the absence (A) and presence of $TPPS_4$, 1.3 mM (B), 2.5 mM (C), 5.0 mM (D), and 10.0 mM (E).

Proton NMR spectra were run on a Bruker AC-200 spectrometer (resonant frequency, 200.13 MHz) at 23 °C. In aqueous samples, the residual water peak was presaturated by gated irradiation during the pre-excitation delay of 2 s. For diamagnetic compounds TMPyP, $TPPS_4$, $ZnTPPS_4$, and surfactants, the NMR protocol was as follows. The 11° pulses of 1-μs duration were repeated every 4.8 s, the bandwidth was 2940 Hz, and the memory size was 16K data points. For paramagnetic $FeTPPS_4$, the pulse repetition time was 2.41 s, the bandwidth was 20 kHz. To reveal the highly broadened peaks of porphyrin moieties in paramagnetic samples, the postprocessing exponential filter with a line-broadening factor of at least 10 Hz was applied in time domain. Chemical shifts were referenced to tetramethylsilane (TMS) (in $CDCl_3$) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, in D_2O).

Nonselective relaxation times T_1 were measured with the inversion−recovery pulse sequence (t-π-τ-π/2-acquire) with a π/2 pulse duration of 8.2 μs. Selective T_1 relaxation times were measured with the same pulse scheme, where the π pulse was given by the proton decoupler gated at the chosen frequency for 36 ms. The residual water resonance in aqueous samples was irradiated except during the pulses and acquisition. Paramagnetic relaxation enhancement by $FeTPPS_4$ is given as $\Delta R_1 = 1/T_1 - 1/T_1^{(0)}$, where $T_1^{(0)}$ is a relaxation time without $FeTPPS_4$.

Water content in RM was estimated by measurements of its ^1H NMR peak area relative to the internal reference TMS (1%) and residual $CHCl_3$ peaks after proper calibration. The accuracy of data is justified by the absence of water presaturation, and by the use of recycle times always exceeding $5T_1$ for the water peak (T_1 was below 450 ms for the samples containing $FeTPPS_4$, and below 650 ms for the diamagnetic samples).

Results

Magnetic resonance techniques are suitable to address the issue of aggregation of $FeTPPS_4$. Aggregated species lack paramagnetism,[12−14] and therefore may be readily distinguished from paramagnetic $FeTPPS_4$ monomers in high-resolution ^1H NMR spectra and by magnetic relaxation measurements. We compared the data obtained for $FeTPPS_4$ with data obtained for the related compounds, $ZnTPPS_4$ and metal-free $TPPS_4$, which are nonparamagnetic, and do not form μ-oxo-bridged aggregates.

Binding of the Porphyrin Derivatives to Aqueous Micelles. The incorporation of $TPPS_4$ and its metal derivatives into the cationic AM resulted in dramatic effects in the spectra of surfactants. The most noticeable effect in the spectra of CTAC was an upfield shift of the peaks belonging to the terminal methyl and neighboring methylenes of the hydrocarbon chain (Figure 1). Some decrease was observed in T_1 for all CTAC groups, seemingly, because of the increase in their rotational correlation time τ_c. The effects induced by $TPPS_4$ and $ZnTPPS_4$ were almost identical, whereas $FeTPPS_4$ produced additional

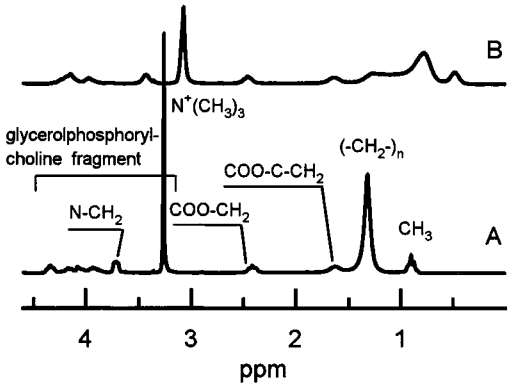

Figure 2. ^{1}H NMR spectra of 20 mM LPC in D_2O at pH 5.5 in the absence (A) and presence (B) of 10 mM TPPS$_4$.

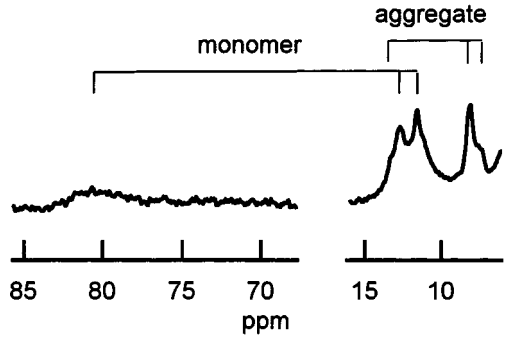

Figure 3. ^{1}H NMR spectrum of 10 mM FeTPPS$_4$ in D_2O at pH 5.5 in the presence of 100 mM CTAC.

upfield shift and significant broadening of the peaks of the terminal part of the surfactant hydrocarbon chain because of the proximity of the paramagnetic iron ion. The T_1 values of all CTAC protons were markedly shortened by the presence of the Fe(III) ion, especially the shifted and broadened peaks of the protons closest to it.

The effects induced by TPPS$_4$ in the spectra of CTAC and LPC were qualitatively similar (Figure 2). As opposed to the anionic TPPS$_4$, cationic TMPyP incorporated only into anionic SDS micelles, again with the same effects on the terminal part of the SDS hydrocarbon chain, the interaction even with zwitterionic HPS and LPC being negligible.

pH-Dependent Aggregation of the Porphyrin Derivatives in Aqueous Micelles. In the spectra of FeTPPS$_4$ solutions in the presence of 100 mM CTAC at pH 5.5 (Figure 3), one can see two types of compounds: monomeric with the peaks at 11.6, 12.7, and about 80 ppm, and aggregated with the peaks at about 7.5, 8.1, and 13 ppm. Peak positions differ from those of the free species in aqueous solution (at 10.3, 14.2, and about 52 ppm for the monomer, and 7.0, 8.1, and 12.7 for the dimer[12,14]) and are concentration-independent. Line widths of the FeTPPS$_4$ signals in the relatively mobile micellar systems (about 70 Hz for the narrowest peaks) were much lower than in highly immobilized complexes with BSA (about 200 Hz[13,14]). At submillimolar concentrations, FeTPPS$_4$ was bound to AM as a monomer, the aggregates being formed at higher concentrations. However, because of the low concentration and still broadened lines, the signal/noise ratio in the ^{1}H NMR spectra of FeTPPS$_4$ was very low, so that their use for quantitative measurements was hardly practicable. Therefore, we opted for chemical shifts and relaxation times of water and CTAC protons influenced by the FeTPPS$_4$ magnetism.

The dose−effect curves for paramagnetic relaxation enhancement of water protons at pH 5.5, as well as of the protons of

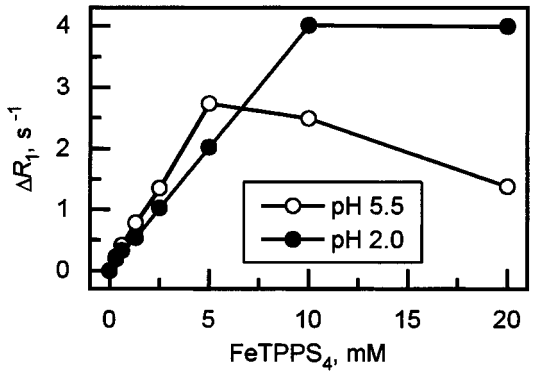

Figure 4. Paramagnetic contribution of FeTPPS$_4$ to the spin−lattice relaxation rate of water protons in the presence of 100 mM CTAC at pH 5.5 and 2.0.

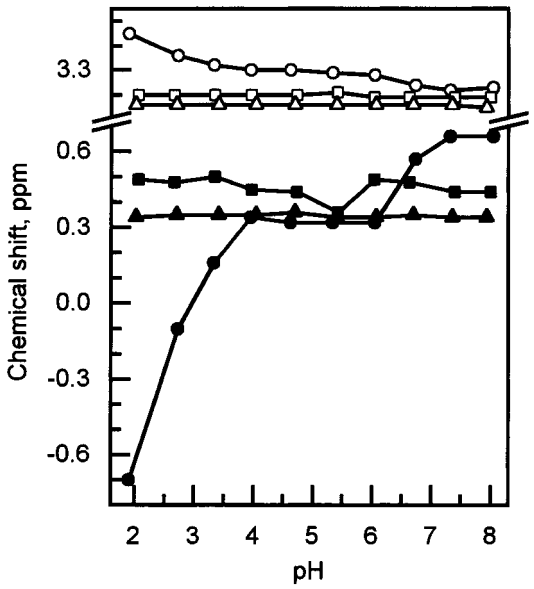

Figure 5. The pH dependence of chemical shifts of 10 mM CTAC protons in the presence of 1 mM FeTPPS$_4$ (circles), ZnTPPS$_4$ (squares), or TPPS$_4$ (triangles). Solid and open symbols represent chain terminal methyls and headgroup methyls, respectively.

CTAC, have a maximum at relatively low FeTPPS$_4$ concentration (Figure 4). In homogeneous solution, the paramagnetic relaxation rate ΔR_1 is proportional to the concentration of paramagnetic centers. In biological systems, the immobilization of paramagnetic ions on macromolecules and membranes causes departure from linearity, yet ΔR_1 is still roughly proportional to their concentration. Therefore, the decrease in ΔR_1 of about one-half from 5 mM FeTPPS$_4$ to 20 mM, instead of the expected about 3-fold increase, suggests the strong aggregation at higher concentrations. At pH 2, the aggregates partially dissociated back into monomers (Figure 4), the plateau above 10 mM being caused by the residual aggregation at higher concentrations. The positions of FeTPPS$_4$ peaks in ^{1}H NMR spectra confirmed the predominance of bound monomers at pH 2.

The pH dependencies of chemical shifts of the 10 mM CTAC resonances in the presence of 1 mM FeTPPS$_4$ clearly demonstrate two transitions: about pH 6.5 and below pH 4 (Figure 5, circles). Similar experiments with TPPS$_4$ and ZnTPPS$_4$ did not show any pH dependence, the chemical shifts of the chain terminal methyls being about 0.35 and 0.46 ppm and of the headgroup methyls being 3.16 and 3.20 ppm for TPPS$_4$ (triangles) and ZnTPPS$_4$ (squares), respectively, between pH 2 and 8. The ^{1}H NMR peaks of TPPS$_4$ and ZnTPPS$_4$ also remained unchanged throughout the whole pH range.

Table 1. Nonselective (T_1^{ns}) and Selective (T_1^{sel}) Relaxation Times (ms) of $TPPS_4$ Protons in Homogeneous and Micellar Aqueous Solutions

	pyrrole		phenyl	
	T_1^{ns}	T_1^{sel}	T_1^{ns}	T_1^{sel}
$TPPS_4$, pH > 5	330 ± 10	280 ± 20	790 ± 30 and 340 ± 30[a]	770 ± 40 and 340 ± 40[a]
$TPPS_4$ + CTAC, pH 5[c]	840 ± 30	420 ± 20[b]	870 ± 20	510 ± 10[b]
$TPPS_4$ + CTAC, pH 1[c]	970 ± 120	410 ± 20[b]	760 ± 40	500 ± 40[b]
$TPPS_4$ + LPC, pH 4.5[d]	460 ± 30	210 ± 20[b]	470 ± 10	240 ± 10[b]

Data are presented as mean ± SEM. The significance of the difference between the group means was tested by the two-tailed independent Student's *t* test. [a] For *ortho*- and *meta*-phenyl protons. [b] $p < 0.05$ vs T_1^{ns}. [c] 5 mM $TPPS_4$/100 mM CTAC. [d] 10 mM $TPPS_4$/20 mM LPC.

$TPPS_4$ itself has a propensity to aggregate in slightly acidic media both in homogeneous solution[12,28,29] and in the presence of cationic surfactants[30] because of its transition to dication, H_2TPPS_4. Here, we observed the dication formation of $TPPS_4$ in aqueous solution in the presence of cationic CTAC (5 mM $TPPS_4$/100 mM CTAC) at pH < 2.5, and in the presence of zwitterionic LPC (10 mM $TPPS_4$/20 mM LPC) at pH < 5. The transition to H_2TPPS_4 manifested itself in the disappearance of peaks belonging to the unbound fraction of $TPPS_4$ as well as in a smaller disturbance in surfactant chains. Proton relaxation times T_1 of a small ligand are often affected by intermolecular interactions more strongly than chemical shifts, and are related to the molecular mobility of the corresponding chemical groups. Pathways of magnetic relaxation are different when only the signal of interest (selective relaxation) or all transitions in the spectrum (nonselective relaxation) were perturbed. The micelle protons are seemingly in the intermediate motional regime $\omega_0\tau_c \sim 1$ (ω_0 is a Larmor frequency); in this case, the selective and nonselective T_1 do not differ significantly, as shown previously for CTAC[31] and confirmed in this study for LPC (data not shown). However, for a small molecule undergoing significant immobilization, selective inversion of its spins may provide an additional index because of the different dependence of nonselective and selective spin−lattice relaxation rates on the correlation time τ_c.[21,32,33] Indeed, the much shorter selective than nonselective T_1 values of $TPPS_4$ bound to AM indicated slow molecular motion ($\omega_0\tau_c > 1$) of the complex (Table 1). However, the T_1 values of $TPPS_4$ bound to CTAC appeared to be pH-independent, i.e., the same for $TPPS_4$ and H_2TPPS_4.

Porphyrin Derivatives in Reversed Micelles. Chloroform is a common solvent for preparing ionic RM in biomimetic chemistry.[21,34,35] It is suitable for our study, because $TPPS_4$ and its derivatives are not soluble in chloroform, and therefore partition exclusively in the RM or in the aqueous phase in the RM interior. A CTAC (100 mM) micellar solution solubilized $TPPS_4$ and $FeTPPS_4$ in limited amount, about 10 mM. The bound forms of $TPPS_4$ have similar NMR peak positions in RM and AM. The spectra of $FeTPPS_4$ in RM have some peculiarities. The monomeric species yielded only two peaks because of phenyl protons at 13.5 ppm and a very broad pyrrole resonance at about 80 ppm. The aggregated species had the spectrum similar to that in AM with phenyl peaks at 7.6 and 8.2 ppm, and a pyrrole peak at 13.4 ppm, however; the *meta*-phenyl line (8.2 ppm) was considerably narrower than in aqueous media (cf. Figure 3).

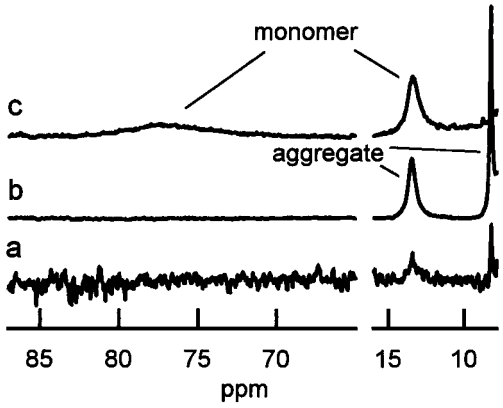

Figure 6. ^1H NMR spectra of $FeTPPS_4$ in reversed micelles of 100 mM CTAC in $CDCl_3$. (a) 0.16 mM $FeTPPS_4$; (b) 5.0 mM $FeTPPS_4$; (c) 5.0 mM $FeTPPS_4$ at pH = −1 in the micelle interior. For spectral assignments see text. The *ortho*-phenyl line at 7.6 ppm is not shown because it is obscured by the wing of the residual $CHCl_3$ resonance at about 7.3 ppm.

The spectra shown in Figure 6 exemplify that, contrary to proteins and AM, $FeTPPS_4$ in 100 mM CTAC RM was aggregated predominantly at all metalloporphyrin concentrations used down to 0.16 mM, the transition to the monomer form occurring upon strong acidification of the water RM interior by a small amount of HCl to about pH −1 (as estimated according to El Seoud[36]). Accordingly, no nonmonotonical behavior of the water relaxation has been found in nonacidified RM.

The influence of $TPPS_4$ and $FeTPPS_4$ on the resonances of CTAC (100 mM) in nonacidified RM is marginal. Even though at 5−10 mM $FeTPPS_4$ all resonances became somewhat broadened with shorter T_1 values, in no case did any chemical shift change more than 0.08 ppm. The acidification of RM interior, bringing $FeTPPS_4$ to the highly paramagnetic monomeric state, resulted in much greater concentration-dependent effects in the spectra (Figure 7). At 5 mM $FeTPPS_4$, all line widths increased several times, T_1 values shortened about an order of magnitude, and the span of chemical shifts of the CTAC protons increased by 0.2 ppm. RM acidification in the presence of $TPPS_4$ or $ZnTPPS_4$, although it also caused significant broadening of the CTAC resonances, did not bring marked alterations in chemical shifts and T_1 values. The line broadening in the absence of paramagnetic Fe(III) ion has a different nature, and may be tentatively attributed to the sticking together of the protonated porphyrin molecules.

(28) Ribó, J. M.; Crusats, J.; Farrera, J.-A.; Valero, M. L. *J. Chem. Soc., Chem. Commun.* **1994**, 681−682.
(29) Pasternack, R. F.; Schafer, K. F.; Hambright, P. *Inorg. Chem.* **1994**, *33*, 2062−2065.
(30) Maiti, N. C.; Mazumdar, S.; Periasamy, N. *Curr. Sci.* **1996**, *70*, 997−999.
(31) Yushmanov, V. E.; Imasato, H.; Perussi, J. R.; Tabak, M. *J. Magn. Reson. B* **1995**, *106*, 236−244.
(32) Valensin, G.; Kushnir, T.; Navon, G. *J. Magn. Reson.* **1982**, *46*, 23−29.
(33) Bonechi, C.; Donati, A.; Picchi, M. P.; Rossi, C.; Tiezzi, E. *Colloids Surf. A* **1996**, *115*, 89−95.
(34) Seno, M.; Sawada, K.; Koji, A.; Iwamoto, K.; Kise, H. *J. Colloid Interface Sci.* **1980**, *78*, 57−64.
(35) Datta, G.; Parvathanathan, P. S.; Rao, U. R. K.; Deniz, K. U. *Physiol. Chem. Phys. Med. NMR* **1992**, *24*, 51−61.
(36) El Seoud, O. A. In *Reverse Micelles. Biological and Technological Relevance of Amphiphilic Structures in Apolar Media*; Luisi, P. L.; Straub, B. E., Eds.; Plenum Press: New York, 1984; pp 81−93.

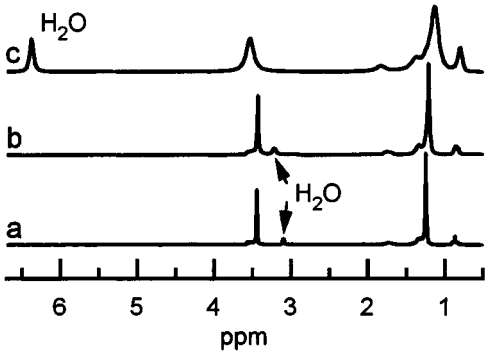

Figure 7. ^{1}H NMR spectra of reversed micelles of 100 mM CTAC in $CDCl_3$ in the presence of FeTPPS$_4$. (a) 0.16 mM FeTPPS$_4$; (b) 10.0 mM FeTPPS$_4$; (c) 5.0 mM FeTPPS$_4$ at pH = −1 in the micelle interior. The dramatic displacement of the water resonance in the chart (c) is mainly caused by acidification. Assignments of other peaks are similar to those in Figure 1.

Discussion

The results of this study, together with some previous reports of others,[26,27,30] show that charged porphyrins strongly interact with ionic micelles and, therefore, may not exclusively partition in the aqueous compartments, as generally supposed,[25] but also may interact with cellular membranes. In general, interaction of charged porphyrins with ionic micelles is determined mainly by electrostatic forces. It is evident from two facts. First, the anionic species (TPPS$_4$ and its metal derivatives) incorporated into cationic (CTAC, CTAB[26]), zwitterionic (LPC), and neutral (Triton X-100[26]) rather than anionic (SDS) micelles. In contrast, the cationic species (TMPyP and its metal derivatives) incorporated only into anionic SDS rather than cationic (CTAC, CTAB[27]), zwitterionic (LPC, HPS), and neutral (Triton X-100[27]) micelles. Second, in all cases when an interaction takes place, its character is the same, the terminal part of the surfactant hydrocarbon chain being the most influenced (Figures 1 and 2). Upfield shifts in this spectral region are most probably due to the effect of the ring currents in the conjugated electron system of the porphyrin, because the alternative possibility, the effect of charged groups, should lead to the higher polarity, which is usually associated with downfield shifts.

The issue of monomer−aggregate equilibrium of the porphyrin derivatives in micellar systems has been addressed repeatedly. Micelles are usually considered only as a means to solubilize aggregates into monomers.[24,26,27,30,37] To the best of our knowledge, so far there has been no sound witness to the ferric porphyrin aggregation induced by micelles. The local maximum of relaxation efficiency (Figure 4) is an unambiguous indication of the predominance of nonparamagnetic bound aggregates at high FeTPPS$_4$/micelle ratio, where not only the fraction, but even the absolute amount of the monomer form decreased. This pattern reproduces the behavior of FeTPPS$_4$ upon its binding to BSA,[13,14] thus suggesting that a universal mechanism of FeTPPS$_4$ aggregation on biological structures may exist. However, when working with BSA, little may be said about the nature of aggregation, because the bound aggregates of FeTPPS$_4$ hardly may be distinguished by NMR from free oxo-dimers at physiological pH. In AM, the breakdown of aggregates into paramagnetic monomers in acidic media shows that the aggregation is a pH-dependent process.

Relaxation measurements alone are not capable of discriminating between free and bound FeTPPS$_4$ species. Contrary to T_1, chemical shifts of the CTAC peaks are influenced mainly by the bound species, and therefore are suitable to study just the bound form and to compare its effects with effects of nonparamagnetic analogues. In the experiment depicted in Figure 5, the concentration of FeTPPS$_4$ (1 mM) is low enough to avoid the formation of dimers in solution at pH < 5.[12] The free dimer formation occurs at pH > 6.5, and this transition manifests itself in Figure 5. Most probably, the monomer−dimer transition in solution influences the chemical shifts of CTAC protons indirectly, through the different binding properties of monomers and dimers, similarly to what has been found for the FeTPPS$_4$ binding to BSA.[14] Another transition occurs below pH 4. This transition is due to the monomer−aggregate equilibrium for the bound species, which was also evidenced by the spectra of FeTPPS$_4$ (data not shown). Thus, the FeTPPS$_4$ aggregation is really induced by micelles at pH below the dimerization point for the free metalloporphyrin. In acidic media (pH < 4), however, bound aggregates disaggregate, which is accompanied by the strong upfield shift of the micelle protons.

Most importantly, no pH-dependent changes have been found for TPPS$_4$ and ZnTPPS$_4$, both for the micelle protons (Figure 5) and porphyrin protons. It is not clear whether TPPS$_4$ and ZnTPPS$_4$ are subject to aggregation in the given experimental conditions. TPPS$_4$ is known to form aggregates of both J-type (with horizontally displaced parallel molecular planes) and H-type (stacks) in its dication form[28−30]; this aggregation would be enhanced in acidic media. The aggregation of the TPPS$_4$ dication might lead to the reduction in its signal intensity[12] and to the smaller displacement of micelle peaks. However, in accordance with earlier data for cetyltrimethylammonium bromide,[30] the dication experienced a transition to the free-base monomer at CTAC concentration above its critical micelle concentration (>1 mM[19]) at moderately acidic pH. In our study, we observed a significant TPPS$_4$ dication formation at 100 mM CTAC only below pH 2.5 because of the pK shift to lower pH upon association with cationic surfactant. Alterations in pK usually indicate complex formation between charged molecules.[38,39] The TPPS$_4$ free base has also been shown to aggregate on BSA, although the underlying mechanism has not been discussed.[16,17]

In ZnTPPS$_4$, the central nitrogens are not available for protonation because of the metal coordination. Therefore, no metalloporphyrin aggregation was reported at low pH.[29] Neither is ZnTPPS$_4$ expected to form dimers with a μ-oxo-bridge between metal ions.[40] At any rate, even if TPPS$_4$ and ZnTPPS$_4$ were partially aggregated in the presence of CTAC, this did not lead to pH-dependent effects typical for FeTPPS$_4$.

The strong pH dependence of the FeTPPS$_4$ bound aggregate formation apparently indicates the involvement of the μ-oxo-bridge in that process. An alternative explanation would be the variations in ion concentrations, because higher ionic strength favors aggregation of porphyrins.[29,41] However, the only possible source of ionic strength variations was the addition of aliquots of acid or base for pH adjustment, which actually was more significant to decrease pH. Thus, the putative effect of ions would be just opposite to that actually observed.

We suppose that the pH-dependent μ-oxo-bridging may also be operative in the FeTPPS$_4$−BSA interaction, when no direct

(37) Simplicio, J. *Biochemistry* **1972**, *11*, 2525−2529.
(38) Rabenstein, D. L.; Bratt, P.; Schierling, T.; Robert, J. M.; Guo, W. *J. Am. Chem. Soc.* **1992**, *114*, 3278−3285.
(39) Yushmanov, V. E.; Perussi, J. R.; Imasato, H.; Ruggiero, A. C.; Tabak, M. *Biophys. Chem.* **1994**, *52*, 157−163.
(40) Mikros, E.; Gaudemer, A.; Pasternack, R. *Inorg. Chim. Acta* **1988**, *153*, 199−200.
(41) White, W. I. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. 5, pp 303−339.

proof may be obtained, because at pH < 5 the protein passes through its isoelectric point and changes its properties. Some geometric issues of the formation of large $FeTPPS_4$ stacks have been addressed elsewhere.[14] Obviously, the mechanism of free-base $TPPS_4$ aggregation on BSA should be different, inasmuch as an oxygen bridge is possible only between metal ions.

Contrary to proteins and AM, in 100 mM CTAC RM a significant amount of aggregated $FeTPPS_4$ form existed at all metalloporphyrin concentrations used down to 0.16 mM, i.e., much less than an average of one $FeTPPS_4$ molecule per RM. A transition to the monomer form occurred upon severe acidification of the water RM interior. This is consistent with the participation of the μ-oxo-bridge in the formation of aggregates. Whether the porphyrin molecules are incorporated into the RM structure, or are free in the interior water, still must be clarified. The similarity of the spectra of the monomeric $FeTPPS_4$ in RM to those when $FeTPPS_4$ is bound to BSA rather than is free in water (such as the absence of the peak for the *meta*-phenyl protons)[13,14] lends substance to the assumption that the porphyrin is incorporated into the RM. Moreover, substituted metalloporphyrins are very bulky, and it is not easy to imagine how they may fit the RM interior without penetrating the RM structure. The radius of a RM depends on the water/surfactant ratio W.[42] In our experimental conditions, the W value was about 1.[21] At this W the contribution of the aqueous phase is negligible, and the hydrodynamic radius of RM is determined mainly by the size of the surfactant molecule[42] that is comparable with the size of the porphyrin, i.e., about 2 nm. Yet AM were much more sensitive than RM to the presence of the bulky porphyrins, seemingly, because of the more restricted motion of surfactant chains in AM.

Further experiments with RM and AM are under way to clarify the nature of the porphyrin aggregation upon binding to biological structures, completing the data obtained on proteins and DNA. Characteristic pH dependence of the $FeTPPS_4$ aggregation in micelles (which was not observed for $TPPS_4$ and $ZnTPPS_4$) lends substance to the hypothesis that aggregated units are bound through the μ-oxo-bridge. Similar mechanism may be operative in other systems, such as porphyrin-protein. Thus, chemical modification of metalloporphyrins altering their aggregation properties may serve as a fine tool influencing their relaxation efficiency and biodistribution. The pH dependence of aggregation and, hence, relaxation efficiency, is also important in light of the most recent interest to the design of pH-sensitive, tumor-specific MRI contrast agents.[43]

Acknowledgment. This work was supported by CNPq, FAPESP, and FINEP. A visiting research grant to the author from CNPq is appreciated. The author is grateful to Prof. Dr. M. Tabak for his interest and advice. The author also acknowledges a stimulating critical attitude of Dr. I. E. Borissevitch and some useful hints of Dr. B. R. McGarvey (Windsor, Ontario, Canada) and Dr. B. E. Mann (Sheffield, UK).

IC980377U

(42) El Seoud, O. A. In *Organized Assemblies in Chemical Analysis*; Hinze, W. L., Ed.; JAI Press: Greenwich, 1994; Vol. 1, pp 1−36.

(43) Mikawa, M.; Miwa, N.; Brautigam, M.; Akaike, T.; Maruyama, A. *Chem. Lett.* **1998**, 693−694.