Oriented Self-Association of Copper(II) Tetraphenylporphine in Liquid-Crystalline Lipid Bilayer Membranes: An EPR Study

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Abstract: Copper(II) tetraphenylporphine (CuTPP) has been found to partition favorably into dimyristoylphosphatidylcholine (DMPC) bilayer membranes. Electron paramagnetic resonance (EPR) spectra of oriented liquid-crystalline DMPC membranes indicate that for a high (≈1/25) CuTPP/DMPC molar ratio CuTPP is oriented with the plane of the molecule *parallel* to the bilayer surface. This result is in clear contrast to our previous observation that at low (≤1/100) CuTPP/DMPC molar ratio the plane of the molecule is preferentially *perpendicular* to the membrane surface (Pasenkiewicz-Gierula, M.; Subczynski, W. K.; Antholine, W. E. *J. Phys. Chem.* **1997,** *101*, 5596−5606). Measurements of the collision rate between CuTPP and the nitroxide moiety of stearic acid spin labels located at different depths in the membrane using saturation-recovery EPR spectroscopy lead to the conclusion that the change of the CuTPP orientation at a high CuTPP/DMPC molar ratio is coupled with self-association by planar stacking of CuTPP molecules near the lipid bilayer center. Additional confirmation of this conclusion comes from comparison of EPR spectra of CuTPP in liquid-crystalline DMPC membranes with those of the polycrystalline form of CuTPP and aggregates, as well as computer simulations of EPR spectra. The self-association process depends both on the CuTPP/DMPC molar ratio and on the time after sample preparation.

Introduction

Porphyrins with various side-chain groups have been used in cancer photodynamic therapy.[1−4] It is believed that the major site for the photosensitized reactions in vivo is in cell membranes.[1,5] Utilization of photosensitizers in therapy relies on their selective uptake and retention by cancerous cells[6,7] or their selective interaction with plasma membranes of certain cancerous cells.[8,9] Thus an understanding of basic processes involved in the interaction between photosensitizers and the membrane is important.

In the study of the interaction of porphyrins with model membranes, copper porphyrins were used.[10,11] Insertion of a copper ion into the porphyrin ring is intended to make it a suitable paramagnetic probe that allows the use of different electron paramagnetic resonance (EPR) techniques. As a probe, copper porphyrins are more favorable than iron porphyrins because EPR measurements can be conducted over a wider range of temperatures, including physiological temperatures. Copper(II) tetraphenylporphine (CuTPP) and di-spin-labeled copper(II) hematoporphyrin IX are readily taken up by membranes composed of saturated and unsaturated phosphatidylcholines (PC).[10,11] The present study was performed in analogy to earlier studies of the interaction of planar copper complexes of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone), which belong to a class of potent antitumor metallodrugs,[12] and of pyruvaldehyde bis(N^4-methylthiosemicarbazone), which has shown promise as a radiopharmaceutical in positron emission tomography,[13,14] with model and biological membranes.[15−18] It was shown that parameters describing motion and orientation of copper complexes in the lipid bilayer can be determined.

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Rotational motion of copper complexes was described by an empirical Cu-motion parameter.[10,16,17] This parameter is sensitive to membrane fluidity and changes abruptly at the main-phase transition of the membrane. Slow motion theory[19] together with the postconvolution method to simulate EPR spectra of CuTPP in viscous solvents and membranes[11] was also applied. This enabled us to describe the rotational motion of CuTPP in an isotropic solvent (paraffin oil) and in an anisotropic environment (liquid-crystalline dimyristoylphosphatidylcholine (DMPC) bilayer). EPR spectra of various copper porphyrin derivatives in membranes have also been reported by other groups.[20,21] Complexes were specially designed to orient the porphyrin ring either parallel or perpendicular to the membrane surface.

In CuTPP, copper(II) is coordinated to four nitrogen ligand atoms. The EPR spectrum of CuTPP consists of four hyperfine lines. The four nitrogens (the nuclear spin of nitrogen ^{14}N is 1) give rise to a rich superhyperfine structure in the EPR spectrum that is resolved both in solution and in liquid-crystalline membranes when the CuTPP/lipid ratio is $\leq$1/100. The EPR spectra of CuTPP in oriented liquid-crystalline DMPC membranes were simulated, which confirmed that CuTPP is well-oriented with the plane of the molecule preferentially perpendicular to the membrane surface.[11]

Porphyrins and metalloporphyrins are known for their ability to undergo aggregation at low concentrations in the aqueous phase.[22,23] Recently it has been shown that hemin affects membranes differently in monomeric and aggregated forms. In the monomeric form it catalyzes lipid and protein oxidation, and in the aggregated form it disorganizes the lipid bilayer structure.[24] The main driving force for planar stacking or aggregation in porphyrins and metalloporphyrins is $\pi-\pi$ interaction.[25] Hydrophobic components of porphyrin derivatives used in phototherapy also tend to aggregate in the aqueous phase. Their integration into membranes, the major site for the photosensitized reactions, occurs as a result of dissolution of the aggregated state.[4,5] In the present paper we extended the study of porphyrin self-association to the hydrophobic hydrocarbon environment of lipid bilayer membranes. We believe that our findings could be of help in the elucidation of the role of porphyrin complexes in phototherapeutic processes.

We observed that the orientation of CuTPP in the liquid-crystalline DMPC bilayer is controlled by the CuTPP/lipid ratio. For a CuTPP/lipid ratio of 1/25, the orientation of the CuTPP molecule is no longer *perpendicular* but *parallel* to the membrane surface. The application of different EPR techniques (continuous wave and pulse EPR) as well as computer simulation of EPR spectra enabled us to determine the spatial arrangement of CuTPP in the membrane. We conclude that, at high CuTPP concentration, self-association occurs by planar stacking of CuTPP molecules near the lipid bilayer center. This is the cause of CuTPP reorientation relative to the membrane surface. To our knowledge, this is the first report describing the supramolecular arrangement of guest porphyrin molecules in the host lipid bilayer membrane.

Experimental Section

Materials. CuTPP was purchased from Aldrich (Milwaukee, WI), 5-doxylstearic acid spin label (5-SASL) and 16-deoxylstearic acid spin label (16-SASL) from Molecular Probes (Eugene, OR), and DMPC from Sigma (St. Louis, MO). All other reagents were analytical grade.

Sample Preparation. Liposomes used in this work were multilamellar dispersions of DMPC. The mixture of lipid (1.0×10^{-5} mol), spin label (1.0×10^{-7} mol), and different amounts of CuTPP in chloroform was dried under a stream of nitrogen and further dried at reduced pressure. A buffer (0.1 mL of 0.1 M borate at pH 9.5) was added to the dry lipid at 40 °C (above the main-phase transition of DMPC membranes) and vortexed vigorously. High pH ensured that all carboxyl groups of SASL were ionized in DMPC membranes.[26,27] The lipid suspension was centrifuged briefly at 4 °C and a portion of the fluffy pellet (~20% lipid wt/wt) transferred to a capillary made of a methylpentene polymer called TPX (Westlake Plastic Co., Lenni, PA) for EPR measurements.

Oriented multibilayers were obtained according to the method of Schreier et al.[28] A mixture of DMPC (1.0×10^{-6} mol) and CuTPP (4.0×10^{-8} mol) in chloroform (total volume of 0.2 mL) was placed inside a quartz EPR flat cell. Chloroform was evaporated with a stream of wet nitrogen. The sample was then hydrated by letting the film, formed on the inner surface of the flat cell, equilibrate with the aqueous phase of buffer (0.2 mL of 0.1 M phosphoric acid at pH 7.0) for about 30 min. Excess buffer was drained off. In this way, multibilayers were obtained in excess water such that membrane properties are independent of the water content.

CuTPP crystals were obtained by allowing chloroform to evaporate slowly at room temperature from the saturated chloroform solution of CuTPP positioned in a quartz capillary of 1 mm i.d. After complete evaporation of chloroform (about 2 days), fine crystals were deposited on the inner wall of a capillary. To obtain a polycrystalline form of CuTPP with evenly oriented monocrystals, fine crystals were removed from the inner wall and collected on the bottom of the capillary. Similarly, CuTPP crystals were obtained during chloroform evaporation from the filter papers (Whatman, No. 1) saturated with the 1 mM CuTPP−chloroform solution. Quick drying (less than 30 min) of CuTPP from the chloroform solution in the flat cell or the capillary with a stream of nitrogen produced aggregates of CuTPP.

Conventional EPR. EPR spectra were obtained with a Varian E-109 X-band spectrometer with a Varian E-231 multipurpose cavity (rectangular TE 102 mode). EPR spectra of oriented multibilayers were recorded with the quartz flat cell parallel or perpendicular to the applied magnetic field.

Simulation of the Powder Spectrum. The EPR powder spectrum of CuTPP was simulated with the software program "EPR" written by F. Neese (QCPE program no. QCMP 136[29]). This program contains several minimization routines and allows for simulations in field and *g*-space. The program has an empirical expression for the spectral line width.

Simulation of Motionally Averaged X-Band Spectra. The simulation program is a modified version of the program obtained from Prof. J. H. Freed, Cornell University, Ithaca, NY. This program is based on the stochastic Liouville equation formalism.[30] The rotational diffusion for a square-planar copper complex is assumed to be axially symmetric. Thus, the rotational diffusion tensor, D, has two components: $D_{\|}$ is the component parallel to the *z*-axis, and $D_{\perp}$ is the component perpendicular to the *z*-axis (an in-plane component). In the membrane environment, the complex is subject to a restoring (orienting) potential.

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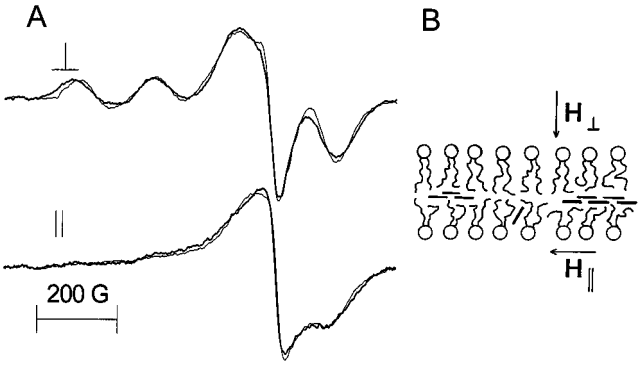

Figure 1. (A) EPR experimental spectra (thick line) of CuTPP in oriented DMPC bilayer membranes (CuTPP/DMPC molar ratio of 1/25) at 25 °C with the magnetic field perpendicular (⊥) and parallel (||) to the membrane surface. Spectrometer conditions: microwave power, 50 mW; modulation amplitude, 5 G; modulation frequency, 100 kHz. The magnetic field range is 2500−2500 G. The microwave frequency is about 8.7 GHz. Simulated spectra (thin line) are superimposed on experimental ones. (B) Schematic drawing showing ordered CuTPP molecules (planar stacks) in the DMPC bilayer.

The strength of the potential is given by a coefficient ϵ_{20}. The exact form of ϵ_{20} is given by eq A23 in Meirovich et al. (1982).[30] Details concerning the model of rotational diffusion of CuTPP in the membrane have been described earlier.[11]

Saturation-Recovery EPR. Spin−lattice relaxation times were measured at X-band by using the saturation-recovery technique as described previously.[17,18] A relatively low level of observation power (8 μW, with the loop-gap resonator delivering an H_1 field of 3.6×10^{-5} G) was used for all experiments. All saturation-recovery decays were measured on the central line of the EPR spin label spectra. Under the experimental conditions employed here, all recovery curves could be fitted to single-component exponential recovery curves.

All EPR spectra and saturation-recovery curves were recorded at 25 °C after samples were thoroughly deoxygenated, enabling us to avoid oxygen-induced line broadening and to obtain correct spin−lattice relaxation times of spin labels.[31]

Results and Discussion

Orientation of CuTPP in Lipid Bilayers. In our previous paper,[11] we showed that computer-simulated EPR spectra of CuTPP in oriented liquid-crystalline DMPC bilayer membranes at CuTPP/DMPC molar ratio 1/100 reproduced experimental spectra for the magnetic field, both perpendicular and parallel to the membrane plane. Spectra for which the magnetic field is perpendicular to the membrane surface can be simulated only if a strong ($\epsilon_{20} = 8.5$) ordering potential is introduced. These results indicated that in the lipid bilayer, CuTPP is well-oriented with the plane of the molecule perpendicular to the bilayer surface ($S_{mol} = 0.88$).

For a high (1/25) CuTPP/DMPC molar ratio, the features of EPR spectra of CuTPP in oriented liquid-crystalline multibilayers differ from those with a CuTPP/DMPC molar ratio 1/100. As can be seen from Figure 1, the rich superhyperfine structure in the EPR spectrum, observed for low molar ratio, is missing. The hyperfine structure (four lines of the $g_{||}$ component) is present in the spectrum for the magnetic field perpendicular to the membrane surface (Figure 1A, top). For the magnetic field oriented parallel to the membrane surface, the $g_{||}$ component is nearly gone (Figure 1A, bottom). This unexpected result can be explained only by assuming that, at this CuTPP concentration, the orientation of the plane of the complex is *parallel* to the membrane surface.

In our studies of other planar copper complex−membrane interactions, the complexes (3-ethoxy-2-oxobutyraldehyde bis-(N^4,N^4-dimethylthiosemicarbazonato))copper(II);[17] copper(II) dibenzotetraaza[14]annulenes[32] at complex/lipid ratio 1/25, as well as CuTPP at CuTPP/lipid ratio 1/100[11]) were always oriented in the liquid-crystalline membrane with the plane of the molecule *perpendicular* to the membrane surface. This was manifested by the presence of lines in the $g_{||}$ region of the EPR spectrum of copper complexes for orientation of the magnetic field parallel to the bilayer surface and their absence for the perpendicular orientation. Orientation of the porphyrin ring parallel to the membrane surface was reported for specially designed steroidal copper(II) porphyrin.[20] In this complex, two steroid moieties were appended from either side of the porphyrin ring, which allowed placement of the porphyrin ring in the bilayer center parallel to the membrane surface. The features of EPR spectra of oriented multibilayers at 77 K clearly indicated such an orientation. In another study,[21] orientation of the porphyrin ring parallel to the membrane surface was achieved by attachment of four evenly distributed anionic substituents to the CuTPP molecule. In that case, the complex was located on the top of the polar head groups of oriented anhydrous ammonium bilayer membranes in the gel phase.

Polycrystalline Powder and Aggregates of CuTPP. Spectra of magnetically pure polycrystalline samples of CuTPP can be easily distinguished from those of CuTPP aggregates. In the crystal, CuTPP molecules are arranged, as indicated by X-ray diffraction data,[33] with planes parallel. The shortest Cu−Cu distance is 8.3 Å; thus, one would expect a strong dipolar magnetic interaction between the nearest copper ions and broadening of the EPR lines. However, the dipolar interaction is reduced due to the out-of-plane phenyl ring shielding of the copper ion from the nearest neighbors. This gives rise to a well-resolved hyperfine structure of the copper EPR spectra.[34−36] Superhyperfine splitting from nitrogen atoms is, however, not resolved, indicating a presence of some dipolar broadening.[35] In aggregates, CuTPP molecules are oriented randomly, giving rise to a broad single-line EPR spectrum.

To ensure that the EPR spectra of CuTTP in the DMPC bilayer membrane shown in Figure 1 are not a result of CuTPP crystallization on the inner surface of the flat cell, we recorded EPR spectra of CuTPP quickly dried from chloroform solution (without DMPC) in the flat cell. Spectra obtained for the cell surface oriented perpendicular and parallel relative to the magnetic field are shown in Figure 2A. For both orientations, hyperfine lines are not observed, indicating that CuTPP crystals are not formed. The broad single line is evidence of random packing (random aggregation) of CuTPP with effective dipolar and exchange interactions. A small difference between the spectra for parallel and perpendicular orientations may be due to some oriented crystallization of CuTPP close to the quartz surface. Figure 2B shows a schematic explanation of these situations.

The EPR spectrum of a polycrystalline powder of CuTPP (powder spectrum) (Figure 3A) from fine crystals collected in a quartz capillary (1 mm i.d.) after slow evaporation (over a

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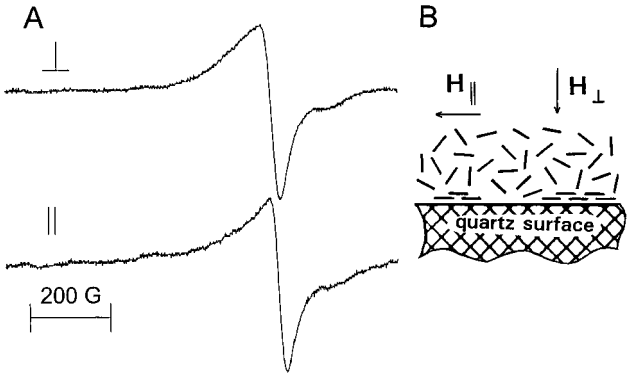

Figure 2. (A) EPR spectra of CuTPP dried from a chloroform solution on the surface of a quartz flat cell. Spectra were recorded with the magnetic field perpendicular (⊥) and parallel (∥) to the surface of the quartz cell. (B) Schematic drawing showing random aggregation of CuTPP molecules.

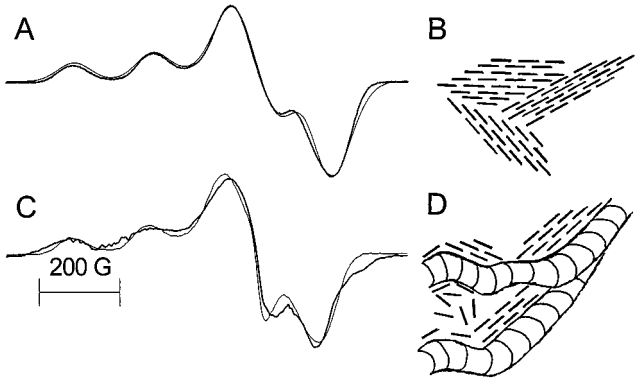

Figure 3. EPR experimental spectrum (thick line) of polycrystalline powder of CuTPP in the quartz capillary superimposed on the simulated spectrum (thin line) (A) and CuTPP crystals and aggregates obtained for CuTPP dried on filter paper (thick line) superimposed on the simulated spectrum (thin line; see text for details) (C). The schematic drawing shows the polycrystalline form of CuTPP (B) that gives the spectrum shown in A and microcrystals of CuTPP formed on the surface of the paper fibers together with CuTPP aggregates (D) that give the spectrum shown in C.

period of 2 days) of chloroform from a CuTPP−chloroform solution shows, as expected, very well resolved hyperfine structure. During chloroform evaporation from the filter paper saturated with the CuTPP−chloroform solution, a portion of CuTPP also formed crystals. The EPR spectrum (Figure 3C) is the sum of two fractions, from polycrystalline powder and aggregates. In this sample, paper fibers provided many micro-surfaces for CuTPP crystallization. Figure 3B,D contains schematic drawings of CuTPP polycrystals and aggregates formed during chloroform evaporation.

Localization of CuTPP in the Lipid Bilayer. Bimolecular collision of CuTPP (a fast-relaxing species) and nitroxide (a slow-relaxing species) induces spin exchange, which leads to a faster effective spin−lattice relaxation of nitroxide.[17,18] According to Hyde and Sarna,[37] the exchange process is of a strong-encounter type in which every collision causes spin exchange. Thus, the difference of the electron relaxation probability (the inverse of T_1) in the presence and absence of the relaxing agent (CuTPP) should be proportional to this collision rate. On the basis of the Smoluchowski equation for isotropic diffusion, this difference can be expressed as

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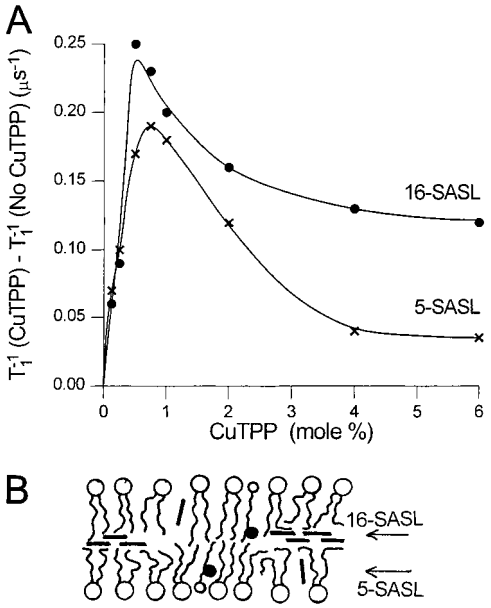

Figure 4. (A) Difference of T_1s in the presence and absence of CuTPP in the membrane (which is proportional to the collision rate between CuTPP and spin label—see eq 1) plotted as a function of mole fraction of CuTPP added to the DMPC suspension. Data obtained at 25 °C just after sample preparation for 5-SASL (×−×) and 16-SASL (●−●) are presented. (B) Schematic drawing showing the positions of the nitroxide moiety of 5-SASL (in the near polar head group region) and 16-SASL (in the membrane center), as well as localization and orientation of monomeric and self-associated forms of CuTPP in DMPC bilayer.

$$T_1^{-1}(\text{CuTPP}, x) - T_1^{-1}(\text{NoCuTPP}, x) = A(D_{\text{SL}} + D_{\text{Cu}}(x))C_{\text{Cu}}(x) \quad (1)$$

Here, T_1s are the spin−lattice relaxation times of the nitroxide in the presence and absence of CuTPP in the membrane. This difference is proportional to the local concentration of CuTPP, $C_{\text{Cu}}(x)$, and the sum of the diffusion coefficients of spin label, D_{SL}, and CuTPP, $D_{\text{Cu}}(x)$, at a "depth" x in the membrane at which the nitroxide moiety of spin label is located. Parameter A is proportional to the interaction distance and to the probability that an observable event occurs when a collision occurs. The effects of different concentrations of CuTPP on spin label T_1s in liquid-crystalline DMPC membranes in DMPC liposome suspension were examined by measuring T_1 using the method of saturation recovery. The results for 5-SASL (the nitroxide moiety located near the polar head group region) and 16-SASL (the nitroxide moiety located near the membrane center) are shown in Figure 4. For both spin labels, the collision rate between the nitroxide moiety and CuTPP increases (almost linearly) up to 0.5 mol % of CuTPP, reaching a maximum in the range of 0.5−0.75 mol %. A further increase in CuTPP concentration causes a decrease in the collision rate. For 6 mol % CuTPP concentration, the collision rate is ∼50% of its maximum value for 16-SASL and ∼20% of its maximum value for 5-SASL. For low CuTPP concentrations (<0.5 mol %), CuTPP-5-SASL and CuTPP-16-SASL collision rates are similar, but at high CuTPP concentrations (4−6 mol %), the collision rate of CuTPP with 16-SASL is about 3.5 times higher than that with 5-SASL.

For low CuTPP concentrations, the CuTPP−nitroxide collision rates are similar to those observed earlier for another hydrophobic square-planar copper complex, (3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato))-copper(II) in liquid-crystalline DMPC membranes.[18] For this

Table 1. EPR Spectral Parameters for CuTPP Obtained from Polycrystalline Powder and a Frozen Solution of Paraffin Oil

sample	g_x	g_y	g_z	A_x (G)	A_y (G)	A_z (G)	w_x (G)	w_y (G)	w_z (G)
polycrystalline powder	2.051	2.051	2.1806	15.0	15.0	214.0	75	75	47
frozen solution of paraffin oil[a]	2.047	2.047	2.1890	29.2	29.2	199.5	4.1[b]	4.1[b]	3.7[b]

[a] Data from ref 11. [b] Because of the dipole−dipole broadening, the line width used for the simulation of spectra in Figure 1 was 30 G (see Table 2).

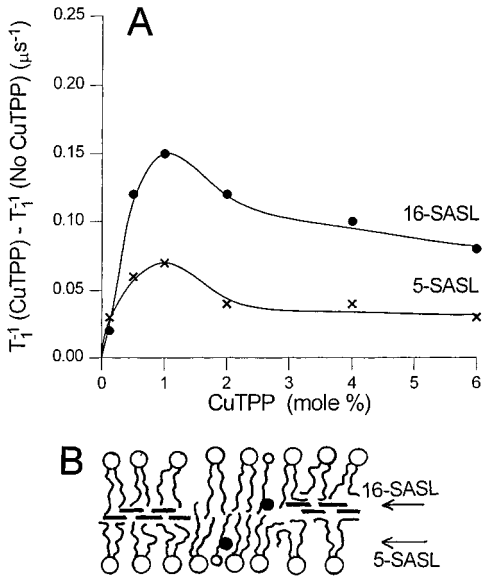

Figure 5. (A) Difference of T_1s in the presence and absence of CuTPP in the membrane plotted as a function of mole fraction of CuTPP added to the DMPC suspension. Data obtained at 25 °C 1 week after sample preparation for 5-SASL (×−×) and 16-SASL (●−●) are presented. (B) Schematic drawing showing a greater degree of CuTPP self-association in the membrane center compared with the sample just after preparation (see schematic drawing for Figure 4).

complex a linear dependence of the collision rate was observed up to 2 mol % (maximum concentration used). Another relaxing agent, oxygen, showed a linear dependence of the collision rate with nitroxide in liquid-crystalline PC membranes over a wide range of concentrations.[31,38,39] Because CuTPP does not affect the membrane structure and dynamics (see next subsection), a possible explanation of these results is that, at higher CuTPP concentration, self-association of CuTPP molecules occurs, which decreases their effective collision rate with nitroxides. These associations are thought to be located in the membrane center because the CuTPP-16-SASL collision rate is much greater than the CuTPP-5-SASL collision rate.

Taken together, data presented in Figures 1 and 4 allow us to put forward a hypothesis that at high CuTPP concentrations (>1 mol %) self-association of CuTPP molecules in the membrane takes place. The associated CuTPP molecules are located in the bilayer center with molecular planes parallel to the membrane surface. The associates are CuTPP planar stacks presumably formed of a few CuTPP molecules that are arranged in a similar way as in CuTPP crystals. CuTPP molecules in these supramolecular structures still have significant contact with lipid alkyl chains. Schematic drawings (Figures 1B and 4B) illustrate our hypothesis.

As it can be seen from Figure 5A, this self-association (planar stacking) process depends not only on CuTPP concentration but also on the time after sample preparation. The collision rate profiles measured 7 days after sample preparation showed broad flat maxima at 1 mol % CuTPP with collision rates much lower compared to rates measured immediately after sample preparation (Figure 4A). This indicates that after 7 days the degree of self-association of CuTPP is greater (compare schematic drawings in Figures 4B and 5B).

Some effort has previously been made to establish distributions of porphyrins in PC liposomal membranes[40] using quenching of fluorescence by stearic acid spin labels. However, the pH value used in that experiment (6.8) was too low to ensure full ionization of the SASL carboxyl groups in the membrane.[26,27] Because the position of the nitroxide moiety in the membrane is different for protonated and ionized forms of SASL, the results of ref 40 were subject to some error. Nevertheless, the experiment indicated that different compositions of the membrane (presence of cholesterol, saturated and unsaturated alkyl chains) may cause different displacements of porphyrins—either toward or out of the membrane center.

Computer Simulation. Magnetic parameters for CuTPP polycrystals were determined by simulating the EPR spectrum in Figure 3A. The experimental spectrum arises from randomly oriented domains of magnetically pure crystals (Figure 3B). This allowed us to obtain components of *g*- and *A*-tensors and the line width tensor, *w*. Significant line width broadening was assumed to arise from Cu−Cu dipole interactions.[35] In Figure 3A, the simulated spectrum is superimposed on the experimental powder EPR spectrum. The components of the magnetic tensors (*g*, *A*, and *w*) are given in Table 1. In Table 1, these values are compared to those obtained by us previously for the EPR spectrum of a frozen solution of CuTPP in light paraffin oil.[11]

The spectrum of CuTPP solution dried on filter paper shown in Figure 3C can be reproduced as a sum of a CuTPP polycrystal spectrum in Figure 3A and an aggregate spectrum in Figure 2A. The best match is obtained when the ratio of intensities (polycrystalline/aggregate) is 2.5.

The simulated (thin line) spectrum of CuTPP in an oriented DMPC bilayer when the magnetic field is perpendicular (Figure 1A, top) and parallel (Figure 1A, bottom) to the membrane surface is superimposed on an experimental spectrum (thick line). These are not single-component spectra. They arise from three types of CuTPP associations: (1) associations of CuTPP molecules with the molecular planes parallel to the membrane surface as shown in Figure 1B (planar stacks of a few CuTPP molecules in an arrangement similar to that in CuTPP crystals); (2) associations located (most likely) outside the membrane, possessing some freedom of rotational motion, but without a specific spatial arrangement (the signal from this species was observed by us previously in EPR spectra from CuTPP in oriented membranes at a low (1/100) CuTPP/lipid ratio,[11] and in that paper, it was called the "background" signal); (3) aggregates of randomly oriented CuTPP molecules giving a spectrum as in Figure 2A (top) for the magnetic field perpendicular to the membrane surface and as in Figure 2A (bottom)

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Table 2. Best Fit Parameters for CuTPP in the Membrane[a]

component	magnetic field orientation	$D_\perp$ (rad/s)	$D_\parallel$ (rad/s)	ϵ_{20}	line width (G)	intens ratio
(1) planar stacks	⊥	2.0 ± 10^6	2.0 ± 10^6	10	30	1.0
(2) background[b]	⊥	5.9 ± 10^7	1.5 ± 10^8	0	20	1.2
(3) aggregate[c]	⊥					4.3
(1) planar stacks	∥	2.0 ± 10^6	2.0 ± 10^6	10	30	1.0
(2) background[b]	∥	5.9 ± 10^7	1.5 ± 10^8	0	20	0.8
(3) aggregate[c]	∥					2.4

[a] $D_\parallel$ is the component parallel to the *z*-axis, and $D_\perp$ is the component perpendicular to the *z*-axis (an in-plane component) of the axially symmetric diffusion tensor. ϵ_{20} is the strength of the membrane-restoring potential (cf. Experimental Section). [b] Data from ref 11. [c] Spectrum from Figure 2A.

for the magnetic field parallel to the membrane surface. The relative intensities of the three components are given in Table 2.

Experimental spectra in Figure 1A could be roughly simulated with parameters obtained from a simulation of the polycrystalline spectrum (Figure 3A, Table 1). However, excellent fits (shown as thin lines in Figure 1A) were obtained when the spectrum was simulated with the magnetic parameters obtained from the frozen solution spectrum of CuTPP in light paraffin oil[11] (Table 1). This agrees with the conclusions derived on the basis of EPR measurements, that the associates of CuTPP planar stacks are made of a few CuTPP molecules and that the hydrophobic environment provided by lipid alkyl chains significantly affects the spectral characteristics. The associates possess very limited rotational freedom around the in-plane axes due to the strong restoring potential ($\epsilon_{20} = 10$, $S_{mol} = 0.90$). Also, the residual line width of 30 G used for simulation of EPR spectra from these associates is smaller than that for the powder spectrum, which confirms the conclusion that the associates are small. The best values for CuTPP motion and order parameters are listed in Table 2.

Effect of CuTPP on Membrane Structure and Dynamics. Using conventional EPR spectroscopy, we checked the effect of CuTPP on the order parameter of 5-SASL and 16-SASL and on the rotational correlation time of 16-SASL in the liquid-crystalline DMPC bilayer. No changes in either of these parameters were observed up to 6 mol % of CuTPP. Similarly, planar copper complexes of dibenzotetraaza[14]annulenes did not affect similar parameters in DMPC membranes.[32] Monitoring the main-phase transition of DMPC membranes with 16-SASL,[41] which is a sensitive probe, showed that for all samples (from 0 to 6 mol % CuTPP) the main-phase transition occurs at 23.5 ± 0.1 °C with the width of the transition, $\Delta T_{1/2}$, not greater than 0.2 °C ± 0.1 °C (data not shown). This weak effect of CuTPP on membrane characteristics may be explained by the following: (1) The computer simulation indicates that more than 50% of CuTPP added to the membrane suspension forms aggregates, presumably in the aqueous phase, so the real concentration of CuTPP in the membrane is less than half of that added to the sample. (2) At a high concentration, self-association of CuTPP in the membrane center decreases CuTPP contact with alkyl chains of lipids. (3) CuTPP molecules do not possess any polar group, which would anchor it at the membrane surface. The anchoring enhances the effect of modifiers on membrane structure and dynamics, as in the case of cholesterol or polar carotenoids.[42]

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General Discussion

Although EPR spectra of various copper−porphyrin derivatives in oriented multibilayer membranes have been reported in the literature,[20,21] to our knowledge, there has not yet been any attempt to measure the behavior of copper complexes in the liquid-crystalline membranes made of phospholipids of biological origin with the exception of our own efforts.[11,17,32] Orientation of liquid-crystalline lipid bilayers made of PCs in excess of water (conditions relevant to physiological conditions) can be achieved only with limited precision. This difficulty was probably the reason Ishikawa and Kunitake[21] used unhydrated gel-phase ammonium bilayers and Groves and Neumann[20] used DMPC bilayers oriented in the presence of a small amount of water with EPR measurements performed at 77 K. Nevertheless, we succeeded, with the assistance of Dr. Schreier, in achieving satisfactorily oriented liquid-crystalline DMPC membranes.[17] This allowed us to investigate the dynamics of square-planar copper complexes in PC membranes at low complex concentrations. Substantial help in our studies came from computer-based spectra simulations[11] in which various motional and orientational modes were tested. In the present work, EPR techniques were used to study self-association of planar copper complexes in the liquid-crystalline membrane. This process is accompanied by the change in the orientation of the complex from *perpendicular* to *parallel* to the membrane surface with the final location of the complex in the membrane center.

Certain orientations of porphyrin molecules in lipid bilayer membranes can be achieved by chemical modifications of these molecules. In one case, anionic groups were chemically attached to the CuTPP complex. Their number and position can change the orientation of the complex from parallel to tilted, or to perpendicular relative to the membrane surface.[21] The anionic groups always locate at the membrane surface, anchoring the complex to it. In another case, two sterol molecules were attached to each side of the porphyrin ring, placing the porphyrin ring in the membrane center and orienting it parallel to the surface.[20] In the present series of papers we showed that orientation of CuTPP molecules in liquid-crystalline membranes can be controlled by the concentration of CuTPP in the membrane. At low concentrations, CuTPP is in the monomeric form and is oriented with the plane perpendicular to the membrane surface.[11] At high concentrations, self-association leads to the formation of CuTPP supramolecular structures by planar stacking. Due to nonconformability with membrane lipids, these supramolecular structures (crystal-like stacks of a few CuTPP molecules) change their orientation in the membrane in such a way that the molecular planes are parallel to the membrane surface. At this stage we are unable to unequivocally determine the size of the associate for which the change of the orientation occurs. However, on the basis of EPR experimental data and the results of computer simulations, we can estimate that these associates are small and presumably formed of a few CuTPP molecules.

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