Rotational Motion of Square Planar Copper Complexes in Solution and Phospholipid Bilayer Membranes

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Electron spin resonance (ESR) spectra of 3-ethoxy-2-oxobutyraldehydebis(*N,N*-dimethylthiosemicarbazonato)-copper(II) (CuKTSM$_2$) and copper(II) tetraphenylporphyrin (CuTPP), in paraffin oil at various temperatures, as well as in oriented dimyristoylphosphatidylcholine bilayer membranes, at 25 °C, were simulated using the stochastic Liouville equation formalism and a postconvolution method. It has been recognized that because nitrogen hyperfine couplings are nearly isotropic, they can be easily introduced after a simulation that omits ligand nuclear parameters, by convoluting a stick diagram for the nitrogen splitting with the copper spectrum. For the whole range of temperatures studied (from −10 to +120 °C for CuTPP and from −60 to +120 °C for CuKTSM$_2$), the line shapes of ESR spectra for these square planar copper complexes can be accounted for by motion. The ESR spectra of Cu(II) in oriented bilayers are well reproduced for the magnetic field both perpendicular and parallel to the membrane plane. Spectra, for which the magnetic field is perpendicular to the membrane plane, can be simulated only if an ordering potential is included.

Introduction

Subczynski and collaborators have shown[1−4] that electron spin resonance (ESR) spectra of square planar copper complexes in the membrane are sufficient to characterize motional behavior of the complexes in the membrane. Such studies are of pharmaceutical and photobiological relevance.[5] While primarily nitroxides and vanadyl complexes have been used as molecular probes for investigating the structure and dynamics of membranes by ESR, few studies have used the active complexes, for example copper complexes. Particularly, copper complexes of known antitumor agents, such as 3-ethoxy-2-oxobutyralde-hydebis(thiosimicarbazonato)copper(II) (CuKTS), have been shown to be effective antineoplastic agents due to their ability to cross cell membranes and react with intracellular thiols.[6,7] A derivative of CuKTS, pyruvaldehydebis(*N*-4-methylthiosemi-carbazonato)copper(II), i.e., CuPTSM, has shown promise as a radiopharmaceutical, used in positron emission tomography for evaluation of myocardial, cerebral, and tumor blood flow.[8−15]

Retrieving detailed information about the motion of the complex from ESR spectra is cumbersome. Even at room temperature with an isotropic solvent of membrane-like viscosity, copper ESR spectra must be analyzed by the slow motion theory[16] because of a large anisotropy of the hyperfine interaction of Cu(II) in these complexes ($A_{xx} - A_{zz}$ is about 500 MHz). When the rotational motion of a paramagnetic probe fulfills the fast motion condition, $|H_{SL}|\tau_R \ll 1$, where H_{SL} is the largest anisotropy in the spin Hamiltonian and τ_R is the rotational correlation time of the probe,[17] an analytical expression for the ESR line shape can be used and the information about the motional state of the system can be obtained in a straightforward way. For most square planar copper complexes dissolved in an isotropic solvent, the condition requires τ_R to be less than 2 × 10^{-9} s, but for viscous solvents like paraffin oil even at 45

°C, τ_R is already more than 2 × 10^{-9} s. In anisotropic solvents (membranes) the Cu(II) spectrum should be analyzed by the slow motion theory at any temperature.[18,19]

The slow motion theory for an ESR spectrum, based on the stochastic Liouville equation (SLE), was developed by Freed and co-workers.[16] The theory has been applied to paramagnetic metal ions in solutions only in a limited number of cases. The first metal complex tested by this theory was a vanadyl complex in isotropic solvents.[20] The shapes and positions of the hyperfine lines in the experimental ESR spectra of VO^{2+} were well reproduced by simulation for correlation times between 8 × 10^{-11} and 10^{-9} s. For longer times, the agreement between experimental and simulated spectra was worse due to an incomplete perturbative treatment of the nonsecular terms in the spin Hamiltonian. Complete treatment of the nonsecular terms, introduced later, significantly improved the agreement, but the simulation time became very long.[21] Significant reduction of the simulation time was obtained by applying the Lanczos algorithm to the spectra computation.[22] This permits the inclusion of nonsecular terms in most calculations of Cu(II) ESR spectra and leads to a better agreement with experiment (cf. Result section).

The slow motion theory of ESR spectra treats Zeeman and hyperfine interactions explicitly.[23] But, it does not include superhyperfine interactions, as it would impose unrealistic computer memory and calculation time requirements. The square planar copper complexes used in the present studies are 3-ethoxy-2-oxobutyraldehydebis(*N,N*-dimethylthiosemicar-bazonato)copper(II) (CuKTSM$_2$) and copper(II) tetraphenyl-porphyrin (CuTPP). In these complexes Cu(II) is coordinated to nitrogen ligand atoms, which results in a rich superhyperfine structure in the ESR spectra.

In this paper an extension to the slow motion theory is used. ESR spectra of these copper complexes in viscous solvents and membranes are simulated by application of a "postconvolution" method. The slow motion spectrum simulation is realized in two steps: (1) a copper spectrum that omits ligand nuclear parameters is generated; (2) the spectrum is then convoluted with a stick diagram for the nitrogen superhyperfine splitting

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(postconvolution).[2,24,25] Time required to perform convolution is negligibly short compared with spectrum simulation time, and there is no extra memory requirement. The method was used to describe quantitatively rotational motion of the two copper complexes in an isotropic solvent (paraffin oil) and in an anisotropic environment (dimyristoylphosphatidylcholine (DMPC) membrane). Although ESR spectra of various copper–porphyrin derivatives in solution[26,27] and the membrane[27,28] have been reported in the literature and in some cases the spectra have been analyzed by computer simulation, to our knowledge, there has not yet been any attempt to use the slow motion theory, with the exception of our own efforts[2,24,25] and that of Basosi's group,[29] to simulate motional spectra of Cu(II) coordinated to nitrogen ligand atoms.

In our previous works, the interaction of various hydrophobic copper complexes with model phosphatidylcholine membranes was considered. Both $CuKTSM_2$ and CuTPP partition favorably into the membrane.[1,3] Using oriented multibilayers, it was demonstrated that $CuKTSM_2$ in the bilayer is well oriented with its plane parallel to the alkyl chains.[1] Rotational motion of the complexes in the membrane was described by an empirical Cu-motion-parameter.[1] The parameter is sensitive to membrane fluidity and changes abruptly at the fluid/gel phase transition.[1,3,30] The behavior of $CuKTSM_2$ in Ehrlich ascites tumor cells and nuclei isolated from the cells[31] has also been studied. $CuKTSM_2$ in these samples locates in at least two regions that vastly differ in motional properties. Thus, $CuKTSM_2$ proved to be a sensitive probe to study membranes. In general, the side groups of the square planar copper complex determine whether it can be a useful membrane probe–changes in side groups affect significantly the partitioning and mobility of the complex in the membrane.[4,31] Using a T_1-saturation recovery ESR method, translational diffusion of $CuKTSM_2$ in phospholipid bilayers has been measured.[30]

The main two objectives in this study are (1) to test the application of the slow motion theory, together with the postconvolution procedure, on square planar copper complexes, and (2) to find details of the motional behavior of the copper complexes in solution over a broad range of temperatures as well as in a fluid-phase phospholipid bilayer. For this purpose, two copper complexes, $CuKTSM_2$ and CuTPP, are used as motional probes. Their chemical structures are shown in Figure 1. In $CuKTSM_2$ and CuTPP, Cu(II) is coordinated to two and four nitrogen ligand atoms, respectively. Since the nuclear spin of nitrogen ^{14}N is 1, the ESR spectra of these complexes are characterized by a rich superhyperfine structure. The appearance of the structure is indicative of the motional state of the complex.[32]

Experimental Section

Materials. CuTPP was purchased from Aldrich, Milwaukee, WI, and $CuKTSM_2$ was generously supplied by Dr. H. G. Petering, University of Wisconsin, Milwaukee. Light paraffin oil was purchased from MCM, Manufacturing Chemists Inc., Germany, and DMPC from Sigma, St. Louis, MO.

Sample Preparation. Solutions (2 mM) of $CuKTSM_2$ and CuTPP in paraffin oil were used. The samples (0.2 mL) were deoxygenated and contained in a disposable pipette sealed with clay. Oriented multibilayers were obtained according to the method of Schreier et al. (1973).[33] A mixture of DMPC (10^{-6} mol) and copper complex ($CuKTSM_2$, 4×10^{-8} mol, or CuTPP, 10^{-8} mol) in chloroform (total volume of 0.2 mL) was placed inside of a quartz ESR 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,
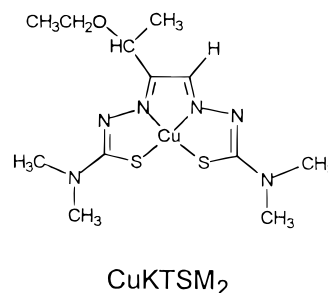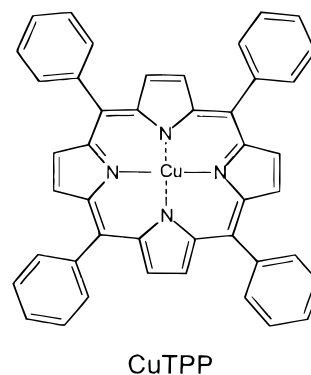

Figure 1. Chemical structures of copper(II) tetraphenylporphyrin (CuTPP) and 3-ethoxy-2-oxobutyraldehydebis(*N,N*-dimethylthiosemi-carbazonato)copper(II) ($CuKTSM_2$).

equilibrate with the aqueous phase of buffer (0.2 mL of reagent grade 0.1 M phosphoric acid at pH 7.0) for about 30 min. Excess buffer was drained off. CuTPP is less soluble in a phospholipid bilayer than $CuKTSM_2$, which has a large 1-octanol/H_2O partition coefficient[34] and is readily taken up in cell membranes.[35]

In the method applied here, ordered multibilayers were obtained in excess water, i.e., when the membrane properties are already independent of the water content. ESR measurements were performed at 25 °C, which is above the main phase transition temperature for DMPC bilayer. In the liquid-crystalline phase, the average tilt angle of the hydrocarbon chains is zero.[36–38] A better alignment of DMPC molecules in the ordered multibilayers could be achieved by a pressure-annealing procedure,[39,40] but samples prepared in that way have low water content (2–7%) and their characteristics (membrane thickness, tilt angle, transition temperature) differ significantly from those for membranes in excess water and depend on the water content.[40,41]

ESR Measurements. ESR spectra were obtained with a Varian E-109 X-band spectrometer with Varian temperature control accessories and an E-231 Varian multipurpose cavity (rectangular TE102 mode). ESR spectra of oriented multibilayers were recorded with the quartz flat cell parallel, or perpendicular, to the applied magnetic field. All samples were thoroughly deoxygenated to avoid oxygen-related line broadening.

Simulation

Simulation of the Rigid Limit X-Band Spectra. The powder spectrum simulation program is a modified version of the program Powder obtained from Prof. J. R. Pilbrow, Monash University, Australia. The spectrum calculation is based on the perturbation theory to the second order in the hyperfine terms.[42] It takes into account *g*- and *A*-strain contributions to the line width and generates the spectrum in the frequency-swept domain.[43] The superhyperfine interaction is assumed to be much

smaller than the hyperfine one, and it consists only of the first-order term, which merely splits the hyperfine lines. The magnetic parameters for CuTPP were obtained by a nonlinear least-squares fitting method. To account for the line broadening due to overmodulation, a pseudomodulation method for calculating first harmonic spectra was employed.[27] Even though the fit between a simulated and an experimental spectrum was excellent, the CuTPP spectrum was simulated with the QPOW program[45,46] to check whether there is any contribution from the nuclear quadrupole interaction. Including the quadrupole terms in the spin Hamiltonian did not improve the fit, and it was assumed that the interaction is insignificant in the complexes we studied. For $CuKTSM_2$, the spectral parameters were published by us elsewhere.[32]

Simulation of the Motionally Averaged X-Band Spectra.
a. Line Shape. The simulation program was 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 (SLE) formalism.[23] SLE is the equation of motion for the magnetization operator of a collection of non-interacting paramagnetic molecules undergoing random re-orientations described by a stochastic process. In this formalism the ESR spectrum intensity is given by

$$I(\Delta\omega) = 1/\pi \ \mathrm{Re} \ \langle\mathbf{v}|[i(\Delta\omega\mathbf{1} - L) + \Gamma]^{-1}|\mathbf{v}\rangle \qquad (1)$$

where: $\Delta\omega = \omega - \omega_0$ is the sweep variable (ω is the microwave frequency and ω_0 is the resonance frequency), L is the Liouville superoperator associated with the orientation dependent spin Hamiltonian, Γ is the symmetrized diffusion superoperator modeling the classical reorientational motion of the paramagnetic molecule, $|\mathbf{v}\rangle$ is the so-called starting vector constructed from the spin operator and the probability distribution function for the orientation of the paramagnetic molecule, $\mathbf{1}$ is the identity operator; $i = \sqrt{-1}$, and Re means the real part of the complex expression.

The spin Hamiltonian consists of electron Zeeman and hyperfine interactions which are grouped into secular, pseudo-secular, and nonsecular terms. Due to a large anisotropy of the copper $\mathbf{g}$- and $\mathbf{A}$-tensor, the nonsecular term in the Hamiltonian cannot be neglected. Otherwise it leads to an incorrect ESR spectrum. However, the Hamiltonian does not include superhyperfine interaction.

b. Rotational Diffusion Model. It is not obvious what type of rotational motion a square planar copper complex undergoes in solution, but Brownian diffusion has been assumed as a model for reorientation. For an isotropic solvent, this model does not require reintroducing an extra parameter (residence time), which is the case for jump and free diffusion,[47] whereas it is the only model that can be conveniently solved for the membrane.[48] The rotational diffusion for square planar copper complexes is assumed to be axially symmetric; the rotation about the z-axis is expected to be faster than that about the x- and y-axes due to a smaller viscosity effect. Thus, the rotational diffusion tensor, $\mathbf{D}$, is axially symmetric. However, since the magnetic tensors ($\mathbf{g}$- and $\mathbf{A}$-tensor) of the complex are axially symmetric, the ESR spectrum of a cupric complex in an isotropic solvent depends only on the rotational diffusion about the in-plane (perpendicular) axes, i.e., $D_\perp$.

In an isotropic solvent (paraffin oil), the diffusion operator for an axially symmetric probe is given by

$$\Gamma_{\mathrm{iso}} = D_\perp\nabla^2 + D_\| - D_\perp\nabla_z^2 \qquad (2)$$

where $D_\|$ and $D_\perp$ are the parallel (along the z-axis) and perpendicular (along an in-plane axis) components of the

rotational diffusion tensor, ∇^2 is the Laplacian operator, and ∇_z^2 is the z-component of the Laplacian operator.

A membrane in which DMPC molecules have a preferred orientation is an anisotropic solvent (of axial symmetry) for the probe. Such a situation is modeled by assigning a restoring (orienting) potential, U, to the membrane. The potential U for a probe in an axially symmetric environment is given by a single term[23]

$$U(\theta) = -kT\{\lambda_0^2\ P_2(\theta)\} \qquad (3)$$

where $P_2(\theta)$ is Legendre polynomial of order 2, θ is an angle between the bilayer normal and an in-plane axis of the complex, and λ_0^2 is a coefficient defining the strength of the potential.

The diffusion operator, Γ (eq 1), for a probe undergoing Brownian rotational diffusion in the presence of the potential U is a sum of Γ_{iso} (eq 2) and a potential dependent term, Γ_u, i.e., $\Gamma = \Gamma_{\mathrm{iso}} + \Gamma_u$. The form of Γ_u, for the potential given by eq 3, is[23]

$$\Gamma_u = \epsilon_{20}P_2(\theta) \qquad (4)$$

where the coefficient ϵ_{20} (strength of the restoring potential) is expressed by λ_0^2 and other constants. The exact form of ϵ_{20} is given by equation A23 in Meirovitch et al. (1982).[23]

c. Superhyperfine Splitting. As stated above, the spin Hamiltonian for slow motion ESR does not include the superhyperfine interactions. Including these interactions directly in the Hamiltonian would result in a significant increase in the dimension of the starting vector (eq 1), making it practically impossible to calculate an ESR spectrum, even for moderately slow motion. To overcome this problem, we have developed methodology to include the superhyperfine interaction in the slow motion ESR spectrum.[2,24,25]

The superhyperfine splitting for a single orientation or orientation independent ESR spectrum can be introduced by convoluting the spectrum with a stick diagram representing the relative positions and intensities of the superhyperfine lines, as long as other magnetic interactions in the system are significantly stronger than the superhyperfine one.

In the rigid limit case, the powder-like spectrum is a sum of the single orientation spectra and convoluting and summing up cannot be interchanged. For the fast motion spectra, where all magnetic anisotropies are effectively averaged out, the spectrum requires only one convolution. In the case of slow motion, averaging of anisotropies is incomplete so the order of first convoluting and then averaging is important. However, as we show here, for square planar copper complexes with nitrogen atoms as ligands, changing the order of convoluting and averaging may introduce a small error only when the rotational correlation time is less than about 10^{-7} s.

If we symbolically denote convolution by $\otimes$ and averaging by $\langle\rangle$, the correct expression for the slow motion ESR spectrum of Cu(II) complexed to nitrogen ligands, S_{cu}, is given as

$$S_{\mathrm{cu}} = \langle\mathrm{CU} \otimes \mathrm{N}^n\rangle \qquad (5)$$

where CU is the orientation dependent copper spectrum (before averaging), N is the orientation dependent stick diagram for a nitrogen ligand splitting, and n is the number of equivalent nitrogen ligands. The anisotropy ($A_{zz} - A_{xx}$) of the CuTPP hyperfine tensor, $\mathbf{A}$, is about 170 G (510.0 MHz—the conversion of units is according the following formula: $A_i(\mathrm{MHz}) = 2.9979 \times A_i(\mathrm{G}) \times g_i \times 0.466\ 88$) (see Table 1). This anisotropy is effectively averaged by the rotational motion with a correlation time less than 2×10^{-9} s. Thus, for correlation times less than

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TABLE 1: Spin Hamiltonian Parameters Obtained from Frozen Solution Spectra for CuTPP and CuKTSM$_2$

complex	g_x	g_y	g_z	A_x (G)	A_y (G)	A_z (G)	AN$_x$ (G)	AN$_y$ (G)	AN$_z$ (G)	w_x (G)	w_y (G)	w_z (G)
CuKTSM$_2$[a]	2.0520	2.0510	2.1399	39.00	39.00	190.31	17.69	14.31	14.29	2.99	3.31	3.74
							AN$_{iso}$ = 15.43					
CuTPP	2.0466	2.0466	2.1890	29.17	29.17	199.50	17.46	17.46	14.06	4.13	4.13	3.71
							AN$_{iso}$ = 16.33					

[a] Data from Pasenkiewicz-Gierula et al. (1987).[32]

2×10^{-9} s (fast motion region) eq 5 becomes

$$S_{cu} = CU_{iso} \otimes N_{iso}{}^n \qquad (6)$$

where CU_{iso} is the fast motion copper spectrum and N_{iso} (**AN**$_{iso}$) is an isotropic nitrogen splitting. The anisotropy of the nitrogen superhyperfine tensor, **AN**, is about 3 G (9.0 MHz) (see Table 1) and is effectively averaged by the rotational motion with a correlation time less than 10^{-7} s. As the superhyperfine interaction is assumed to contribute neither to the line position nor to the line width, it can be averaged first and independently of the larger interactions. For correlation times less than 10^{-7} s and more than 10^{-9} s, the spectrum of the complex is given by

$$S_{cu} = \langle CU \rangle \otimes N_{iso}{}^n \qquad (7)$$

For correlation times longer than 10^{-7} s, a single value of N_{iso} can no longer be used to calculate the spectrum because the anisotropy of the nitrogen splitting becomes pronounced.

In practice, introduction of the nitrogen superhyperfine splitting into the copper slow motion spectrum is done by performing the Fourier transform (FT) of both the copper spectrum and the stick diagram for the nitrogen ligand. For n ligand atoms, the FT of the stick diagram is multiplied by itself n times and then multiplied by the FT of the slow motion spectrum. The result is back Fourier transformed.

d. Goodness of Fit Parameter and Simulation Error. The motion-related parameters for CuTPP in solution and in the membrane were obtained by a nonlinear least-squares fitting method. χ^2, given by eq 8, was used as the goodness of fit parameter

$$\chi^2 = 1/N \sum_{i=1}^{N} (y^{e_i} - y^{t_i})/\sigma_i^2 \qquad (8)$$

where N is the number of points representing the spectrum ($N = 1000$), y^{e_i}, y^{t_i} are the ith intensity of the experimental and simulated spectrum at minimum, respectively ($i = 1, ..., N$), and σ_i^2 is the experimental error (the error was assumed to be the base line noise and the same for each i).

The simulation error, $\Delta\sigma$, of a best fit parameter is given as a diagonal element of the variance–covariance matrix[49]

$$\Delta\sigma^2(x_i) = (\mathbf{B})_{ii}{}^{-1} \qquad (9)$$

where x_i is the ith fitted parameter and **B** is the matrix given as

$$\mathbf{B} = \mathbf{A}^T \mathbf{W} \mathbf{A} \qquad (10)$$

where **A** is the matrix of partial derivatives of the simulated spectrum with respect to the fitted parameters $\{x_i\}$ and **W** is the diagonal matrix whose diagonal elements are $W_{ii} = 1/\sigma_i^2$, where σ_i^2 are experimental errors (the same as in eq 8). Thus, $\Delta\sigma(x_i)$ depends both on the sensitivity of the simulated spectrum to the ith parameter (the smaller the sensitivity (function gradient), the bigger simulation errors) and on the actual

experimental error (a bigger experimental error causes bigger simulation errors).

In the case of CuKTSM$_2$ we could not perform the curve fitting as the experimental spectra were not digitized; instead, we used the trial and error method to find the best fit.

Simulations were carried out using a MC5500 Masscomp microcomputer to calculate the rigid limit and CuKTSM$_2$ motional spectra and DEC-Kubota TITAN workstation to calculate the motional spectra of CuTPP.

Results and Discussion

1. Determination of Magnetic Tensors Components. Prior to performing any detailed analysis of the motional behavior of copper complexes in isotropic (solution) and anisotropic (membrane) environments, magnetic parameters were determined by simulating the frozen solution ESR spectra. Light paraffin oil was the solvent. It was chosen because it resembles the hydrophobic environment of the membrane and also because its viscosity can change with temperature over a wide range, enabling the study of motion with correlation times spanning almost 4 orders of magnitude.[3,32]

When simulating the first harmonic ESR spectra of CuTPP, either an analytical expression for the first derivative line or the pseudomodulation method, as described by Hyde et al. (1990),[44] was used. The latter should be superior to the former, as it takes into account possible overmodulation of the experimental spectrum. Nevertheless, the differences in the values of the magnetic parameters obtained for the two methods of spectrum calculation were negligible. Although introduction of slight rhombicity to the magnetic tensors and nonequivalency to the nitrogen donor atoms improved the fit of the frozen solution experimental spectrum of CuKTSM$_2$,[32] in the motional spectra simulations, it is assumed that the **g**- and **A**-tensor for the copper complexes are axially symmetric and nitrogen ligands are equivalent. The first assumption introduces a negligible error, as observed previously by Campbell and Freed (1980),[21] in the case of VO(acac)$_2$ in toluene. The second assumption is a natural consequence of our way of treating the superhyperfine interactions (see Methods). For these reasons, when simulating the frozen solution spectrum of CuTPP, the **g**- and **A**-tensor are assumed to be axially symmetric and the nitrogen ligands are assumed to be equivalent. The components of the magnetic tensors (**g**, **A**, **AN**, and the line width tensor, **w**) for CuKTSM$_2$ and CuTPP are given in Table 1. The values for CuTPP are similar to previously published data.[26]

Since the copper complexes used in this study contain naturally abundant isotopes of ^{63}Cu (69%) and ^{65}Cu (31%), the simulated frozen solution spectra are the superposition of the spectra arising from ^{63}Cu and ^{65}Cu. However, the **g**-tensor components for both isotopes are identical, and the **A**-tensor components for ^{65}Cu are only 1.068 times larger than those for ^{63}Cu. Therefore, in the motional spectra simulations, only one copper species (^{63}Cu) is assumed. This assumption is the cause of a small discrepancy between simulated and experimental spectra, particularly for correlation times longer than 10^{-8} s.

2. Determination of Motional Parameters of Copper Complexes. Since the hyperfine interaction in square planar
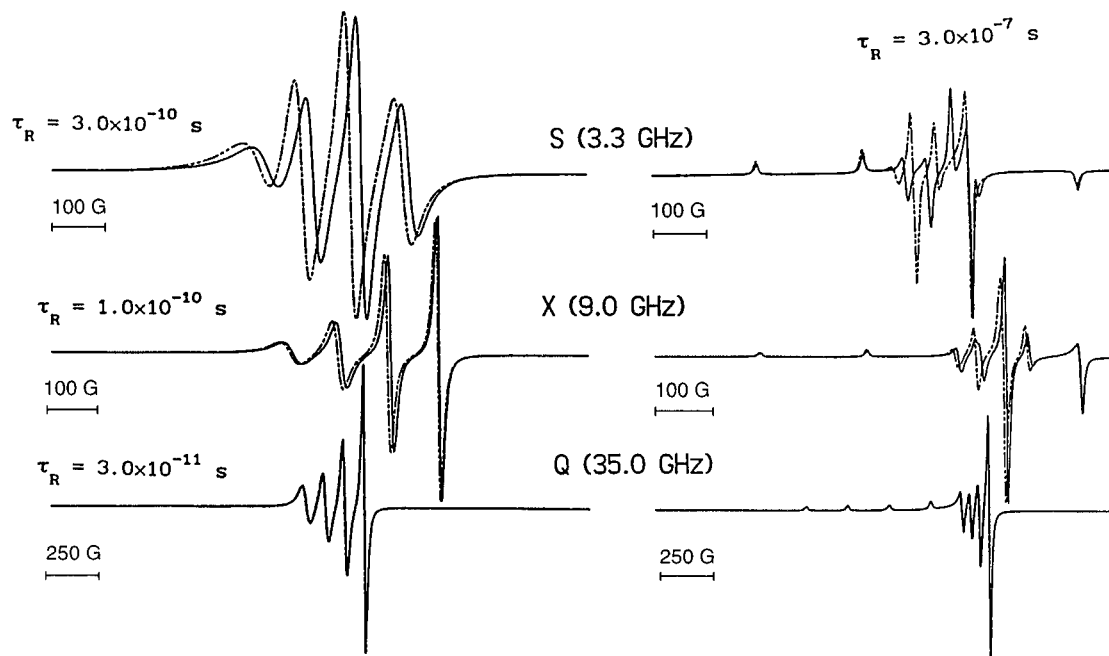
Figure 2. Simulated spectra of Cu(II) ESR (without superhyperfine structure) when nonsecular terms in the spin Hamiltonian are omitted (solid line) and included (dash line) at three different microwave frequencies, ω (S, X, and Q bands), for two different rotational correlation times, τ_R. The longer correlation time (3×10^{-7} s) is the same for all frequencies; the shorter correlation time was chosen to fulfill, for each frequency, the condition $\tau_R^{-1} \approx \omega$, under which the nonsecular term has the largest contribution to the fast motion line shape.

copper complexes is large ($A_{zz} \approx 200$ G), the contribution of the nonsecular terms to the X-band spin Hamiltonian is expected to be nonzero. Figure 2 shows the comparison of ESR spectra of Cu(II) (without superhyperfine structure) in an isotropic solvent at three different microwave frequencies, ω, for two different correlation times, τ_R, calculated when nonsecular terms are omitted and included. The shorter correlation time for each frequency was chosen to fulfill the condition $\tau_R^{-1} \approx \omega$ under which the nonsecular term has the largest contribution to the fast motion line shape. It is clear from Figure 2 that for Cu(II) at X-band, especially for longer correlation times, the nonsecular terms in the spin Hamiltonian should not be omitted.

2.a. CuTPP in Light Paraffin Oil. Simulated spectra superimposed on experimental ones for CuTPP in light paraffin oil over a range of temperatures are shown in Figure 3. When calculating the spectra, the rigid limit values for **g**- and **A**-tensor components for copper and the trace of **AN**-tensor, $AN_{iso} = \frac{1}{3}(AN_{xx} + AN_{yy} + AN_{zz})$, for the nitrogen superhyperfine splitting (listed in Table 1) were used as input parameters. There were only two adjustable parameters: $D_\perp$ (in-plane component of rotational diffusion tensor) and T_2^{-1} (the residual line width, i.e., the width not dependent on the rotational modulation of the components of the **g**- and **A**-tensor). As a model for reorientation, Brownian rotational diffusion was assumed. Because the **g**- and **A**-tensors are axially symmetric, the motional spectra cannot provide any information about rotational diffusion around the complex z-axis ($D_\parallel$ component). This rotation is expected to be faster than the rotation around the x- or y-axis (see Simulation section). But, since it cannot be determined in the ESR experiment, for simplicity, we assumed that $D_\parallel$ is equal to $D_\perp$—the rotational correlation time, $\tau_R = (6D_\perp)^{-1}$. The best fit values for $D_\perp$ and T_2^{-1}, together with their simulation errors (eq 9) and the goodness of fit parameters, χ^2, (eq 8) are given in Table 2.

Although the fit between the simulated and experimental spectra for CuTPP in paraffin oil is excellent over the whole range of temperatures studied, one can observe some discrepancy between the two spectra and, subsequently, larger values

for the χ^2 at the lowest temperatures of 0 and -10 °C. This discrepancy arises mainly from neglecting the contribution of ^{65}Cu and also from using a single (isotropic) value for the nitrogen superhyperfine splitting at these temperatures. The larger χ^2 values are additionally caused by 2–3 times higher signal to noise ratio compared with the higher temperature spectra (at lower temperature σ_i is smaller, due to better S/N, while ($y^{e_i} - y^{t_i}$) is larger, due to model inadequacy; thus χ^2 is larger than at higher temperatures (cf. eq 9)).

2.b. CuKTSM₂ in Light Paraffin Oil. Experimental and corresponding simulated ESR spectra for CuKTSM₂ in light paraffin oil over a range of temperatures are shown in Figure 4. The simulation was performed under the same assumptions as in the case of CuTPP.

Since the experimental spectra were not digitized, the comparison between them and the simulated spectra can only be qualitative; nevertheless, one can see from Figure 4 that the simulated spectra reproduce the experimental ones very well over the entire range of temperatures studied.

The values of $D_\perp$ in Table 2 show that, in light paraffin oil at the same temperature, rotational diffusion of CuTPP is about four times slower than that of CuKTSM₂. As a result, even at the highest temperature of 120 °C, the spectrum of CuTPP (Figure 3) does not have characteristic features of the motionally narrowed, four-line spectrum, which is observed for CuKTSM₂ (Figure 4). There is a range of temperatures (between 50 and 80 °C for CuKTSM₂ and between 20 and 45 °C for CuTPP) when the spectra of both complexes have no resolved superhyperfine structure, but the structure reappears when the temperature is lowered. For CuKTSM₂, the superhyperfine structure appears first in the perpendicular region of the spectrum, whereas for CuTPP, it appears in the high-field line. The difference may be due to a larger (ca. 10 G) value of A_{xx} ($=A_{yy}$) for CuKTSM₂ than for CuTPP (Table 1). In effect CuKTSM₂ spectrum is better resolved in the perpendicular region.

2.c. CuTPP in an Oriented DMPC Bilayer. Figure 5a,b shows experimental and simulated spectra of CuTPP in an

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TABLE 2: Best Fit Parameters for CuTPP and CuKTSM$_2$ in Light Paraffin Oil

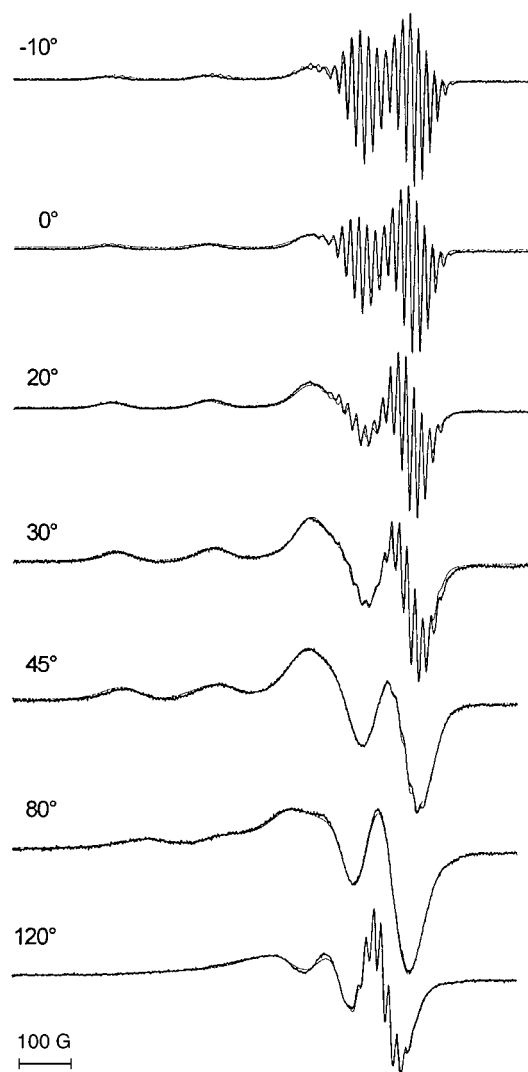| | $D_\perp$ (rad/s) | | T_2^{-1} (G) | | |
| | CuKTSM$_2$ | CuTPP | CuKTSM$_2$ | CuTPP | χ^2 CuTPP |
T (°C)					
120	2.0×10^9	$5.6 \times 10^8 \pm 0.37 \times 10^8$	4.0	2.80 ± 1.03	7.7
80	8.0×10^8	$2.4 \times 10^8 \pm 0.18 \times 10^8$	4.0	2.95 ± 1.85	7.3
50/45	2.5×10^8	$5.6 \times 10^7 \pm 1.00 \times 10^7$	4.0	4.43 ± 2.17	5.9
30	9.5×10^7	$1.9 \times 10^7 \pm 0.79 \times 10^5$	4.0	5.00 ± 0.63	13.9
20	4.5×10^7	$7.9 \times 10^6 \pm 0.64 \times 10^5$	4.0	4.11 ± 0.20	25.3
0	1.0×10^7	$3.5 \times 10^6 \pm 0.38 \times 10^4$	4.0	3.23 ± 0.14	95.3
−10/−60	6.0×10^5	$1.7 \times 10^6 \pm 0.21 \times 10^4$	4.0	3.49 ± 0.15	105.2

Figure 3. X-band simulated spectra are superimposed on experimental ones for CuTPP in light paraffin oil over a range of temperatures. The thicker and the thinner lines represent the experimental and the simulated spectra, respectively. Temperatures at which the experimental spectra were recorded are indicated. The magnetic field range is 2500–3500 G; the microwave frequency is about 8.7 GHz.

oriented DMPC membrane when the magnetic field is perpendicular (Figure 5a) and parallel (Figure 5b) to the membrane surface. In these simulations, for the reasons explained below, there are four adjustable parameters: $D_\perp'$, $D_\parallel'$ (the components of rotational diffusion tensor), T_2^{-1} (the residual line width), and ϵ_{20} (the strength of the restoring potential).

Subczynski et al. (1987)[1] showed that CuKTSM$_2$ locates in the membrane with its plane parallel to the hydrocarbon chains of the phospholipids. A similar parallel orientation of CuTPP in the bilayer is assumed. The axis that is parallel to the hydrocarbon chains is chosen to be the x-axis. In effect the diffusion axes of the complex do not coincide with the principal

axes. Rotation of the complex about its in-plane x- and y-axes is not equivalent: rotation about the y-axis (parallel to the membrane surface) is restricted due to the ordered structure of the membrane, whereas rotation about the x-axis (perpendicular to the membrane surface) is unrestricted due to axially symmetric form of the potential U (eq 3). Rotation about the z-axis (parallel to the membrane surface and perpendicular to the complex plane) is also unrestricted.

ESR experiments were conducted in two geometries: with the magnetic field perpendicular to the membrane surface (perpendicular spectrum) and with the magnetic field parallel to the membrane surface (parallel spectrum). As the restoring potential of the membrane is defined relative to the membrane normal (eq 3), perpendicular geometry allows for observation of restricted reorientation of the complex about its y-axis. The degree of confinement of the reorientation depends on the strength of the potential, ϵ_{20} (eq 4). For a very large ϵ_{20} the ESR spectrum is a single-crystal spectrum along the x-axis. For ϵ_{20} equal to zero, it is an isotropic solvent spectrum. For an intermediate ϵ_{20} the spectrum arises from an incomplete averaging of the x-, y-, and z-components. There is no restoring membrane potential defined relative to the in-plane axes, and reorientation of the complex about its x-axis (parallel to the membrane normal) is unrestricted. Thus, for parallel geometry one observes the spectrum with a full contribution from the y- and z-components. There is also a contribution from an incomplete averaging with the x-axis; however, the effect depends on the relative orientation between the magnetic field direction and the z-axis of the complex. When the angle is 0°, the effect is largest; when it is 90°, the effect is zero. As there is no preferred orientation of the complex in the y,z-plane, the total contribution from the x-axis is small.

For practical reasons the principal axes of the complex are relabeled. The x-axis that is parallel to the potential symmetry axis (membrane normal) was labeled z'. The z-axis that is perpendicular to the normal was labeled x'. The y-axis remained unchanged. The diffusion constant for the rotation about the z'-axis was denoted by $D_\parallel'$ and about the y-axis was denoted by $D_\perp'$ (Figure 6).

The model for copper complex reorientation in a phospholipid bilayer proposed here is in line with the model proposed by Antholine et al. (1987).[31] They argued that rotation of the complex about the z'-axis is accompanied by coordinated displacement of neighboring lipids and causes little energy change in the system. The rotation about the x'-axis does not require displacement of lipids and encounters little hindrance. However, the rotation about the y-axis raises the energy of the system and therefore is hindered.

The extended ring system of the CuTPP molecule (Figure 1) makes the structure flat, rigid, and of large area. Therefore, in the highly packed and relatively ordered structure of the membrane, the rotation of the complex about the z'-axis is expected to be more strongly coupled to the collective diffusions of the adjacent DMPC molecules than it is in the case of CuKTSM$_2$. On the other hand, the complex is expected to
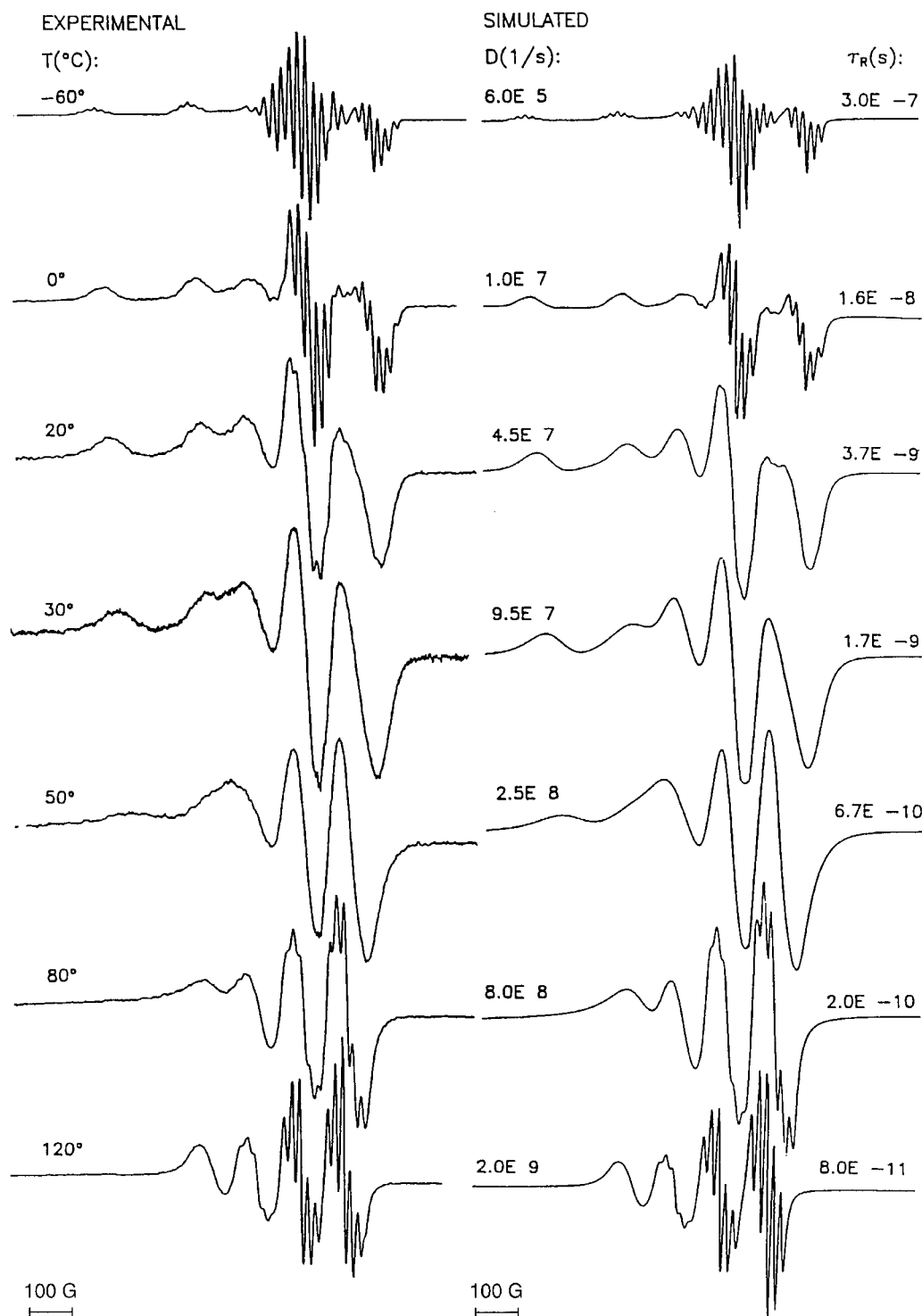
Figure 4. Comparison of X-band experimental (the left-hand side) and corresponding simulated (the right-hand side) ESR spectra for CuKTSM$_2$ in light paraffin oil over a range of temperatures. Temperatures at which the experimental spectra were recorded, as well as calculated rotational correlation times, are indicated. The magnetic field range is 2500−3500 G; the microwave frequency is about 8.7 GHz.

undergo frequent, small amplitude reorientation about its *y*-axis caused by local motions of segments of DMPC molecules. Such reorientations will be characterized by a relatively large diffusion constant, $D_\perp'$, and large ordering parameter, ϵ_{20}.

There are no data about the distribution of the CuTPP molecule in the membrane along the vertical axis. Nevertheless, we assumed that it is uniform and that the ordering potential felt by the complex in the membrane is independent of the exact location of the complex in the hydrocarbon core. The latter gets support from similar dimensions for the complex and for the effective hydrocarbon chain length in the liquid crystalline state.

As was stated in the Experimental Section, CuTPP does not dissolve in the membrane well; therefore, the quality of the experimental spectra is not very high, particularly in the case of the magnetic field that is parallel to the membrane surface.

2.c.1. Magnetic Field Perpendicular to the Membrane Surface. For this experiment the *z′*-axis is parallel to the magnetic field. This axis is not the axis of axial symmetry of the complex. This fact and the necessity of including the potential make the problem very computationally demanding (cf. paragraph 3 and Table 4). Therefore, the nonsecular terms are omitted when calculating the spectrum.
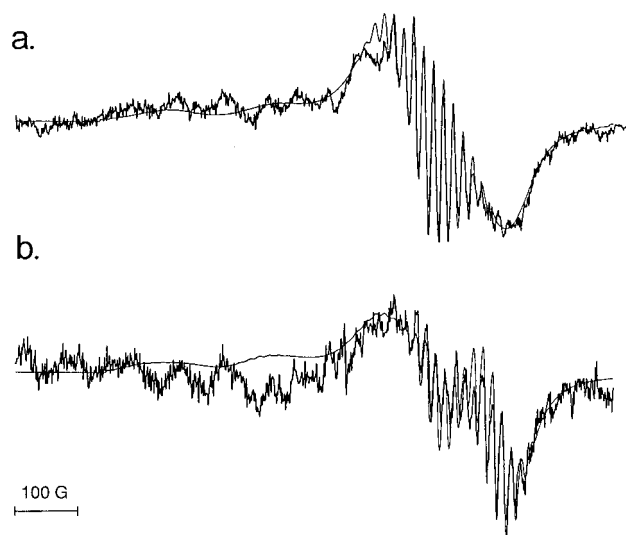
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Figure 5. X-band simulated spectra are superimposed on experimental ones for CuTPP in oriented DMPC bilayer membrane at 25 °C, when the magnetic field is perpendicular (a) and parallel (b) to the membrane surface. The magnetic field range is 2500−3500 G; the microwave frequency is about 8.7 GHz.
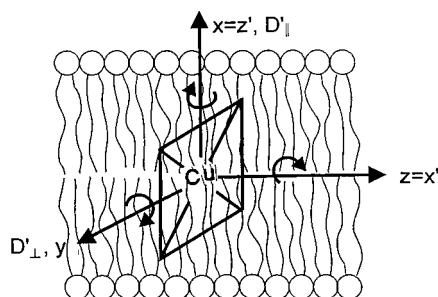

Figure 6. Schematic drawing of an oriented lipid bilayer containing a square planar copper complex. The relabeled principal axes and the rotational diffusion axes of the complex in the bilayer are indicated. Arrows show the possible rotational motion.

The effect of omitting the nonsecular terms when calculating the ESR spectrum of copper complex in an oriented bilayer membrane was tested for ϵ_{20} not greater than 5.0. For larger values of ϵ_{20} the program failed to reliably calculate the spectrum. Test calculations showed that alteration in the spectrum caused by the inclusion of the nonsecular terms is similar for both isotropic and anisotropic solvents; namely, the "perpendicular" lines in the spectrum are nonuniformly shifted toward lower values of the magnetic field and their relative intensities slightly change as compared to the spectrum generated when nonsecular terms are omitted. However, they also showed that the effect dose not significantly depend on ϵ_{20}, i.e., the shift and intensity change are similar for ϵ_{20} equal to 2.0 and 5.0. These would suggest that in the subsequent calculations both the restoring potential and the components of the diffusion tensor might be slightly underestimated.

Initially the spectrum was treated as arising from a single paramagnetic species. However, with this assumption, the simulated spectrum was not able to correctly reproduce intensities of the superhyperfine lines in the middle nor in the high-field portions of the experimental spectrum. Therefore, another species had to be introduced, which gave a satisfactory result (Figure 5a). The best fit values for both lines are given in Table 3. The main spectrum arises from the complex located in the membrane, undergoing relatively fast ($D_{\perp}' \approx 3.1 \times 10^7$ rad/s), but with a small amplitude due to a strong restoring potential ($\epsilon_{20} \approx 8.5$), reorientation about the y-axis. Its reorientation about

the z'-axis is very slow ($D_{\parallel}' \approx 8.7 \times 10^5$ rad/s). As $D_{\parallel}'$ is much smaller than $D_{\perp}'$, there is practically no averaging between the y and z-directions during the time of perpendicular rotation; therefore, the restricted reorientation about the y-axis mixes x-component with practically unaveraged z (x')-component. However, a very large simulation error of $D_{\parallel}'$ (see Table 3) makes determination of this parameter rather imprecise. The second spices signal (background spectrum lacking the super-hyperfine structure) is a nonspecific (large simulation errors) and broad Cu(II) spectrum from an aggregated complex located, most likely, outside the membrane. The ratio of the intensities of the main to background signal is about 1.0.

2.c.2. Magnetic Field Parallel to the Membrane Surface. For this geometry the z'-axis is perpendicular to and the y- and x'-axis are coplanar with the magnetic field vector. To calculate the spectrum for such geometry the membrane system has to be rotated 90° relative to the magnetic field. This requires a very large basis set (minimum truncation scheme, MTS (cf. paragraph 3)) and a very long computation time. Therefore we returned to the original principal axes of the copper complex and conducted calculations with the magnetic field parallel to the original z-axis (axial symmetry axis). Test calculations gave similar best fit values of motional parameters for the two above geometries, but the simulation errors were smaller for the second geometry. The best fit parameters are given in Table 3.

In the calculation of the parallel spectrum, the same background signal and intensity ratio as in the perpendicular spectrum case were used. The quality of the experimental spectrum is poor, and there is a large discrepancy between the spectra in the parallel region; nevertheless, in the perpendicular region the number of the superhyperfine lines, their positions, and in most cases their intensities are well reproduced by the simulated spectrum (Figure 5b).

2.d. CuKTSM₂ in an Oriented DMPC Bilayer. Since CuKTSM$_2$ dissolves in the phospholipid membrane much better than CuTPP, the experimental spectra of CuKTSM$_2$ are of a single component. Although a trial-and-error method was used to fit the spectra instead of curve fitting, both the perpendicular and parallel spectrum reproduce the experimental ones very well (Figure 7a,b). The best fit parameters are given in Table 3.

Even though CuKTSM$_2$ is much smaller than CuTPP, it was also assumed that the restoring potential acting on the probe in the membrane is independent of its location in the hydrocarbon core. Reorientation of the complex about the z'-axis is similar to that about the y-axis and relatively fast ($D_{\perp}' = D_{\parallel}' = 5 \times 10^7$ rad/s). The rotation about the y-axis is restricted ($\epsilon_{20} = 1.5$) but much less than in the case of CuTPP ($\epsilon_{20} \approx 8.5$).

Comparison of the entries in Table 3 for CuTPP and CuKTSM$_2$ shows that $D_{\perp}'$ for the two complexes are similar, whereas, ϵ_{20} for the perpendicular rotations as well as $D_{\parallel}'$ are vastly different. The rigid part of CuKTSM$_2$ is much smaller than that of CuTPP. Therefore, the rotation about the z'-axis of CuKTSM$_2$ is believed to require less collective motion in relocation of adjacent phospholipids than in the case of CuTPP; thus, the value of $D_{\parallel}'$ is larger for CuKTSM$_2$. On the other hand, the reorientation about the y-axis of a complex is believed to be activated by local motions of phospholipids, so its rate should be of the same order for both CuTPP and CuKTSM$_2$; thus, the values of $D_{\perp}'$ are similar for both complexes. However, because CuTPP is larger and stiffer (Figure 1), its rotation about the perpendicular axis is much more hindered than that of CuKTSM$_2$; thus, the value of ϵ_{20} is much larger for CuTPP. It is also possible that the CuKTSM$_2$ molecule, which has the polar oxygen in the ethoxyethyl side chain and the bulky methyl groups, disturbs the local structure of the membrane to

TABLE 3: Best Fit Parameters for CuTPP and CuKTSM$_2$ in the Membrane

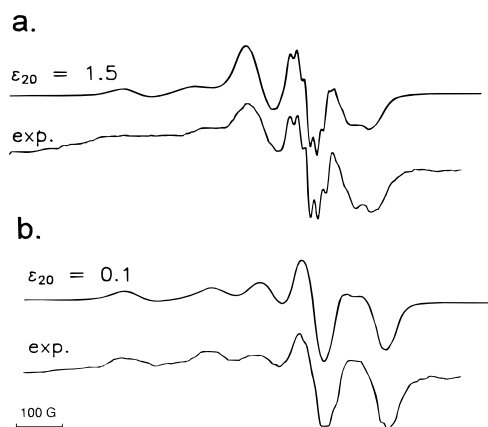| complex	magn. field orientation	$D'_\perp$ (rad/s)	$D'_\parallel$ (rad/s)	ϵ_{20}	T_2^{-1} (G)	χ^2
CuTPP	$\perp$	$3.1 \times 10^7 \pm 1.4 \times 10^7$	$8.7 \times 10^5 \pm 8.8 \times 10^7$	8.51 ± 1.7	3.5 ± 1.2	4.24
CuKTSM$_2$	$\perp$	5.0×10^7	5.0×10^7	1.5	4.0	
background	$\perp, \parallel$	$5.9 \times 10^7 \pm 7.5 \times 10^7$	$1.5 \times 10^8 \pm 1.2 \times 10^8$	0.0 ± 2.0	20.0 ± 16.9	intensity ratio $= 1.1 \pm 0.3$
CuTPP	$\parallel$	$2.4 \times 10^6 \pm 3.6 \times 10^6$	$2.4 \times 10^6 \pm 3.6 \times 10^6$	0.0	4.0 ± 4.0	1.94
CuKTSM$_2$	$\parallel$	5.0×10^7	5.0×10^7	0.1	4.0	

Figure 7. Comparison of X-band experimental (lower) and corresponding simulated (upper) ESR spectra for CuKTSM$_2$ in oriented DMPC bilayer membrane at 25 °C, when the magnetic field is perpendicular (a) and parallel (b) to the membrane surface. The magnetic field range is 2500−3500 G; the microwave frequency is about 8.7 GHz.

a larger extent than the CuTPP molecule. This gives CuKTSM$_2$ more free space to reorient—in effect, the value of ϵ_{20} is smaller.

Because of the axial symmetry of the square planar copper complexes, the spectra cannot provide any information about the rate of reorientation about the x'-axis (z-axis). However, one can expect that, because the structure of CuTPP is highly symmetric, the rotation about the z-axis is practically unrestricted. In the case of CuKTSM$_2$, due to the presence of the ethoxyethyl side chain, the rotation about the z-axis might be slower and hindered.

Very poor base lines in the spectra for CuTPP in oriented membranes inhibit correct determination of the level of noise. For this reason, the experimental errors, σ_i, are overestimated. This results in underestimated χ^2 (cf. eq 8) and overestimated simulation errors (cf. eq 9), particularly in the case of the parallel spectrum.

3. Numerical Parameters. The results of numerical calculations of ESR spectra by the slow motion program depend strongly on the choice of (1) the minimum truncation scheme (MTS) that determines the dimension, N, of the matrix representation of the superoperators L and Γ in eq 1 (so called matrix $\mathbf{A}$[48]), and (2) the number of recursive steps, ns, in the Lanczos algorithm[22] (see Introduction) that tridiagonalizes the matrix $\mathbf{A}$.[50,51] MTS consists of the indices, L, K, M, of the Wigner rotation matrices, which are eigenfunctions of the diffusion operator Γ (eq 1). General requirements are $L \geq 2$, $L \geq K$, $M \geq 0$, and K is even. For axially symmetric $\mathbf{g}$- and $\mathbf{A}$-tensors, $K = 0$. In the case when a paramagnetic probe is dissolved in an isotropic solvent or membrane without tilt, $M \geq 2I$, where I is the nuclear spin of the probe. In the presence of tilt, M may be larger than $2I$, but always smaller than L. The expansion of the starting vector $|\mathbf{v}\rangle$ (eq 1) into the Wigner matrices is performed separately for the even and odd L and, in general, the required minimum value for odd L, L_{odd}, is smaller than that for even L, L_{even}.

TABLE 4: Numerical Parameters Used To Simulate ESR Spectra of CuTPP in Light Paraffin Oil (l.p.o) and a DMPC Bilayer

l.p.o. temp (°C)	L_{even}[a]	L_{odd}[a]	K[a]	M[a]	ns[a]	nonsecular[b]
120	4	1	0	3	23	+
80	4	1	0	3	28	+
45	8	1	0	3	55	+
30	12	1	0	3	58	+
20	16	1	0	3	58	+
0	20	1	0	3	50	+
−10	30	1	0	3	160	+

membrane	L_{even}[a]	L_{odd}[a]	K[a]	M[a]	ns[a]	nonsecular[b]
DMPC $\perp$	24	5	24	3	180	−
DMPC $\parallel$[c]	14	5	8	14	180	−
DMPC $\parallel$[d]	38	1	0	3	182	+

[a] Definitions of the indices are given in paragraph 3. [b] When nonsecular term in the spin Hamiltonian is included then +, otherwise −. [c] The largest MTS which could be used when magnetic field is perpendicular to the z'-axis and the system is rotated by 90° (cf. paragraph 2.c.2). [d] MTS when magnetic field is parallel to the z-axis (cf. paragraph 2.c.2).

The values for MTS and ns used in our simulations were established by the trial-and-error method and are given in Table 4.

Conclusions

Successful simulations of ESR spectra of CuKTSM$_2$ and CuTPP in an isotropic solvent over a wide range of temperatures and in oriented phospholipid bilayer membranes prove that the slow motion theory can be applied to a $^1/_2$ spin system with a large anisotropy of magnetic tensors even in extreme motional conditions encountered in a solvent of high viscosity as well as in an ordered solvent. They also prove that the postconvolution method developed to introduce the superhyperfine splitting into the slow motion copper spectrum is reliable and useful.

To calculate the spectrum for the Cu(II) complex in an isotropic solvent, the nonsecular terms in the spin Hamiltonian can be included even for a very slow reorientation. However, because the complex in the membrane orients with its plane parallel to the lipid alkyl chains, the diffusion axis parallel to the membrane normal (the potential symmetry axis) is not the axis of axial symmetry. This necessitates the use of a large basis set (MTS) and prevents the inclusion of the nonsecular terms in the spectrum calculation. Test calculations indicate that omitting these terms may result in slight underestimation of the restoring potential and the components of the diffusion tensor. However, since the quality of the experimental spectra of Cu(II) in oriented phospholipid bilayer membranes is not high (CuTPP does not dissolve in the membrane well) these parameters cannot be estimated precisely anyway.

When the components of $\mathbf{g}$- and $\mathbf{A}$-tensors, determined from the frozen solution spectra, were allowed to vary in the simulation of the motionally averaged spectra in an isotropic solvent, their values never departed from the starting values more than the simulation errors. Thus, our results show that the ESR spectra of copper complexes at different temperatures and ordering conditions depend mainly on the diffusion-tensor

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components and the restoring potential. This result is very reassuring as it shows that the principal values of the magnetic tensors have no significant temperature dependence and obtaining them from the frozen solution spectra is the correct way to follow.

To obtain the best fit spectra in an isotropic solvent both the in-plane component of rotational diffusion tensor, $D_\perp$, and the residual line width, T_2^{-1}, had to be varied. With increasing temperature T_2^{-1} is first constant ($-60-0$ °C) and then increases ($0-20$ °C). For higher temperatures ($45-120$ °C) the errors in determining the line width are too large to make any meaningful comparison; nevertheless, the line width decreases. Temperature dependent residual line width of VO^{2+} in toluene was observed by Wilson and Kivelson (1966)[52] in the motional narrowing region, and by Campbell and Freed (1980)[21] in the slow motion region. The increase with temperature is due to motional modulation of spin-rotational coupling which causes the decrease of the spin-rotational relaxation time.[53] The increase of CuTPP residual line width can be explained by spin-rotational interaction, while the subsequent decrease might arise from effective averaging of anisotropic contributions to the line width, like unresolved hyperfine splitting, and g- and A-strain.[54] A decrease in the line width when going from the slow motion to the motional narrowing region was observed for VO^{2+} by Campbell and Freed (1980);[21] however, the decrease was much smaller than that observed in this study for CuTPP.

The Brownian rotational diffusion proved to be a satisfactory model for reorientation of these complexes in both the isotropic and ordered solvent.

A large anisotropy of the spectral parameters poses a problem in obtaining the field-swept spectrum. The actual sweeping variable in the calculation is the microwave frequency ($\omega - \omega_0$) (eq 1), and only in the final step is the spectrum converted to the magnetic field-swept one by using a single, isotropic value of the g-factor (ignoring the **g**-tensor anisotropy across the spectrum). Such a way seems satisfactory for spectra from nitroxide spin labels but may be problematic in the case of paramagnetic metal complexes. This problem was discussed by Campbell and Freed (1980).[21] They estimated that, in the case of a vanadyl complex, the error due to a single g-value spectrum conversion may be as large as 20%. The sweep width of the copper spectrum is two times smaller than that of the vanadyl one; however, the **g**-tensor anisotropy, Δg, is larger (for vanadyl complexes Δg is about 0.04,[21] for $CuKTSM_2$ is 0.09, and for CuTPP is 0.14 (Table 1)); therefore, the error introduced to the copper spectrum might be similar or larger. The error obviously depends on the rate of reorientation: for fast reorientation it is negligible because the anisotropy of the **g**-tensor is averaged out, but the slower the reorientation the larger the error. Although our simulations give very good fits to the experimental spectra, the procedure is still imperfect.

The problem of the field-swept spectra simulation was solved by Vasavada et al. (1987),[51] who developed a generalization of the conjugate gradient method to calculate the slow motion spectra. This method not only enables one to calculate the spectra directly as a function of the magnetic field but also gives the optimal basis set, MTS, and recursive steps, ns. An arbitrary determination is no longer necessary. Although, at the time when our simulations were performed, the version of the computer program utilizing the conjugate gradient method aimed at simulating the spectra from spin labels was not suitable for large anisotropy systems such as square planar copper complexes, the use of Vasavada's method is not expected to change the conclusions from this study.

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