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Chemistry and Physics of Lipids 124 (2003) 69–80
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

DMPG gel–fluid thermal transition monitored by a phospholipid spin labeled at the acyl chain end

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Received 8 January 2003; received in revised form 24 March 2003; accepted 24 March 2003

Abstract

Low ionic strength aqueous dispersion of dimyristoyl phosphatidylglycerol (DMPG) presents a rather peculiar gel–fluid thermal transition behavior. The lipid main phase transition occurs over a large temperature interval (ca. 17 °C), along which several calorimetric peaks are observed. Using lipids spin labeled at the acyl chain end, a two-peak electron spin resonance (ESR) spectrum is observed along that temperature transition region (named intermediate phase), at three different microwave frequencies: L-, X- and Q-bands. The intermediate phase ESR spectra are analyzed, and shown to be most likely due to spin labels probing two distinct types of lipid organization in the DMPG bilayer. Based on the ESR spectra parameters, a model for the DMPG intermediate phase is proposed, where rather fluid and hydrated domains, possibly high curvature regions, coexist with patches that are more rigid and hydrophobic.

Keywords: DMPG; Gel–fluid transition; ESR; 16-PCSL; α-MSH

1. Introduction

Due to the presence of acidic lipid headgroups, most cell membranes have a negative surface charge. Hence, anionic phospholipids have been widely used as model membrane systems. Phosphatidylglycerol (PG), being the most abundant anionic phospholipid headgroup present in prokaryotic cell membranes, has been extensively studied as a model for negatively charged membrane domains (see, for instance, Seelig et al., 1987; Heimburg and Biltonen, 1994; Biaggi et al., 1997; Fernandez and Lamy-Freund, 2000).

Dimyristoyl phosphatidylglycerol (DMPG) was shown to be a very interesting anionic lipid system, displaying a thermal behavior strongly dependent on dispersion conditions (Salonen et al., 1989; Heimburg and Biltonen, 1994; Lamy-Freund and Riske, 2003). In a range of sodium salt concentration (below ca. 100 mM) and pH values (above ca. 6), DMPG shows a large gel–fluid transition region, accompanied by an unusually low turbidity and high viscosity. This transition region was named in our previous studies the

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doi:10.1016/S0009-3084(03)00046-X

"intermediate phase," even though it is not yet clear to what extent this transition region can be described as a different lipid phase. Under those conditions (pH above 6 and low ionic strength), the differential scanning calorimetry (DSC) profile of DMPG is characterized by the presence of several calorimetric peaks, between a very sharp one ($T_m^{on} \sim 18\,^{\circ}C$), which correlates with the decrease in sample turbidity, increase in sample viscosity and conductivity, and beginning of the chain melting process, and a broad peak ($T_m^{off} \sim 30\,^{\circ}C$), concomitant with the regain in sample turbidity, decrease in viscosity and conductivity and end of the gel–fluid melting process. Within this transition region, a continuous decrease in membrane packing with temperature was measured using both spin and fluorescent probes intercalated into the bilayer (Lamy-Freund and Riske, 2003).

A small-angle X-ray scattering experiment (SAXS) revealed no Bragg peak related to a lamellar repeat distance. The only feature detected in the studied q range was a broad peak due to the electron density contrast of the bilayer with the aqueous medium. This broad peak starts to lose intensity at T_m^{on}, reaches a minimum just below T_m^{off}, and then regains almost all its intensity above T_m^{off}, suggesting an unusual bilayer packing in the intermediate phase. The peak position shifts steadily to higher q values between T_m^{on} and T_m^{off}, reflecting the gradual decrease in membrane thickness, likely to occur during the chain melting process.

The transition region narrows significantly as the ionic strength increases, until a single main phase transition at T_m ($23\,^{\circ}C$) is observed with 100 mM NaCl (Lamy-Freund and Riske, 2003). Around this ionic strength, DMPG presents a thermal behavior quite similar to that displayed by the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC). Further increase in salt concentration causes the DMPG gel–fluid transition temperature T_m to increase to $29\,^{\circ}C$ at 2 M NaCl (Cevc et al., 1980). A decrease in pH, with the complete protonation of the phosphate groups (Watts et al., 1978), or the addition of divalent cations (Van Dijck et al., 1978), also drastically reduce the extent of the gel–fluid temperature transition region, and lead to a much higher bilayer transition temperature, $T_m \approx 42\,^{\circ}C$, related to a more stable gel state. Thus, the extent of the intermediate phase, and its characteristics, are surely related to the presence of charged phosphate groups at the bilayer surface, which may cause a significant repulsion between vesicles as well as between adjacent or opposite lipids in the same bilayer, thus determining its packing. However, the structural characteristics of the intermediate phase are far from being well understood.

Electron spin resonance (ESR) of nitroxides intercalated into DMPG bilayers has been used to monitor the structure of the gel–fluid transition region. So far, the analysis of the ESR spectra of different spin labels evidenced the presence of a sharp decrease in bilayer fluidity at T_m^{on}, followed by a gradual reduction on the lipid chain packing up to T_m^{off} (Riske et al., 2001). In the present work, the analysis of the ESR signal of a phospholipid labeled close to the acyl chain end, at the 16th carbon atom (16-PCSL), incorporated into DMPG bilayers, gives further information on the properties of the DMPG intermediate phase. The modification caused by the cationic peptide α-melanocyte stimulating hormone (α-MSH) on the 16-PCSL ESR spectrum due to peptide/bilayer interaction is used as a tool in the understanding of the 16-PCSL signal in DMPG bilayers.

We have discovered that spin probes labeled near the acyl chain end yield fascinating spectra within the transition temperature region. We show that these spectra are definitely correlated to the behavior of the membrane in this temperature region. The purpose of this work is to analyze these spectra as fully as possible in our continued efforts to deepen our understanding of the intermediate phase. While we are unable as yet to prove it, we show that these spectra are likely due to the spin probe's sampling of two distinct types of lipid organization. A model is suggested which would account for the results.

2. Materials and methods

2.1. Materials

The sodium salt of the phospholipid DMPG (1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-glycerol]), DMPC (1,2-dimyristoyl-*sn*-glycero-3-[phospho-choline]), and the spin labels 14- and 16-PCSL (1-palmitoyl-2-(14- or 16-doxyl stearoyl)-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). α-MSH was purchased from Sigma. The buffer system used was 10 mM HEPES

(4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid) adjusted with NaOH to pH 7.4. The ionic strength was calculated and measured to be 4 mM. Mille-Q Plus water (Millipore) was used throughout.

2.2. Lipid dispersion preparation

A lipid film was formed from a chloroform solution of lipids, dried under a stream of N_2 and left under reduced pressure for a minimum of 2 h, to remove all traces of the organic solvent. Vesicles were prepared by the addition of the HEPES buffer with 2 mM added NaCl, followed by vortexing for about 2 min above T_m or T_m^{off}. The samples were kept at room temperature and used within a few hours after preparation. For the ESR measurements 0.3 mol% of 14-PCSL and 0.2 mol% of 16-PCSL was added to the chloroform lipid solution. At this lipid/spin label no spin exchange linewidth broadening occurred.

2.3. ESR spectroscopy

ESR measurements at X-band were performed with a Bruker EMX spectrometer. A field-modulation amplitude of 1 G and a microwave power of 8 mW were used. The temperature was controlled to about 0.2 °C with a Bruker BVT-2000 variable temperature controller. The temperature was monitored with a Fluke 51 K/J thermometer with the probe placed just above the cavity. The magnetic field was measured with a Bruker ER 035 NMR Gaussmeter. Alternative frequencies were used: Q-band (35 GHz) and L-band (1.280 GHz). The Q-band Varian spectrometer model E110 was used equipped with the Varian Variable Temperature Controller. The L-band system was homemade, equipped with a loop-gap resonator working at a frequency around 1.3 GHz, and adapted to a Varian ESR console and a Variable Temperature Controller (Sartori and Nascimento, 1992). Spectral parameters were measured using the Win-EPR Software.

2.4. ESR simulations

For least-squares analysis of ESR spectra, we used the computer program NLSL developed by Freed and co-workers (Schneider and Freed, 1989; Budil et al., 1996). The use of this program has been extensively discussed in their papers, hence only a few of the most relevant points for performing the simulations will be pointed out. As discussed in the literature (Ge and Freed, 1999), the best-yielded simulation of the spectrum of spin labels in dispersions (microscopic order and macroscopic disorder, MOMD) is strongly dependent on the initial parameters (seed values). Searches with different initial values will lead to distinct local minima. Hence, the least-squares analysis is not intended to be carried out fully automated, but requires the "intuition" of the user. Considering that the variations on the g value with the label local polarity is rather small, the g-tensor components were kept as $g_{xx} = 2.0089$, $g_{yy} = 2.0058$ and $g_{zz} = 2.0021$ (Ge and Freed, 1999; Earle et al., 1994). The hyperfine splitting tensor was considered to be axial, $A_{xx} = A_{yy} = A_\perp$, and the $A_\perp$ and A_{zz} values were allowed to vary within a reasonable interval (Earle et al., 1994). We have not used fixed A_{zz} values, obtained from frozen samples, as there are indications that the hyperfine splitting obtained at this condition is not identical to that obtained at higher temperatures. Indeed, the amount of water inside the bilayer may be dependent on the lipid phase and temperature (Fernandez and Lamy-Freund, 2000), and it is well known that the value of A_{zz} is sensitive to the amount of water. As expected, different Gaussian inhomogeneous broadening parameters were used for the simulations of the spectra, at the three frequencies. Rotational correlation times ($\tau_\perp$ and $\tau_{//}$) were calculated based on the best results obtained for the principal values of an axially symmetric rotational diffusion tensor for the nitroxide moiety attached to the chain segment ($R_\perp$ and $R_{//}$; $\tau = 1/6R$). For the diffusion tensor, the symmetry axis is the axis of the chain segment, z' (Schneider and Freed, 1989) (the molecular frame). The angle between the molecular z' axis and the magnetic z''' axis (by convention, defined parallel to the $2p_z$ orbital of the N atom), the diffusion tilt angle β, was allowed to vary, but no better simulation could be obtained, so β was kept null (see discussions in Ge and Freed, 1993, 1999). The parameters c_{20} and c_{22} are related to the membrane orienting potential, hence with the probe microenvironment microscopic order.

2.5. DSC

The calorimetric data were carried out in a Microcalorimeter Microcal VP-DSC at a scan rate of

20 °C/h. The baseline subtractions, and peak integrals were done using the Microcal Origin software with the additional device for DSC data analysis provided by Microcal.

3. Results and discussion

Fig. 1 compares the ESR spectra of phospholipid spin probes, incorporated in DMPG bilayers, with the paramagnetic nitroxide moiety attached to the acyl chain at two different positions, the 14th and the 16th carbon atoms (14-PCSL and 16-PCSL, respectively), at various temperatures. The start and end points of the gel–fluid transition region, T_m^{on} and T_m^{off} (Riske et al., 2001), are indicated in the figure. The spectra obtained with 14-PCSL (Fig. 1a), and those yielded by phospholipids labeled between the 5th and the 14th carbon atom (results not shown), can be attributed to spin labels residing in one domain only. The ESR spectra were analyzed through the ratio of the amplitudes of the central and low field lines (h_0/h_{+1}), which tends to the unity as the spin label mobility increases. The parameter h_0/h_{+1} indicated a sharp decrease in chain packing at T_m^{on}, followed by a smooth decrease until T_m^{off} was reached (Riske et al., 2001). These results were compared with those obtained with 14-PCSL in DMPC (or DMPG in HEPES buffer + 100 mM NaCl), which shows a sharp decrease in chain packing at a single T_m (23 °C). Above T_m^{off} the microviscosity of the fluid phases of DMPC and DMPG were found to be quite similar.

However, the spectra yielded by 16-PCSL (Fig. 1b) incorporated into DMPG bilayers present an unusual feature between T_m^{on} and T_m^{off}: the high-field peak is split in two. Fig. 2 (DMPG at 25 °C) shows that the two peaks at the high-field position, characteristic of the intermediate phase, are only present in a certain range of ionic strength: increasing amounts of NaCl decrease the intensity of the outer peak, leading finally to the presence of a single peak at 100 mM NaCl, located at an intermediate position. For this high ionic strength condition, and for DMPC, the two-peak feature at the high-field position was not observed over

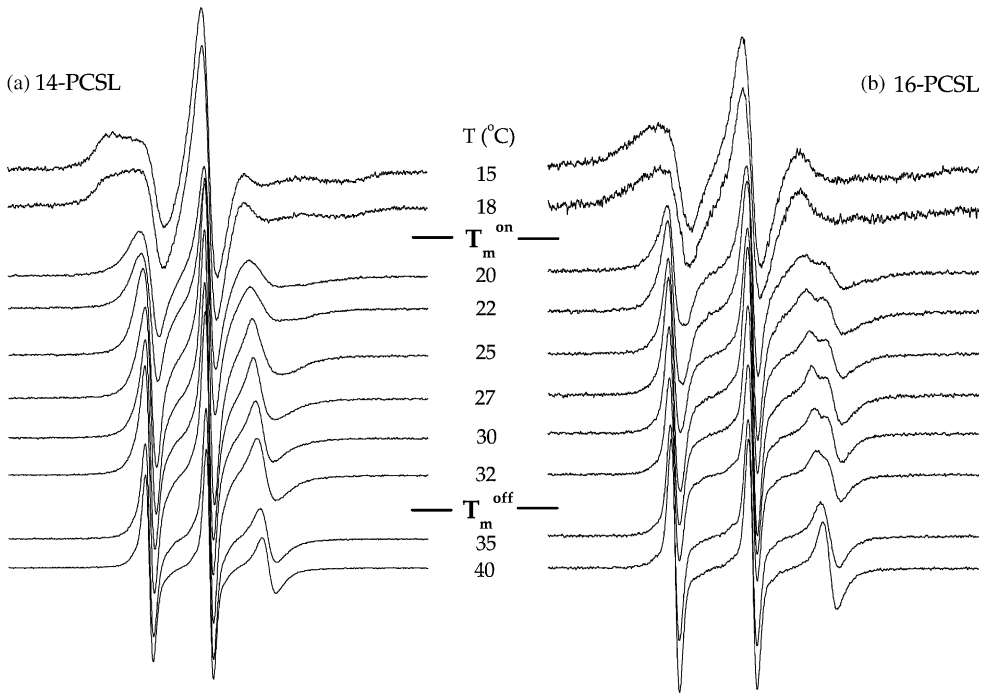

Fig. 1. X-band ESR spectra of (a) 14-PCSL and (b) 16-PCSL in 10 mM DMPG in HEPES buffer + 2 mM NaCl, at different temperatures. Total spectrum width 100 G.

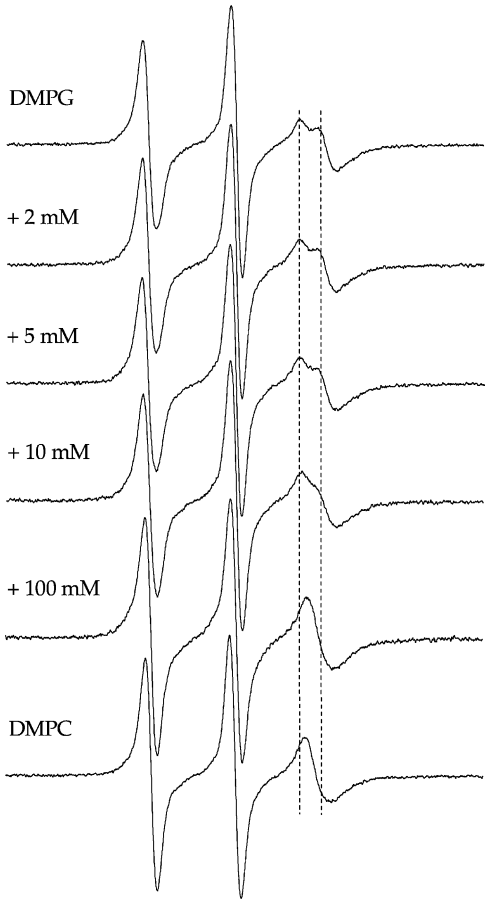

Fig. 2. X-band ESR spectra of 16-PCSL in 10 mM DMPC (bottom spectrum) and in 10 mM DMPG in HEPES buffer at different NaCl concentrations, as indicated, at 25 °C. Total spectrum width 100 G. The dashed lines make evident the two features at high-field position, in low ionic strength DMPG.

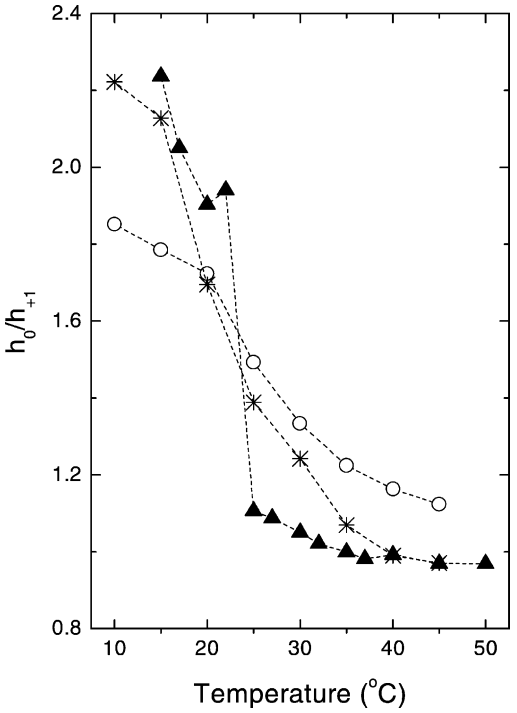

Fig. 3. Temperature dependence of the ratio between the amplitudes of the central and low field resonance lines (h_0/h_{+1}) measured on the ESR spectra of 16-PCSL incorporated in (✳) 10 mM DMPG in HEPES buffer + 2 mM NaCl; (▲) 10 mM DMPG in HEPES buffer + 100 mM NaCl; (○) 10 mM DMPG in HEPES buffer + 2 mM NaCl + 10 mol% α-MSH.

the whole range of temperature studied (results not shown). The disappearance of the two-peak feature in the ESR spectra of 16-PCSL with increasing salt concentration, correlates with the vanishing of the intermediate DMPG phase, seen by DSC and light scattering, and the decrease in the DMPG bilayer surface potential (Riske et al., 1997, 1999, 2001). Fig. 3 shows h_0/h_{+1} measured on the spectra of 16-PCSL in DMPG at low and high ionic strength, at different temperatures. The same trend shown with 14-PCSL (Riske et al., 2001) was obtained: a sharp decrease with temperature in h_0/h_{+1} for the high ionic strength sample at a single temperature ($T_m = 23\,^{\circ}C$), and a gradual decrease in h_0/h_{+1} from T_m^{on} to T_m^{off} for the low ionic strength sample. However, this parameter must be treated with care, because if the spectrum is due to overlapping ESR signals, a changing mixture in the two can be confused with fluidity changes. Between T_m^{on} and T_m^{off}, where the presence of the two peaks might indicate the presence of two different ESR signals, the decrease in h_0/h_{+1} is most likely reflecting a mixture between changes in mobility and in the proportion of these two spin label populations.

The double peak at the high-field position could also be observed with two other spin probes, labeled at the 16th carbon: the stearic acid (16-SASL) and its methyl ether derivative (16-MESL). However, the double peak is not as well defined in the 16-MESL spectra as it is in the 16-PCSL and 16-SASL spectra (Fig. 4). This indicates that the appearance of this double peak is characteristic of a strict spin label depth, since the

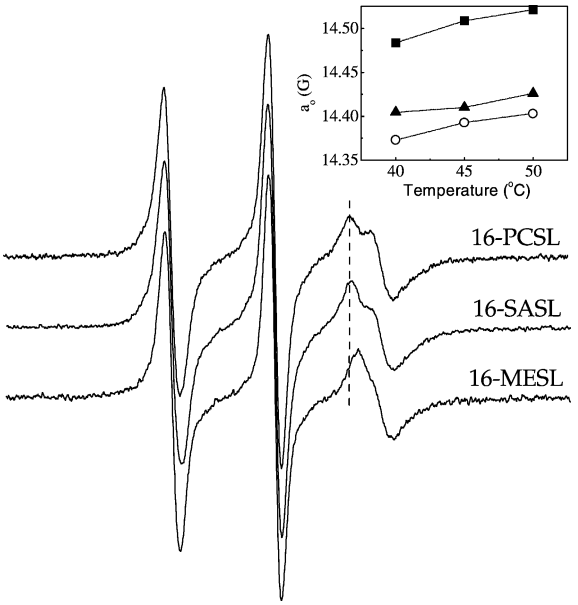

Fig. 4. X-band ESR spectra of probes labeled at the 16th C-atom of the acyl chain of different lipids, in 10 mM DMPG in HEPES buffer + 2 mM NaCl, at 25 °C. Total spectrum width 100 G. Inset: isotropic hyperfine splitting (a_0) directly measured on the ESR spectra, at high temperatures: (▲) 16-PCSL, (○) 16-SASL, and (■) 16-MESL.

relative positions of these three spin labels are slightly different in DMPG bilayers (Turchiello et al., 2000). The difference in bilayer vertical position of the three spin labels can be inferred by the isotropic hyperfine splitting (a_0) measured in the ESR spectra (inset in Fig. 4), which reveals the dielectric constant of the microenvironment of the nitroxide in each label (Griffith et al., 1974). Since the bilayer polarity decreases from the membrane surface to its core, and a greater a_0 value means a higher dielectric constant, it can be concluded that the nitroxide moiety in the 16-PCSL and 16-SASL monitor, on average, rather similar microregions in the bilayer, whereas the methyl ester derivative (16-MESL) monitors a microregion closer to the bilayer surface. Therefore, the presence of the double peak at the high-field position seems to be strongly related to the vertical position of the nitroxide moiety in the bilayer, as it is mainly monitored by 16-PCSL and 16-SASL.

We have been unable to simulate a spectrum yielding two peaks employing a wide range of parameters. This, together with the evidence presented below suggests that the two peaks occur because the spectrum is a composite of two signals arising from the spin probe's sample of distinct regions of the membrane. The occurrence of two different signals would indicate that the spin label is partitioned into two different bilayer microdomains. The fact that two well separated spectra are obtained shows that the exchange rate between the two sites, or the two populations, is slow on the ESR time scale.

It is important to mention that the unusual signal obtained with 16-PCSL in DMPG in the intermediate phase, with the two-peak feature, could not be reproduced by the addition of signals yielded by 16-PCSL in DMPG in the gel and fluid phases, or DMPG with and without NaCl, at any temperature.

3.1. 16-PCSL in DMPG bilayers at different ESR frequencies

In order to get further information about the spectra yielded by 16-PCSL in low ionic strength DMPG dispersions, especially between T_m^{on} and T_m^{off}, the ESR signals were obtained at two other frequencies, 1.3 GHz (L-band) and 35 GHz (Q-band). As the isotropic splitting is nearly independent of the ESR frequency used, and the g values are strongly dependent, the idea was to be able to separate the two possible signals present in the spectra. Fig. 5 compares ESR spectra obtained at the three bands, in low and high salt DMPG dispersions, at 25 and 50 °C. The ESR spectra at the three frequencies at 50 °C (fluid phase for low and high salt conditions) are almost independent of sodium concentration, as has been shown before (Fig. 3, and Fernandez and Lamy-Freund, 2000), and are typical of only one ESR signal. In high salt DMPG dispersions, at 25 °C, the membrane is also in the fluid phase, and the 16-PCSL spectra obtained at the three frequencies are also typical of one ESR signal, related to the spin label monitoring, on average, one microenvironment.

In the gel–fluid transition region (low ionic strength DMPG at 25 °C), it is possible to see the presence of two-peak features in the three bands, although the field positions at which they are well separated are different. In the X-band spectrum, the two peaks are observed in the high-field region; at L-band, in the low- and high-field positions; and at Q-band, in the central- and high-field regions. High frequency ESR

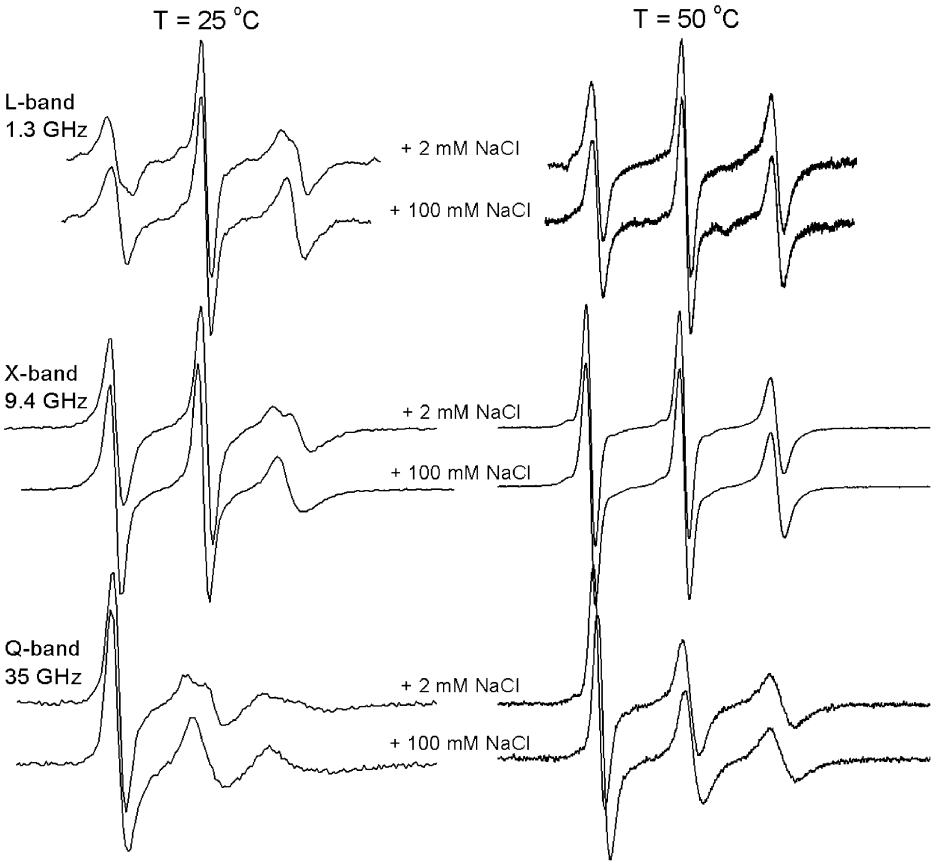

Fig. 5. L-, X- and Q-bands ESR spectra of 16-PCSL in 50 mM DMPG in HEPES buffer + 2 mM NaCl or HEPES buffer + 100 mM NaCl, at 25 and 50 °C. Total spectrum width 70 G (L-band).

(250 GHz) was also tried, but no better resolution was obtained, possibly due to *g*-strain and the slow motion of the probe relative to that frequency.

The presence of two peaks raises the possibility that the spectrum is the superposition of spectra arising from labels residing in different microenvironments, although no clear separation of two such spectra could be achieved at any frequency. In an attempt to separate possible overlapping spectra, the L-band spectrum at 25 °C was analyzed as if it was a sum of two signals in the motional narrowing regime using three Lorentzian hyperfine lines. The L-band spectrum was used because spectral effects due to slow motion are minimized at low frequencies. Moreover, at this frequency, the two-peak features are seen in both low- and high-field positions (Fig. 5, top spectrum at 25 °C), making the spectrum analysis more straightforward. In fact, a satisfactory simulation of the spectrum could be achieved as shown in Fig. 6. Nevertheless, it is very unlikely that the simulated spectrum corresponds to true overlapping spectra because of two facts. First, the doubly-integrated intensities of each of the three hyperfine lines were found to be different. Second, one of spectra presents a rather unusual a_0 value for a doxyl ring, 12.55 G, well below the value of approximately 14 G observed even in pure hydrocarbon environment.

Therefore, as a second attempt, the 16-PCSL ESR spectra obtained at 25 °C were simulated, for the three microwave bands, using the methodology developed by Dr. Freed's group (see Section 2), which has no restriction to rate or amplitude of probe movement. The same fitting parameters were used for the three frequencies. Firstly, it was assumed that the spectra were due to one ESR signal only. In that case, the

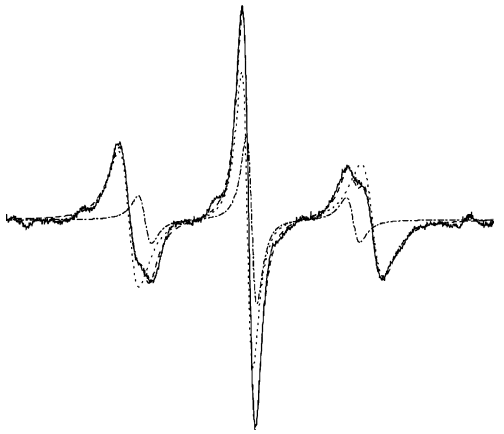

Fig. 6. A tentative interpretation of the L-band 16-PCSL ESR spectrum in 50 mM DMPG in HEPES buffer + 2 mM NaCl, at 25 °C (—) as the sum (- - -) of two signals (-·-·- and ·····) with Lorentzian line shapes.

two-peak feature, present in different field positions for the three bands, was found to be poorly defined in the simulations (not shown). Secondly, a two sites simulation was tried, considering that the spectra were a result of two different 16-PCSL populations, monitoring two different bilayer microregions. Although no better fittings were obtained (not shown), that could be related to the limitations in the theoretical simulation used, particularly, to the impossibility of simultaneously simulating two sites with different g_0 values. On the other hand, the spectra obtained in the presence of high ionic strength (100 mM NaCl, in Fig. 5) could be well simulated as a one-site signal, at the three frequencies (not shown).

3.2. 16-PCSL in DMPG bilayers in the presence of the peptide α-MSH

Our group has been working with the cationic peptide α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) for some time. Using spin labels at different bilayer positions, we have shown that this peptide interacts with low ionic strength DMPG bilayers, changing the membrane packing and altering the amount of water molecules in the bilayer core (Biaggi et al., 1997; Fernandez and Lamy-Freund, 2000). Tryptophan fluorescence and its suppression by nitroxide moieties positioned at different bilayer depths have shown that α-MSH partially penetrates

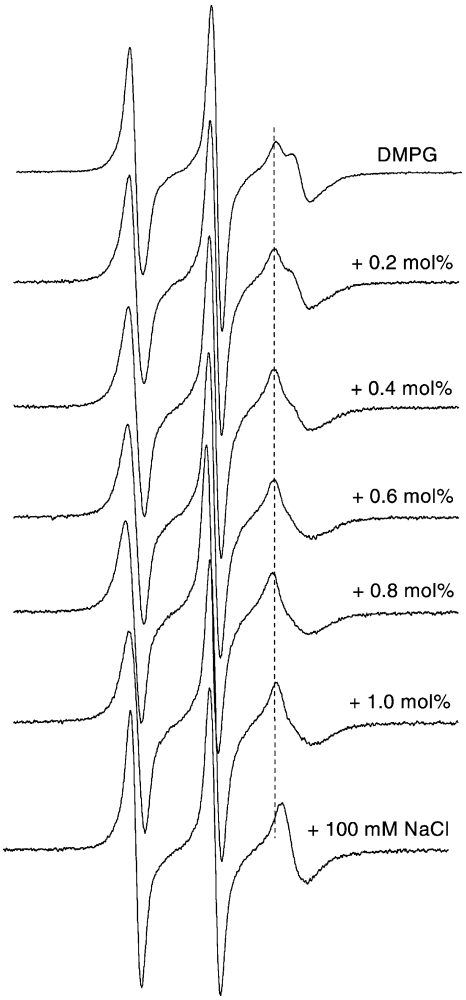

Fig. 7. X-band ESR spectra of 16-PCSL in 10 mM DMPG in HEPES buffer + 2 mM NaCl, with different concentrations of α-MSH, at 25 °C. Total spectrum width 80 G.

DMPG bilayers (Ito et al., 1993; Macêdo et al., 1996). Therefore, it was rather interesting to find that α-MSH changes the ESR spectra of 16-PCSL incorporated in DMPG in the intermediate phase, leading to the disappearance of one of the high-field peaks. Fig. 7 shows that increasing amounts of peptide make the highest field peak gradually disappear. This is a strong evidence that the two-peak features, characteristic of the transition region, reflects the presence of two different ESR signals, one of which being abolished by the presence of the peptide. It is important to point out that addition of salt ([NaCl] above 10 mM) also yields

the presence of one high-field peak only. However, this peak is located between the two peaks present in the high-field region of low ionic strength DMPG (see Fig. 7 bottom spectrum for 100 mM NaCl).

The ESR spectra obtained with 16-PCSL in low ionic strength DMPG dispersions in the presence of 10 mol% α-MSH could be simply analyzed through the ratio of the amplitudes of the central and low field lines (h_0/h_{+1}), considering the presence of one ESR signal only. Fig. 3 shows that, on average, α-MSH increases the bilayer fluidity in the DMPG gel phase (diminishes h_0/h_{+1}), and makes the membrane more packed for temperatures above 25 °C. A similar behavior was obtained with other spin labels (Biaggi et al., 1997; Fernandez and Lamy-Freund, 2000).

However, a more detailed analysis of the effect of α-MSH on the DMPG bilayer is necessary. Considering that the presence of the cationic peptide at the bilayer surface removes one of the high-field peaks, it could be assumed that the peptide eliminates one of the two ESR signals present in the DMPG gel–fluid transition region. Accordingly, the spectrum obtained with 16-PCSL incorporated in DMPG bilayers at the transition region (low ionic strength, 25 °C) was subtracted from that obtained with DMPG + α-MSH, at the three frequencies. Fig. 8 (bottom spectra) show the spectra obtained after subtracting a weighted DMPG + α-MSH signal (Fig. 8, middle spectra) from the composite one (Fig. 8, top spectra). The weight of the DMPG + α-MSH signal was varied until the resulting spectrum looked like a one-component signal (bottom spectra in Fig. 8). This subtraction proceeds by trial and error, judging by eye the quality of the resultant spectrum. For the three frequencies, the resultant spectrum corresponds to the same percentage of the original DMPG spectrum (34%).

The resultant spectra, at the three frequencies, are typical of a spin label in a rather mobile environment, and could be well simulated as a one-component signal, with the same parameters at the three frequencies (see dashed lines in Fig. 8, bottom spectra, and parameters in Table 1). The spectra obtained with DMPG + α-MSH dispersions could also be well simulated as one-component spectrum, at the three frequencies (see dashed lines in Fig. 8, middle spectra, and Table 1). The fact that the subtraction was successful, and consistent in the three bands, is a rather strong indication of the existence of two different ESR signals along the DMPG thermal transition region.

Table 1
Parameters for the best non-linear least-squares[a] fitting of the 16-PCSL ESR spectra shown in Fig. 8, for the three frequency bands

	DMPG + α-MSH	Difference
$A_\perp$ (G)	5.1	5.3
A_{zz} (G)	33.0	34.4
a_0[b] (G)	14.40	15.00
a_0[c] (exp) (G)	14.60	15.04
$\tau_\perp$ (ns)	1.13	0.68
$\tau_{//}$ (ns)	0.003	0.80
c_{20}	−0.60	0.12
c_{22}	0.08	0.20

[a] For the discussion of the simulation program used see Section 2.
[b] $a_0 = (2A_\perp + A_{zz})/3$.
[c] Average values directly measured on the ESR spectra between 40 and 50 °C.

At 25 °C, the resultant spectrum, corresponding to the population removed by the presence of α-MSH, presents *high* mobility and *low* order (low rotational correlation times, $\tau_\perp$ and $\tau_{//}$, and orienting potential coefficients, c_{20} and c_{22}, in Table 1), in accordance with a nitroxide in a quite fluid microenvironment (fluidity similar to that of 16-PCSL in DMPG at 35 °C). However, the obtained a_0 value (15.00 G, in Table 1) is higher than that yielded by a nitroxide bound to the 16th carbon atom (14.40–14.60 G, Table 1 and Fernandez and Lamy-Freund, 2000), lower than the value obtained for free spin label in aqueous medium (15.78 G), being similar to the value obtained for 5-PCSL incorporated in DMPG. Therefore, the resultant spectrum a_0 value is consistent with a nitroxide at the 5th carbon atom position of a DMPG bilayer, though its mobility/disorder are incompatible with this rather ordered bilayer microenvironment (Fernandez and Lamy-Freund, 2000).

Based on the above discussion, the ESR signal obtained with 16-PCSL in DMPG bilayers in the transition region can be interpreted as a composite spectrum, corresponding to the presence of spin labels in two structurally different domains, with different microviscosities and polarities. One domain would be relatively packed and hydrophobic, well suitable for a nitroxide moiety in a bilayer core, and the other one, rather fluid and fairly hydrated, unusual for any depth position in a bilayer. It is important to point out

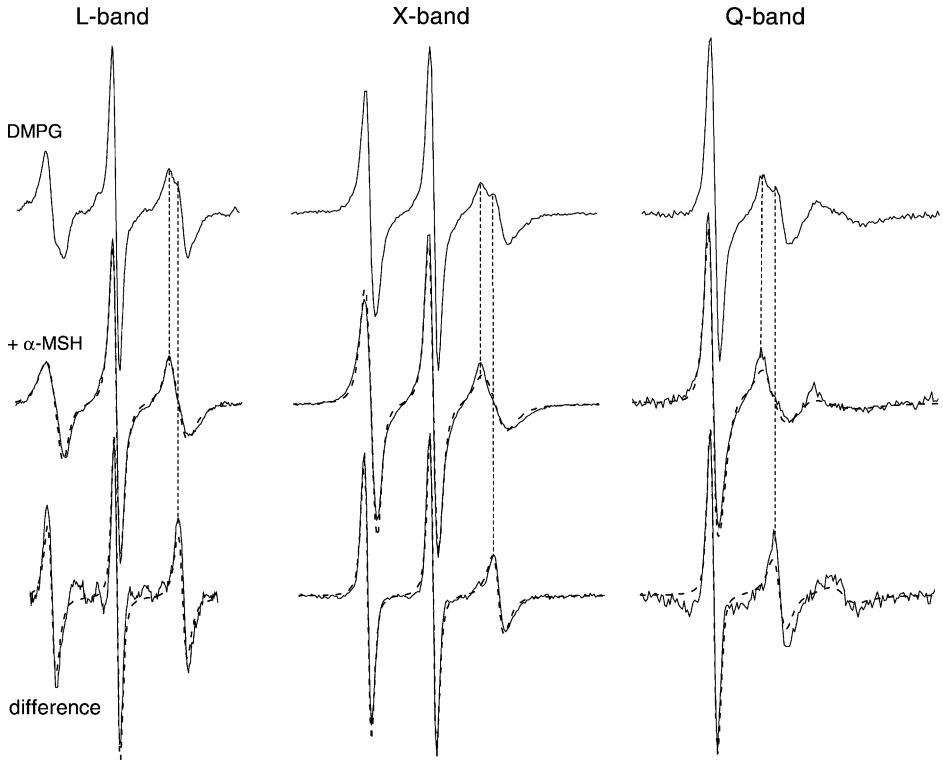

Fig. 8. L-, X- and Q-bands ESR spectra of 16-PCSL in 50 mM DMPG in HEPES buffer + 2 mM NaCl (top spectra); HEPES buffer + 10 mol% α-MSH (middle spectra); top − middle spectra (bottom spectra, difference). $T = 25\,^{\circ}C$. Total spectrum width 70 G. The dashed lines are the spectra best theoretical simulations (parameters in Table 1).

that although the other spin labels (5- to 14-PCSL) could also be partitioned between the two different sites, two distinct signals would only be detected with 16-PCSL due to the faster movement of the nitroxide moiety in this probe, therefore to its ESR narrower lines. The presence of the cationic peptide α-MSH would then change the DMPG polymorphism, nearly extinguishing the more fluid domains.

The coexistence of gel and fluid domains has been observed in other lipid systems, over a short range of temperature (Benatti et al., 2001; Bagatolli and Gratton, 1999). With giant vesicles, it has been suggested that over the chain melting temperature interval there are membrane deformations, with rigid, flat gel domains coexisting with highly curved, fluid patches (Bagatolli and Gratton, 1999). DMPG seems to be a rather peculiar system, due to its relatively short hydrocarbon chains, 14 carbon atoms, and to its charged phosphate groups. There is some cooperative process that happens at T_m^{on}, measured by different techniques, including DSC, but the melting of the acyl chains seems to be only complete at T_m^{off}. For the samples used here, 10 mM DMPG in HEPES buffer + 2 mM NaCl, there seems to be a coexistence of lipid phases over a large temperature interval (17 °C), between T_m^{on} and T_m^{off}.

Alternatively, instead of two different membrane domains, the two signals could correspond to two different sites in a homogeneous membrane where the doxyl moiety could reside. This would only be feasible in the intermediate phase, with rather unusual bilayer packing. For instance, 16-PCSL could be present in two different conformations, with the signal corresponding to high mobility and polarity indicating a spin label population with a bending in the acyl chain to which the nitroxide is attached. As nitroxides are more polar than the paraffin region, it is reasonable to assume that a chain with a nitroxide at

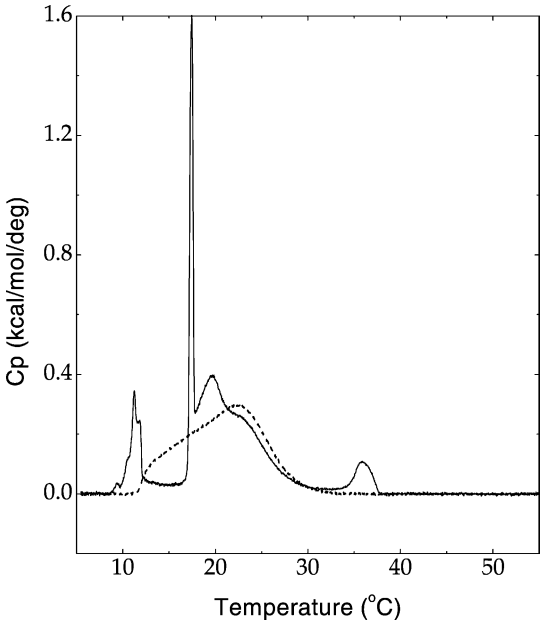

Fig. 9. DSC traces of (—) 10 mM DMPG in HEPES buffer + 2 mM NaCl and (- - -) 10 mM DMPG in HEPES buffer + 2 mM NaCl + 10 mol% α-MSH.

its end could bend so as the nitroxide would reside in a shallower position. However, a signal with high mobility is unlikely to be associated with a N-O around the 5th C-atom position, as indicated by the signal a_0 value (Table 1). Moreover, a quick exchange between the two possible sites in the same membrane region would be expected, resulting in an average ESR signal.

The α-MSH effect on low ionic strength DMPG bilayers can also be monitored via the huge effect the peptide causes on the lipid DSC profile (Fig. 9): it completely eliminates the more cooperative peaks. The ESR parameters also indicate a much less cooperative process for DMPG + α-MSH gel–fluid transition (see Fig. 3).

4. Conclusions

With spin probes labeled at the acyl chain end we have observed a two-peak spectrum intimately associated with the DMPG intermediate phase. Neither the hypothesis of a single odd 16-PCSL ESR signal, nor the possibility of two different spin label populations residing in an unusual homogeneous bilayer packing in the intermediate phase can be completely ruled out. However, based on the arguments presented here, we conclude that the 16-PCSL spectrum yielded by the probe incorporated in DMPG in the intermediate phase is most likely due to the overlap of two spectra. Therefore, we propose a model where rather fluid and hydrated domains, possibly high curvature regions, coexist with patches that are more rigid and hydrophobic (not necessarily in a gel state), along the DMPG intermediate phase large temperature interval. The DSC first cooperative peak would be related to some thermal process, a result of the balance between the charged headgroup repulsion and the acyl chains van der Waals attraction, possibly some initial acyl chain kink, associated with the appearance of curved regions in the DMPG bilayer. Only above T_m^{off} the membrane would achieve the fluid phase, with a homogeneous surface. It is still not clear why α-MSH would eliminate the presence of the highly curved fluid domains, making the vesicle surface more homogeneous, though it has been observed that peptides can change the membrane polymorphism (Epand, 1997). It would certainly be interesting to find out whether this is a specific effect of α-MSH, or if it is related to any molecule that partially penetrates the DMPG bilayer.

Acknowledgements

This work was supported by USP, FAPESP and CNPq. B.L.B. acknowledges support NIH/MBRS S06 GM48680-03. The research group of Dr. J. Freed is acknowledged for the 250 MHz experiment. We thank Dr. M. Peric for the initial efforts in running the ESR simulation programs.

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