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Effects of dirhamnolipid on the structural properties of phosphatidylcholine membranes

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Abstract

Rhamnolipids are biosurfactants produced by *Pseudomonas aeruginosa* which are well known for their potential industrial and environmental uses. Rhamnolipids have gained considerable interest in recent years due to their potential use in cosmetics and pharmaceutics. They also show broad biological activities and have potential applications as therapeutic agents. The amphiphilic nature of rhamnolipids points to the membrane as their hypothetical site of action. We have purified dirhamnolipid and studied its interaction with phosphatidylcholine membranes, using differential scanning calorimetry, X-ray diffraction and infrared spectroscopy. It has been found that dirhamnolipid greatly affects the gel to liquid crystalline phase transition of phosphatidylcholines, broadening and shifting the transition to lower temperatures. Dirhamnolipid increases the interlamellar repeat distance of phosphatidylcholines and reduces the long-range order of the multilamellar systems. The phospholipid hydrocarbon chain conformational disorder is increased and the packing of the phospholipid molecules is perturbed in the presence of dirhamnolipid. The above evidence supports the idea that dirhamnolipid intercalates into the phosphatidylcholine bilayers and produces structural perturbations which might affect the function of the membrane.

Keywords: Dirhamnolipid; Model membranes; DSC; X-ray diffraction; Infrared spectroscopy

1. Introduction

Biosurfactants constitute a diverse group of surface-active molecules synthesized by microorganisms. *Pseudomonas aeruginosa* produces rhamnose containing glycolipids also called rhamnolipids when grown on a number of water immiscible substrates (Benincasa et al., 2002). The structure of the rhamnose containing glycolipids produced by the genus *Pseudomonas* has been widely studied (Sim et al., 1997; Desai and Banat, 1997; Lang and Wagner, 1993). The surfactant properties of rhamnolipids are well documented. They reduce the surface tension of water and the interfacial tension of water/oil systems (Lang and Wullbrandt, 1999). Rhamnolipids have gained considerable interest due to their low toxicity, biodegradable nature and diversity. Their range of potential industrial applications includes enhanced oil recovery, crude oil drilling, lubricants and bioremediation of water insoluble pollutants (Banat et al., 2000).

Besides the environmental and industrial use of rhamnolipids, a significant potential application is emerging for them as fine and specialty chemicals (Ishigami, 1997). In this regard, the use of rhamnolipids as emulsifiers, penetrating agents and drug delivery systems in cosmetics and pharmaceutics is a great developing area of research. Rhamnolipids show a great variety of biological activities as they have been shown to have antimicrobial action (Itoh et al., 1971; Benincasa et al., 2004), antiphytoviral effect (Haferburg et al., 1987) and zoosporicidal activity (Stanghellini and Miller, 1997). There is increasing interest in the effect of rhamnolipids on human and animal cells

Abbreviations: DiRL, dirhamnolipid; DMPC, 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; SAXD, small angle X-ray diffraction; WAXD, wide angle X-ray diffraction

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and cell lines. Rhamnolipids have been shown to affect cellular immunosuppresion (Piljac and Piljac, 1995b), and to display differential effects on human keratinocytes and fibroblast cultures (Stipcevic et al., 2005). Very recently, endotoxin-like properties have been described for rhamnolipids (Rademann et al., 2006). Rhamnolipids have also been assayed in the treatment of psoriasis (Piljac and Piljac, 1995a) and in the process of cutaneous wound healing (Stipcevic et al., 2006).

Considering the amphiphatic nature of rhamnolipids, membranes are expected to be critical not only as point of entry to the cell but also as their hypothetical site of action. Despite the importance that the interaction between rhamnolipids and membranes might play in their biological mechanism of action, very little is known especially regarding rhamnolipids phospholipids molecular interactions. We have very recently shown that dirhamnolipid has a bilayer stabilizing effect, impeding formation of the inverted hexagonal-H_{II} phase of dielaidoylphosphatidylethanolamine (Sánchez et al., 2006). Rhamnolipids are produced as a mixture, and the ratio and composition of homologues, the length of the alkylic chain, and the size of the hydrophilic headgroup can affect their properties. It is important to elucidate the individual contribution of each rhamnolipid homologue to the biological activities in order to understand their mechanism of action and to obtain a rhamnolipid product with desirable properties for specific uses.

We produced rhamnolipid from *P. aeruginosa* and purified and isolate dirhamnolipid (DiRL). DiRL has a complex structure (Fig. 1), the carboxylate group gives DiRL its anionic character, two rhamnosyl groups contribute to the bulky hydrophilic part of their structure, and two hydrocarbon chains constitute its hydrophobic part. In order to understand the influence of DiRL on the lipid component of membranes we made a detailed study of the effect of DiRL on the thermotropic and structural properties of phosphatidylcholine, the most important phospholipid in eucaryotic membranes, using differential scanning calorimetry (DSC), small and wide angle X-ray diffraction (SAXD and WAXD) and infrared spectroscopy.

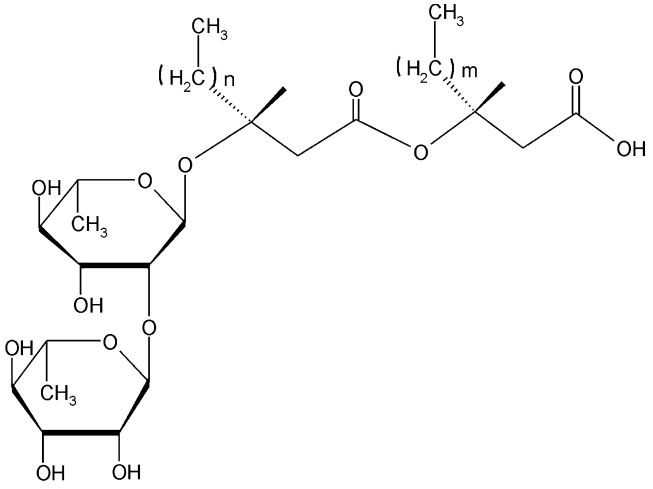

Fig. 1. The chemical structure of the DiRL compounds produced by *P. aeruginosa*. For Rha-Rha-C_{10}-C_{10}: m, $n = 6$ and for Rha-Rha-C_{10}-C_{12}: $m = 8$ and $n = 6$.

2. Materials and methods

2.1. Materials

1,2-Dimiristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). All other reagents were of the highest purity available.

2.2. Bacterial strain and culture conditions

Biosurfactant producer, strain 47T2 (NCIB 40044), was isolated from contaminated soil samples from Barcelona (Spain) and was selected for its capacity to accumulate surface active rhamnolipids from hydrophobic substrates (Robert et al., 1989). This strain was maintained by forthright cultures and preserved in cryovials at −20 °C (Combourg, France). From previous morphological and biochemical tests the isolate was identified as *P. aeruginosa* (Haba et al., 2000). Strain 47T2 produces rhamnolipids when grown in mineral medium of the following composition ($g\,l^{-1}$): $NaNO_3$ 4.64; K_2HPO_4/KH_2PO_4 (ratio 2:1) 1; $CaCl_2$ 0.01; KCl 0.1; $MgSO_4{\cdot}7H_2O$ 0.0074; yeast extract 0.1 $ml\,l^{-1}$; and supplemented with 0.05 $ml\,l^{-1}$ of a trace mineral solution containing ($g\,l^{-1}$): H_3BO_3 0.26; $CuSO_4{\cdot}5H_2O$ 0.5; $MnSO_4{\cdot}H_2O$ 0.5; $MoNa_2O_4{\cdot}2H_2O$ 0.06; $ZnSO_4{\cdot}7H_2O$ 0.7. As carbon source 50 $g\,l^{-1}$ of waste fried oil (1:1, v/v olive/sunflower), which was mainly composed of $C_{18:1}$ (74.4%), $C_{18:2}$ (20%), $C_{16:0}$ (2.7%) and $C_{18:0}$ (1.4%) was used. The strain was incubated at 30 °C and 120 rpm in a reciprocal shaker.

2.3. Dirhamnolipid recovery and purification

Rhamnolipids were recovered from the culture supernatant as follows. Cells were removed from the culture by centrifugation at 8000 × g in a Kontron centrifuge (Milano, Italy) for 20 min at 15 °C. Purification of rhamnolipids was achieved, following a modification of the method of Reiling et al. (1986), by adsorption chromatography on a polystyrene resin, Amberlite XAD2 (Sigma, St. Louis, USA). The resin (60 g) was placed in a glass column (60 cm × 3 cm), yielding a bed volume of 200 ml. The column was equilibrated with 0.1 M phosphate buffer, pH 6.1. The culture supernatant was acidified to pH 6.1 and applied through a sieve placed on top of the resin to prevent whirling up. The adsorption of the active compounds on the resin was monitored measuring the surface tension (γ_{st}) of the column outlet. The saturation of the resin was determined when γ_{st} of the effluent dropped below 40 mN/m. Thereafter, the column was rinsed with three volumes of distilled water until γ_{st} of the effluent approached 72 mN/m, at which no free fatty acids remained in the column. Then, biosurfactants were eluted with methanol and, finally, the solvent was evaporated under vacuum. To check the purity of the extract, i.e. the absence of residual fatty acids, a 5 mg aliquot was resuspended in methanol and analysed by HPLC–MS (Abalos et al., 2001).

The DiRL component was separated from the total mixture as follows. A slurry of silicagel 60 in chloroform was poured onto

a glass chromatography column (2 cm × 40 cm). Two grams of the crude rhamnolipid mixture was dissolved in 4 ml of chloroform and loaded onto the column. The column was washed, at a flow rate of ca. 1 ml min^{-1}, with chloroform until neutral lipids were totally eluted, followed by chloroform/methanol 50:3 and 50:5 (which eluted the monorhamnolipid component), and chloroform/methanol 50:50 and pure methanol (to elute the DiRL component). The composition of the fractions was checked by thin layer chromatography on silica gel plates using chloroform/methanol/H_2O (65:15:2) as mobile phase. The purified DiRL component showed a single spot by thin-layer chromatography. DiRL was quantified by weighing after extensive desiccation under high vacuum until constant weight. The chemical characterization of the compounds were confirmed by ES–MS as previously described (Haba et al., 2003), and it consists mainly of Rha-Rha-C_{10}-C_{10} (ca. 50%) and Rha-Rha-C_{10}-C_{12} (ca. 29%), with small contributions of other three minor species.

2.4. Differential scanning calorimetry

The lipid mixtures for DSC measurements were prepared by combination of chloroform/methanol (1:1) solutions containing 4 μmol of phospholipid and the appropriate amount of DiRL as indicated. The organic solvents were evaporated under a stream of dry N_2, free of O_2, and the last traces of solvents were removed by a further 3 h evaporation under high vacuum. Multilamellar liposomes were prepared in 50 μl of 0.1 mM EDTA, 100 mM NaCl, 10 mM Hepes (pH 7.4) buffer by mixing, using a bench mixer, always keeping the samples at a temperature above the gel to liquid-crystalline phase transition temperature of the phospholipid. Samples were placed into small aluminium pans. Pans were sealed and scanned in a Perkin-Elmer DSC-7 calorimeter, using a reference pan containing buffer. The heating rate was 4 °C/min in all the experiments. In order to normalize the thermograms to the same amount of phosphatidylcholine, the total phospholipid contained in a pan was measured as follow: the pan was carefully opened, the lipid was dissolved with chloroform:methanol (1:1) and the phosphorous contents were determined using the method of Böttcher et al. (1961). The instrument was calibrated using indium as standard. The construction of partial phase diagrams was based on the heating thermograms for a given mixture of phospholipid and DiRL at various DiRL concentrations. The onset and completion temperatures for each transition peak were plotted as a function of the mole fraction of DiRL. These onset and completion temperatures points formed the basis for defining the boundary lines of the partial temperature-composition phase diagram.

2.5. X-ray diffraction

Simultaneous SAXD and WAXD measurements were carried out as described previously (Teruel et al., 2004) using a modified Kratky compact camera (MBraum-Graz-Optical Systems, Graz Austria), which employs two coupled linear position sensitive detectors (PSD, MBraum, Garching, Germany). Nickel-filtered Cu Kα X-rays were generated by a Philips PW3830 X-ray Generator operating at 50 kV and 30 mA. Samples for X-ray diffraction were prepared by mixing 15 mg of phospholipids and the appropriate amount of DiRL in chloroform/methanol (1:1). Multilamellar vesicles were formed as described above. Samples were measured in a thin-walled Mark capillary held in a steel cuvette, which provides good thermal contact to the Peltier heating unit. X-ray diffraction profiles were obtained for 10 min exposure times after 10 min of temperature equilibration.

2.6. Infrared spectroscopy

For the infrared measurements, multilamellar vesicles were prepared in 40 μl of D_2O as described above. Samples were placed in between two CaF_2 windows (25 mm × 2 mm) separated by 50 μm Teflon spacers and transferred to a Symta cell mount. Infrared spectra were obtained in a Nicolet MX-1 FT–IR spectrometer. Each spectrum was obtained by collecting 27 interferograms. Subtraction of D_2O spectra taken as the same temperature was performed interactively using either GRAMS/32 or Spectra-Calc (Galactic Industries, Salem, MA).

3. Results and discussion

3.1. Differential scanning calorimetry

In this study, the nature of the interaction between DiRL and membranes has been investigated using lipid vesicles formed by phosphatidylcholines. DSC was used to characterize the influence of DiRL on the thermotropic properties of phosphatidylcholines of different acyl chain lengths, and the effect of DiRL on the macroscopic organization and structural properties of the phospholipids was studied by means of X-ray diffraction and infrared spectroscopy.

The influence of DiRL on the thermotropic gel to liquid crystalline phase transition of saturated phosphatidylcholines bearing acyl chains with 14 (DMPC), 16 (DPPC) and 18 (DSPC) carbon atoms is depicted in Fig. 2. In the absence of DiRL, phosphatidylcholines exhibited two endotherms upon heating: a lower temperature lower enthalpy pretransition and a higher temperature higher enthalpy main transition. In the pure phospholipids thermograms, the higher temperature tall narrow peaks correspond to the chain melting transition (Lee, 1983) and the lower temperature small peaks correspond to the pretransition which related to the untilting of the phospholipids acyl chains (Lewis et al., 1997). These transitions appeared respectively at 13 and 23.6 °C for DMPC, at 35 and 41 °C for DPPC, and at 49 and 54.3 °C for DSPC in agreement with previous results (Marsh, 1990; Chicano et al., 2001). The thermotropic pretransition of the different phosphatidylcholines was greatly affected by the presence of a very low concentration of DiRL, being already abolished at a DiRL mole fraction of 0.01. Increasing concentrations of DiRL progressively made the transition less cooperative as demonstrated by the increasing in width of the main transition and caused a shift to lower temperatures with the appearance of a second endothermic component in the thermograms. The effect of DiRL on the main phase transition was qualitatively similar for the different phosphatidylcholines, however it was

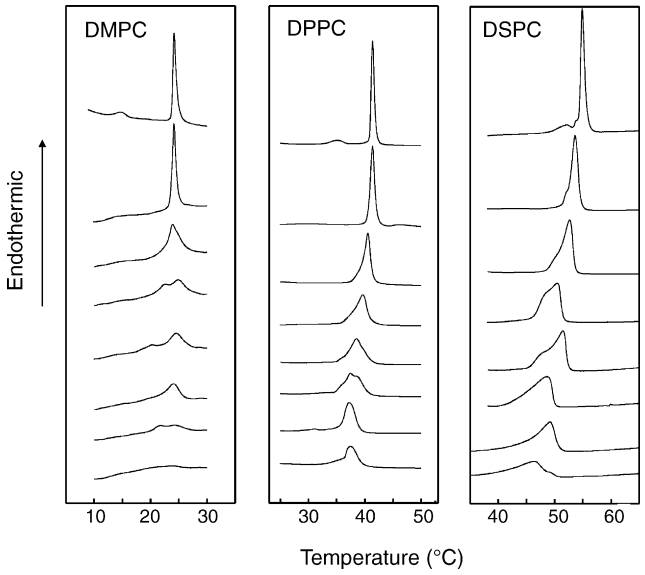

Fig. 2. DSC heating thermograms for DMPC (left), DPPC (center) and DSPC (right) containing DiRL at different concentrations. Molar fraction of DiRL from top to bottom: 0, 0.01, 0.03, 0.05, 0.07, 0.10, 0.15 and 0.20. Thermograms were normalized to the same amount of phospholipid.

larger in the case of the shorter homologue DMPC where the broadening of the transition and the separation between both endotherms were more evident. These effects could be explained by the establishment of a molecular interaction between the phospholipid acyl chains and the DiRL molecule. This interaction would be the consequence of the intercalation of the DiRL molecule between the phospholipids. The alignment of the hydrocarbon chains of DiRL with the phospholipids acyl chains can disrupt the phospholipid packing, reduce the cooperativity of the transition and shift the phase transition temperature to lower values. This is the expected behaviour for a molecule that penetrates into the hydrophobic core of the bilayer and disrupts phospholipid–phospholipid contact as has been described before for other hydrophobic molecules like the toxicant abietic acid (Aranda and Villalaín, 1997) or the antimicrobial peptide LL-37 (Henzler-Wildman et al., 2004). The appearance of a second melting component in the thermograms when the concentration of DiRL is increased can be explained by the formation of DiRL enriched domains. At increasing concentrations of DiRL the shape of the main transition peak become asymmetric. The asymmetric line shape indicates that the phase transition is no longer two-state or there are multiple two-state transitions (Lee, 1983). Making the reasonable assumption that there are two phases that coexist and each undergo a two-state transition, the lower temperature endotherm can attributed to phospholipids in DiRL rich regions or those phospholipids that are near the DiRL molecules and are highly perturbed. The higher temperature endotherm can be attributed to lipids in DiRL poor regions or are far away from the DiRL molecules and thus have less perturbed acyl chains. Similar domain formation has been recently described for antimicrobial peptide LL-37 in phosphatidylcholine systems (Henzler-Wildman et al., 2004). The carboxylate group and the two rhamnosyl groups which form the bulky hydrophilic part of the DiRL structure will tend to locate near the water interface, where they could interact with the polar part of the phosphatidylcholines in a similar way as it has been described for its interaction with phosphatidylethanolamines (Sánchez et al., 2006).

3.2. Small angle X-ray diffraction

Information on the structural characteristics of phosphatidylcholine/DiRL systems was obtained by SAXD. Phospholipids, when organized into multilamellar structures, should give rise to reflections with relative distances of 1:1/2:1/3... (Luzzati, 1968). Fig. 3 shows the small angle X-ray diffraction pattern profiles corresponding to pure phosphatidylcholines and phosphatidylcholines containing DiRL at different temperatures. Pure phosphatidylcholines produced three reflections with relative distances of 1:1/2:1/3, which is consistent with their expected multilamellar organization (Seddon and Cevc, 1993).

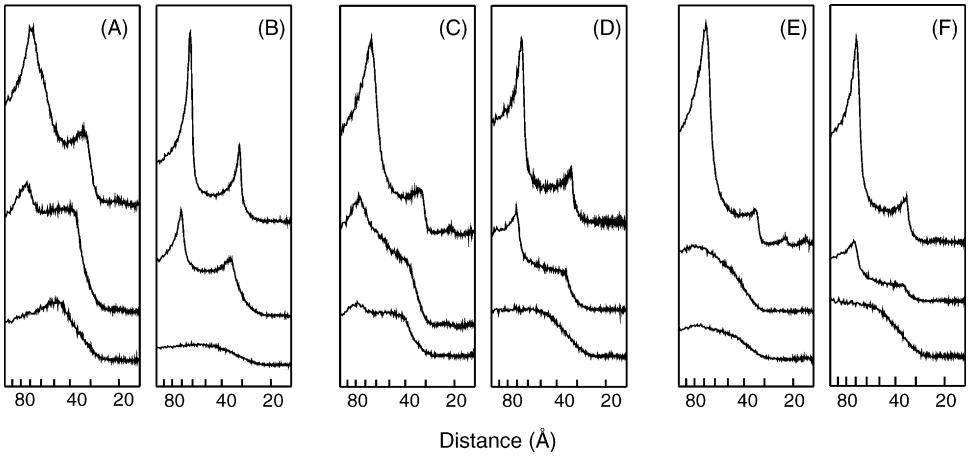

Fig. 3. Small angle X-ray diffraction profiles of phosphatidylcholine systems containing different concentration of DiRL in the gel and the liquid crystalline phase. From top to bottom: pure phosphatidylcholine, phosphatidylcholine containing 0.03 mol fraction DiRL, phosphatidylcholine containing 0.07 mol fraction DiRL. (A) DMPC/DiRL at 10 °C; (B) DMPC/DiRL at 30 °C; (C) DPPC/DiRL at 25 °C; (D) DPPC/DiRL at 50 °C; (E) DSPC/DiRL at 40 °C; (F) DSPC/DiRL at 60 °C.

This technique not only defines the macroscopic structure itself, but also provides the interlamellar repeat distance in the lamellar phase. The largest first-order reflection component corresponds to the interlamellar repeat distance (*d*-value), which is comprised of the bilayer thickness and the thickness of the water layer between bilayers (Rappolt et al., 2003). DMPC gave rise to a first-order reflection with a *d*-value of 66.3 Å in the gel state (Fig. 3A) and 62.0 Å in the liquid crystalline state (Fig. 3B). Observed *d*-values in the gel and liquid crystalline state were 64.0 and 65.1 Å for DPPC (Fig. 3C and D) and 67.5 and 68.7 Å for DSPC (Fig. 3E and F), in agreement with literature results (Marsh, 1990). Samples containing 0.03 mol fraction of DiRL gave rise to two or three reflections which related as 1:1/2:1/3 in the whole range of temperatures under study, confirming that the presence of DiRL at this concentration does not alter the lamellar structural organization of phosphatidylcholines. However, the interlamellar repeat distance was found to be between 5 and 13 Å (depending on phosphatidylcholine acyl chain and temperature) larger in the presence of 0.03 mol fraction DiRL than in the absence of glycolipid, which can be a consequence of the increase of the water layer between the phospholipids bilayers or it can be due to the effective increase of the bilayer thickness. It is interesting to note that the presence or DiRL broadened the reflections and lowered their intensities. This was more evident when the concentration of DiRL was increased to 0.07 mol fraction. In this case, no sharp reflections but only broad scattered bands were observed indicating that the presence of DiRL reduces the long-range order in the multilamellar system. Similar loss of order in the multilamellar phosphatidylcholine system has been described for other amphiphilic molecules like triorganotin compounds (Chicano et al., 2001).

The properties of binary mixtures of compounds can be presented in the form of phase diagrams (Lee, 1977). We used DSC data and information of the phosphatidylcholine structural organization obtained from SAXD to construct phase diagrams of the phospholipids/DiRL mixtures. As expected for a molecule with short hydrocarbon chains, pure DiRL did not show any detectable thermotropic phase transition in the range of temperature under study, and hence the constructed phase diagrams were partial phase diagrams for the phosphatidylcholine component (observed transitions) in mixtures of phospholipid and DiRL. The construction of the phase diagrams were based on the heating thermograms shown in Fig. 2 for the various phosphatidylcholine/DiRL mixtures at different relative compositions. The onset and completion temperatures for each transition peak were plotted as a function of the molar fraction of DiRL. The lines connecting the onset and completion temperatures are the solidus and liquidus, respectively. When DiRL is incorporated into phosphatidylcholine systems, it will change the transition temperature of the phospholipid, if both types of molecules are miscible. The phase diagrams presented in Fig. 4, for all phosphatidylcholines under study, show that the solidus lines display a near ideal behaviour, i.e. the temperature decreases as more DiRL was present in the systems. This indicates that phosphatidylcholine and DiRL are miscible in the gel phase, and that the intercalation of DiRL molecules in the phospholipids palisade perturbs its thermotropic properties.

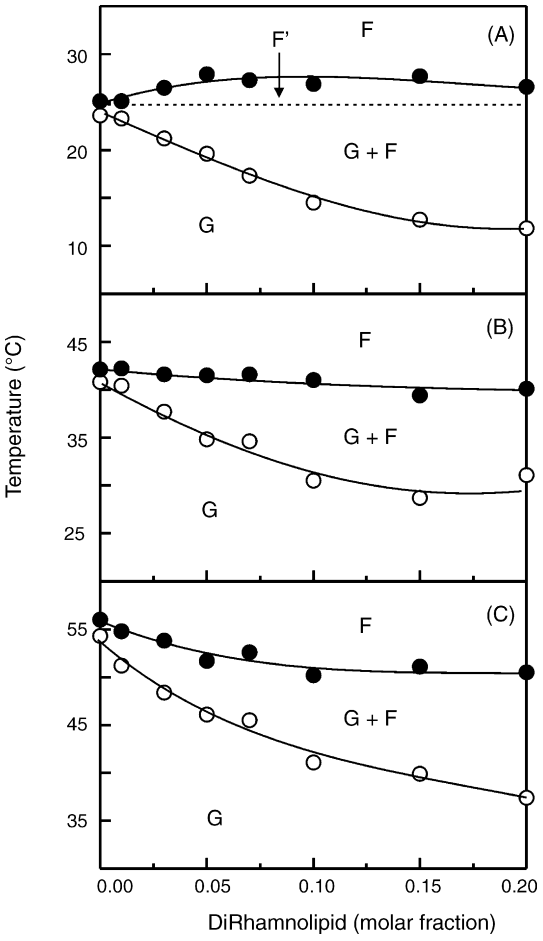

Fig. 4. Partial phase diagrams for phosphatidylcholine in phosphatidylcholine/DiRL mixtures. (A) DMPC/DiRL; (B) DPPC/DiRL; (C) DSPC/DiRL. Open and closed circles were obtained from the onset and completion temperatures of the main gel to liquid crystalline phase transition. The phase designations are as follows: G, gel phase; F, liquid crystalline phase; F′, immiscible phosphatidylcholine/DiRL domains in the liquid crystalline phase.

However, differences in the behaviour of the liquidus line can be observed depending on the acyl chain length of the particular phosphatidylcholine. The presence of DiRL in DSPC systems (Fig. 4C) clearly produces a decrease of the temperature of the liquidus line, indicating that both molecules are miscible in the liquid crystalline state. This decrease is less evident in the case of DPPC (Fig. 4B). For the shorter homologue DMPC (Fig. 4A) the temperature of the fluid line increased slightly and then decreased producing a bell shaped line, indicating that in this range of DiRL concentrations fluid immiscibility occurs. Bell-shaped fluid immiscibilities have been previously described for mixtures of phosphatidylcholine and anaesthetics (De Verteuil et al., 1981) and triphenyltin (Chicano et al., 2001). When temperature is increased the different phosphatidylcholine/DiRL systems evolved from a lamellar gel phase (G phase) to a lamellar liquid-crystalline phase (F phase) through a coexistence region (G + F). In the case of the shorter homologue (Fig. 4A) the system presented a small region which shows a fluid phase immiscibility in which different lamellar liquid-crystalline phases coexist (F′).

Table 1
Frequency at the absorbance maximum of the asymmetric CH_2 stretching vibration band of the infrared spectra of different phosphatidylcholines containing DiRL at different molar fractions (m.f.), in the gel and the liquid crystalline phases

Sample	Frequency (cm^{-1}) (gel phase)	Frequency (cm^{-1}) (liquid crystalline phase)
DMPC	2915.3 (10 °C)	2917.6 (30 °C)
DMPC/DiRL (0.03 m.f.)	2918.0 (10 °C)	2922.2 (30 °C)
DMPC/DiRL (0.07 m.f.)	2920.1 (10 °C)	2923.2 (30 °C)
DPPC	2917.4 (25 °C)	2921.7 (50 °C)
DPPC/DiRL (0.03 m.f.)	2918.0 (25 °C)	2922.1 (50 °C)
DPPC/DiRL (0.07 m.f.)	2919.0 (25 °C)	2923.2 (50 °C)
DSPC	2917.8 (40 °C)	2923.0 (60 °C)
DSPC/DiRL (0.03 m.f.)	2917.6 (40 °C)	2921.1 (60 °C)
DSPC/DiRL (0.07 m.f.)	2917.3 (40 °C)	2921.9 (60 °C)

3.3. Infrared spectroscopy

The effect of DiRL on the phosphatidylcholine acyl chains was examined by monitoring the changes occurring in the CH_2 stretching vibration bands which occur between 2800 and 3000 cm^{-1}. The CH_2 stretching region of the infrared spectrum of phosphatidylcholines contains two major bands centered near 2850 and 2920 cm^{-1}, which arise from the symmetric and asymmetric methylene stretching vibrations, respectively. With most phospholipid bilayers, these vibrations give rise to relatively sharp absorption peaks at temperatures below the phospholipids gel to liquid-crystalline phase transition, and when the phospholipids hydrocarbon chains melt, the absorption bands broaden and shift upward in frequency by 2–3 cm^{-1}. Such behaviour is characteristic of hydrocarbon chain melting phenomena and results from an increase in hydrocarbon conformational disorder and molecular mobility at the chain melting phase transition (Casal and Mantsch, 1984). These changes in frequency were observed with pure phosphatidylcholines and also with their mixtures with DiRL (Table 1) and they were observed over temperatures ranges comparable to those of the thermotropic events detected by DSC (Fig. 2). This observation provides evidence that the thermotropic events observed in the DSC experiments, the two endothermic components detected in the phosphatidylcholine/DiRL systems, all involve the melting of the phospholipids hydrocarbon chains. Fig. 5 compares the infrared CH_2 stretching bands of the gel and liquid crystalline phases of pure DMPC with that of DMPC containing 0.07 mol fraction of DiRL. It is observed that in both the gel (Fig. 5A) and liquid crystalline (Fig. 5B) states, band maxima of the CH_2 stretching vibrations exhibited by the DMPC/DiRL system occur at higher frequencies than those of the pure DMPC. Given that increases in the frequencies of these band maxima are generally correlated with increases in phospholipids hydrocarbon chain conformational disorder (Maroncelli et al., 1985), these results suggest that the incorporation of DiRL into DMPC bilayers results in an overall increase in hydrocarbon chain disorder in both the gel and the liquid crystalline states. It is interesting to note that antimicrobial peptide LL-37 not only perturbs the thermotropic properties of DMPC in the same way that DiRL

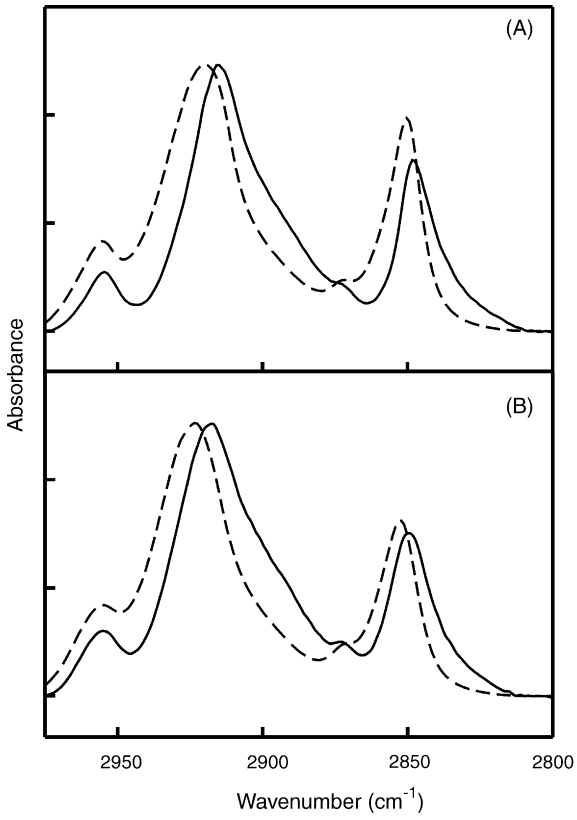

Fig. 5. Infrared spectra of the CH_2 stretching band of DMPC (solid line) and DMPC containing 0.07 mol fraction DiRL (dashed line). (A) 10 °C (gel phase); (B) 30 °C (liquid crystalline phase).

but also has a similar disordering effect on the phospholipids acyl chains (Henzler-Wildman et al., 2004). This disordering effect was less marked when DiRL was incorporated into DPPC and disappeared when DiRL was incorporated into DSPC systems (Table 1). These results indicate that phosphatidylcholines with shorter acyl chains are less able to accommodate the DiRL molecule into the phospholipids palisade than do the ones with larger acyl chains and therefore are more sensible to the presence of the glycolipid.

3.4. Wide angle X-ray diffraction

Measurements in the WAXD region provide information about the packing of the phospholipids acyl chains. Fig. 6 shows the WAXD pattern corresponding to pure DPPC and DPPC containing DiRL at 25 °C (gel phase). In the gel phase, pure DPPC shows a sharp reflection at 4.19 Å surrounded by a broad reflection around 4.10 Å, which is typical for lipid bilayers in the lamellar gel phase with tilted hydrocarbon chains and pseudo-hexagonal chain packing (Tardieu et al., 1973). Above the main transition temperature, a diffuse reflection typical for unordered lipid chains in the liquid crystalline phase was observed (not shown) (Janiak et al., 1979). In the presence of DiRL, the sharp reflection with its shoulder was replaced by a rather symmetric reflection around 4.14 Å, indicating that the hydrocarbon chains are oriented perpendicular to the bilayer plane, which is in accordance with an untilting of the acyl chains (Pressl et

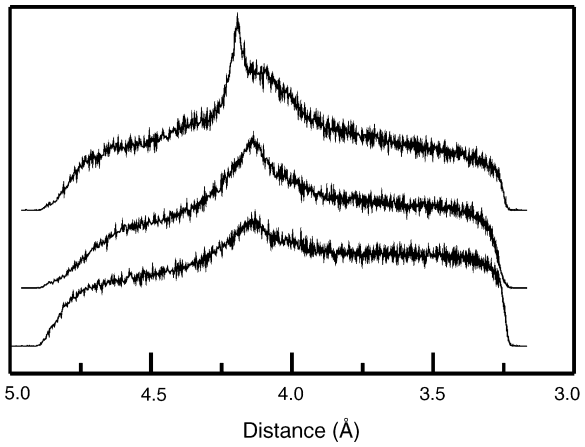

Fig. 6. Wide angle X-ray diffraction profiles at 25 °C, obtained from (top to bottom) pure DPPC, DPPC containing 0.03 mol fraction DiRL, and DPPC containing 0.07 mol fraction DiRL.

al., 1997). The effect of DiRL on the acyl chain packing when incorporated into DMPC or DSPC systems was similar to that commented above for DMPC system (not shown). These results indicate that DiRL is able to perturb the packing of the phosphatidylcholine acyl chains and that this perturbation does not depend on the acyl chain length.

4. Concluding remarks

In this work, we have produced rhamnolipids from *P. aeruginosa*, purified and isolated DiRL, and carried out a study of the molecular interaction between DiRL and phosphatidylcholine membranes. We have shown that DiRL is able to incorporate into phosphatidylcholine bilayers and affect their structural properties. DSC data supported the location of the DiRL molecule intercalated between the phospholipids ones, were it can perturb the phospholipid palisade, reduce the cooperativity of the transition and shift the phase transition temperature to lower values. These effects on the thermotropic properties of phosphatidylcholines are more marked when the acyl chain of the phospholipids is shorter. SAXD data showed that DiRL does not affect the macroscopic bilayer organization of phosphatidylcholines but increases the interlamellar repeat distance and reduces the long-range order in the multilamellar system. Partial phase diagrams showed good miscibility between DiRL and phosphatidylcholines both in the gel and the liquid crystalline phases except for the appearance of fluid immiscibilities in the case of the shorter homologue (DMPC). Infrared experiments evidenced an increase of the phospholipid hydrocarbon chain conformational disorder in the presence of DiRL and WAXD showed that DiRL alters the packing of phosphatidylcholines in the gel phase. The observed interaction between DiRL and phosphatidylcholines promotes physical perturbations, which could affect membrane function and may help to understand the mechanism of action of DiRL.

Dirhamnolipid is known to be antimicrobial (Lang and Wullbrandt, 1999; Benincasa et al., 2004). Many antibacterial compounds act by promoting a negative membrane curvature which can lead to the collapse of the phostphatidylethanolamine-rich bacterial cytoplasmic membrane (El Jastimi and Lafleur, 1999; Willumeit et al., 2005). The mechanism of antimicrobial activity of dirhamnolipid seems to be different, as we have recently reported that dirhamnolipid has a bilayer stabilizing effect impeding the formation of the inverted hexagonal HII phase in phosphatidylethanolamine systems (Sánchez et al., 2006). Recent studies on the mechanism of antimicrobial activity have shown that many antimicrobial compounds which, like dirhamnolipid, induce a positive curvature strain of the membrane (i.e. increase the lamellar to hexagonal H_{II} phase transition temperature of bilayers), disrupt cell membranes through the formation of a transient pore (Henzler-Wildman et al., 2003; Hallock et al., 2003; Thennarasu et al., 2005). Interestingly, the perturbation exerted by DiRL on phosphatidylcholine bilayers, i.e. domain formation and disordering of the phospholipids acyl chains, is very similar to that described for that type of antimicrobial peptides. This similarity is striking as it shows that two molecules with completely different structures (helical peptide and glycolipid) can affect in a similar manner the structure of the phospholipid bilayer and might share a common mechanism of physiological action. Future research is needed to study the effect of DiRL on membrane permeability and to get insight into its mechanism of antimicrobial activity, these studies will be important in order to elucidate whether the hypothetical mechanism of DiRL bilayer disruption would be a detergent-like or it takes place through the formation of a pore like the above mentioned antimicrobial peptides.

Rhamnolipids play a variety of different functions in microbial cells, but there is not consensus on their real physiological role. In general, the main role associate with rhamnolipids is to permit bacteria to grow on hydrophobic substrates by reducing the surface tension allowing the uptake and metabolism of hydrocarbon compounds (Fiechter, 1992). It has been demonstrated that rhamnolipids causes the release of lipopolysaccharide from the bacterial outer membrane resulting in an increase in cell surface hydrophobicity (Al-Tahhan et al., 2000). In the context of antimicrobial activity, it is interesting to note that bacteria with more hydrophobic surface will be more susceptible to the action of hydrophobic antibiotics, thus future research attention should be paid to the possibility that rhamnolipids might potentiate the action of particular antibiotics. Finally, we have previously shown that DiRL behaves as an inverted-cone shaped molecule which opposes the cone shape of phosphatidylethanolamine (Sánchez et al., 2006). In this sense, it would be possible that the presence of DiRL in the outer membrane of biosurfactant producing bacteria might protect the membrane from the action of antimicrobial peptides which induce negative curvature strain. This possibility, which needs to be studied, would be viable due to the complementary shape of both types of molecules and could be explained by the dynamic shape theory of lipid polymorphism (Cullis and De Kuijff, 1979).

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