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Packing behaviour of two predominant anionic phospholipids of bacterial cytoplasmic membranes ☆

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ABSTRACT

Phosphatidylglycerol and cardiolipin represent the most abundant anionic phospholipid components of cytoplasmic bacterial membranes and thus are used as constituents for membrane mimetic systems. In this study, we have characterized the temperature dependent phase behaviour of the binary system dipalmitoyl-phosphatidylglycerol (DPPG) and tetramyristoyl-cardiolipin (TMCL) using microcalorimetry and X-ray scattering techniques. Both lipids exhibited a very similar main transition temperature (~41 °C), showing a minimum (39.4 °C) for the binary mixtures at $X_{DPPG}=0.8$, and exhibited low-temperature phase transitions, which were abolished by incorporation of small amounts (≤10 mol%) of the other lipid component. Therefore, over a wide temperature and composition range a lamellar L_β gel phase is the predominant structure below the chain melting transition, characterized by a relatively broad wide-angle peak for $X_{DPPG}\leq 0.8$. This observation suggests the existence of packing inconsistencies of the TMCL/DPPG hydrocarbon lattices in the gel phase, supported by the small average size of lipid clusters (~50 lipids) within this composition range. The bilayer thickness for the lamellar-gel phase showed a monotonic increase (56 Å for TMCL to about 58 Å for $X_{DPPG}=0.8$ at 30 °C), which may be explained by different degrees of partial interdigitation of the acyl chains to compensate for the differences in the hydrocarbon lengths of DPPG and TMCL in the L_β phase.

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1. Introduction

We face a worldwide increase in pathogenic bacteria that are (multi-)resistant to commercially available antibiotics, while the number of novel antibiotics on the market declines. Several strategies to retrieve control on bacterial infections have been developed in the last decade (e.g. Lohner [1]). One promising approach is based on antimicrobial peptides. They mostly perturb and destroy the structural integrity of the lipid membrane, although some of these peptides seem to pass the membrane barrier and to act on cytosolic targets [2,3]. Of particular interest is in any case the cell specificity of antimicrobial peptides, i.e. to discriminate between the host and bacterial cell membrane. While the outer leaflet of mammalian plasma membranes almost exclusively consists of neutral phospholipids, the bacterial one has a high content of negatively charged phospholipids, which is supposed to be a predominant factor for the affinity of the positively charged peptides to bacterial membranes. However, recently we showed that in respect of membrane-perturbing mechanism lipid net charge is not the decisive factor, but lipid packing density, the ability to form intermolecular H-bonds and lipid molecular shape have to be also taken into account [4]. Therefore, it is of interest to study the properties of membrane mimetic systems such as structure or packing properties.

As indicated above bacterial cell membranes have high contents of negatively charged lipids like phosphatidylglycerol (PG) or diphosphatidylglycerol (DPG or cardiolipin), which are the most abundant anionic phospholipids of cytoplasmic bacterial membranes. These phospholipids are particularly prominent in a number of Gram-positive bacteria (Table 1). Interestingly, the level of cardiolipin may substantially be increased under certain environmental (e.g. high salt) or stress conditions as reported for *S. aureus* [5–8]. The increased amount of cardiolipin may reflect a requirement for enhancement of the structural integrity of the bacterial cell membrane or for the support of stress-related increases in energy transduction, or both [5,9].

Cardiolipin with its quadruple hydrocarbon chains is a unique lipid also found in a significant amount in the inner mitochondrial membrane

Abbreviations: DPG, diphosphatidylglycerol (cardiolipin); TMCL, tetramyristoyl-cardiolipin; (DP)PG, (dipalmitoyl-)phosphatidylglycerol; PE, phosphatidylethanolamine; $L_{c'}$, subgel phase; SGII, subsubgel phase with tilted hydrocarbons; L_{R1}, subsubgel phase with untilted hydrocarbons; L_β, lamellar-gel phase with untilted hydrocarbon chains; $L_{\beta'}$, lamellar-gel phase with tilted hydrocarbon chains; $P_{\beta'}$, ripple-gel phase; L_α, fluid phase with melted hydrocarbon chains; DSC, differential scanning calorimetry; $T_{(m)}$, (main) transition temperature; T_{pre}, pre-transition temperature; $\Delta H_{(m)}$, (main) transition enthalpy; ΔH_{pre}, pre-transition enthalpy; SWAXS, small and wide-angle X-ray scattering; z_H, distance between head group and centre of the bilayer; d_B, thickness of the bilayer; OLV, oligolamellar vesicles; X_{DPPG}, mol fraction of DPPG.

☆ Dedicated to Prof. Dr. Alfred Blume on the occasion of his 65th birthday.

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Table 1
Membrane phospholipid composition of representative Gram-positive bacteria (see K. Lohner [38] and references therein).

Bacteria species	Phospholipid as percentages of the total				
	PG[a]	Cardiolipin	Lysyl-PG	PE	Others
Staphylococcus aureus	57	5	38	0	Trace
Bacillus subtilis	29	47	7	10	6[b]
Micrococcus luteus	26	67	0	0	7[c]

[a] PG, phosphatidylglycerol; PE, phosphatidylethanolamine.
[b] Including phosphatidic acid and glycolipids.
[c] Almost exclusively phosphatidylinositol.

of eukaryotic cells being essential for e.g. insertion and translocation of proteins in the inner mitochondrial membrane [10]. While it seems that a major biological role of cardiolipin is related to the function of membrane proteins [9], Haines and Dencher [11] suggested another important function for cardiolipin due to its high pK_{a2} value (>8), which enables cardiolipin to trap protons at the H^+-uptake pathway of the energy transducing membrane [for details see [12]]. PG does not show such a variety of specific functions, but serves as precursor molecule for the biosynthesis of cardiolipin and other phospholipid molecules [13].

Regarding bacteria, cardiolipin was described to be concentrated in polar and septal regions as visualized by nonyl acridine orange (see Mileyovskaya [14], Schlame [15], and references therein). The ability to form non-lamellar structures is supposed to be required for membrane curvature in the septal region of cytoplasmic membranes, which is related to the cell division process. The propensity to form non-lamellar structures is referred to the small polar head group of cardiolipin (see Matsumoto [16] and references therein). Because of this unique conformation, this lipid is also able to pack tightly forming micro domains [11] and in stack like arrays, which are also stabilized by membrane proteins [16]. Haines et al. [11] described the head group as a bicyclic formation of the two phosphate groups linked to the centred glycerol residue. Thereby, the H-bonding to the hydroxyl residue alters the pK_a to >8 and creates – by trapping a proton – an acid-anion. Therefore, at physiological conditions, cardiolipin has a single negative charge on the head group [11]. PG also carries one net negative charge at physiological pH (pK_a value of ~3 [17,18]), but unlike cardiolipin the cross section of the head group is comparable to the cross section of its hydrocarbon chains. This gives the lipid a cylindrical molecular shape and thus PG will prefer to form flat bilayers.

Although there is a wealth of knowledge on the individual lipids, to our knowledge no study has been reported so far on binary mixtures of PG and cardiolipin, which represents an interesting model system for some Gram-positive cytoplasmic membranes. As lipids we have chosen the saturated species dipalmitoyl-PG (DPPG) and tetramyristoyl-cardiolipin (TMCL). The thermotropic phase behaviour of both lipids has been characterized showing very similar chain melting transition (around 41 °C) under comparable buffer conditions [19,20]. Moreover, both lipids are characterized by a similar membrane hydrophobic core in the lamellar-gel phase, which can be explained by the chain tilt of DPPG that compensates for its longer chain length (C16 vs. C14) as shown in this study. The experiments were performed at physiologically relevant buffer conditions using differential scanning calorimetry (DSC) and small and wide-angle X-ray scattering (SWAXS) techniques to yield thermodynamic and structural parameters of the DPPG/TMCL mixture.

2. Materials and methods

2.1. Preparation of liposomes

1,2-dipalmitoyl-phosphatidylglycerol (DPPG) and tetramyristoyl-cardiolipin (TMCL) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA) as Na-salt powders and used without further purification. Stock solutions were prepared by dissolving the required amounts of DPPG or TMCL in chloroform/methanol, 2/1 (vol/vol). For film preparation, mixtures of calculated stock solution quantities were evaporated under a nitrogen gas stream and at a temperature of 40 °C until a thin lipid film remained. The films were kept under vacuum over night for total solvent evaporation. The hydration procedure comprised an incubation time of 90 min at 65 °C, interrupted by thorough vortex mixing. The hydration buffer consisted of 20 mM NaPi, pH 7.4, 130 mM NaCl prepared from highly purified water (Milli-Q water purification system).

2.2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a VP-DSC high-sensitivity calorimeter from MicroCal, LLC (Northampton, MA, USA). The lipid concentration was 1 mg/ml. At least two sample preparations were performed for each mixture to check on repeatability. Prior to experiments, all samples were degassed for 5 min at room temperature. The scanning temperature range was set between 1 and 60 °C and the scan rate was adjusted at 30 °C/h. The equilibration time before each scan was 15 min. For each measurement the scan program included three heating/cooling cycles showing identical thermograms for the second and third scans, which were used for data evaluation. This was performed with Origin 7 SR4 including a calorimetric modelling package. Recorded DSC thermograms were normalized with respect to the lipid concentration and scan rate. After baseline correction, the calorimetric enthalpies (ΔH) were determined by integrating the peak areas. The calorimetric enthalpy, the maximum heat capacity and the phase transition temperature were used for calculation of the van't Hoff enthalpy [21,22] in order to calculate the cooperativity of the main transition given by the ratio between van't Hoff and calorimetric enthalpy.

2.3. X-ray scattering

Small and wide-angle X-ray scattering (SWAXS) patterns were recorded on a SWAX camera (System 3, Hecus X-ray Systems, Graz, Austria) using a sealed X-ray tube generator from Seifert (Ahrensburg, Germany) working at 50 kV and 40 mA. The X-ray beam was filtered for CuK_α radiation ($\lambda = 1.542$) using a Ni foil and a pulse height discriminator. The SWAXS patterns were recorded in the wave vector ($q = 4\pi \sin\theta/\lambda$, where θ is half the scattering angle) regimes of 10^{-3} Å^{-1} < q < 1 Å^{-1} (SAXS) and 1.2 Å^{-1} < q < 2.7 Å^{-1} (WAXS) using two linear, one-dimensional, position-sensitive detectors (PSD 50, Hecus X-ray Systems, Graz, Austria). Silver stearate and p-bromo-benzoic acid were used for detector calibration of the small and the wide-angle range, respectively. Temperature was controlled with a Peltier heating unit.

Lipid samples (concentration 50 mg/ml) were filled into thin walled 1 mm capillaries (Hilgenberg GmbH, Malsfeld, Germany) and sealed with a two-component adhesive. To prevent sedimentation, the capillaries were rotated at constant speed along their axis within the sample stage. Data sets of pure buffer solution were recorded at 25 °C and used for baseline subtraction. Background corrected SAXS data were evaluated in terms of the Global Analysing Program developed by Pabst et al. [23,24]. In the applied model, the bilayer electron-density profile is given by the summation of three Gaussian distributions, two describing the electron-density of the head group region and one at the centre of the bilayer accounting for the minimum density at the methyl terminus of the hydrocarbon chains.

The main parameters determined by SAXS data evaluation are the distance between head group and centre of the bilayer (z_H), the hydrocarbon chain length and the thickness of the bilayer (d_B), respectively, which were determined as detailed previously [25]. Peak

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