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Stability against temperature and external agents of vesicles composed of archaeal bolaform lipids and egg PC

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Abstract

The bolaform lipid PLE extracted from the thermophilic archaeon *Sulfolobus solfataricus* and its mixtures with egg phosphatidylcholine (egg PC) have been used to prepare sonicated vesicles. The leakage of entrapped calcein was continuously monitored by fluorescence dequenching. The half times of leakage have been used to compare vesicle stability under different conditions of temperature, lipid composition and presence of destabilizing agents like Ca^{2+} ions and poly(ethylene glycol) (PEG). It has been found that leakage is primarily modulated by the monopolar/bipolar lipid ratio. In particular, the half time of leakage for vesicles formed from a mixture of the polar lipid extract (PLE) and egg PC is characterized by a maximum at about 1:2 molar ratio. The free energy of mixing has been evaluated from pressure-area isotherms on monolayers at the air/water interface. The results indicate a non monotonous behaviour of the excess free energy of mixing as a function of the molar ratio and the occurrence of a minimum at a fixed molar ratio. The possible formation of a complex is discussed and compared with previous calorimetric measurements on similar compounds.

Keywords: Vesicle stability; Phosphatidylcholine; Polar lipid extract; External agent

1. Introduction

Sulfolobus solfataricus is a thermophilic archaeon which grows in extreme environments at $T \simeq 87°C$ and $pH \simeq 3$ [1,2]. The plasma membrane of this microorganism must have a unique composition and organization to preserve its integrity and therefore a specific permeability pattern. A key role in membrane stability is played by tetraether lipids, which are endowed of two polar heads and span the entire membrane thickness [3]. The backbone of these lipids consists of two C_{40}-phytanyl chains linked through ether bonds to two glycerols (glycerol-dialkyl-glycerol tetraether, GDGT) or to a glycerol and a nonitol (glycerol-dialkyl-nonitol tetraether, GDNT) (Fig. 1). The properties of dispersed lipids have been analyzed with the aim of understanding the physico-chemical basis of thermostability [4]. The polar lipid extract of *S. solfataricus* and a lipid fraction of *S. acidocaldarius* are able to form closed unilamellar vesicles in aqueous media [5–7]. The stability of liposomes composed of archaeal lipids has been studied by monitoring the release of entrapped carboxifluorescein or calcein [6,8,9]. It has been shown that archaeal liposomes are stable in a broad range of conditions, including temperature and pH. This behaviour is mainly due to the unique structure of their membrane, which is composed by a single monolayer [2,4].

In this paper we have analyzed the stability properties of mixed monolayer/bilayer liposomes under various physical conditions. Data reported so far indicate that it is possible to mix common monopolar lipids, like PC, with archaeal bolaform lipids, although it might be surprising that such different compounds are miscible [10,11]. However, no detailed information on molecular interactions between these two classes of lipids has been provided besides calorimetric data [12]. In order to elucidate this point, we evaluated the free energy of mixing from pressure-area isotherms on monolayers at the air/water interface. The results indicate that there is a preferential ratio of mixing at which the two different classes of lipids gather optimal interactions. Vesicles of such composition display an exceptionally low permeability to calcein, even at high

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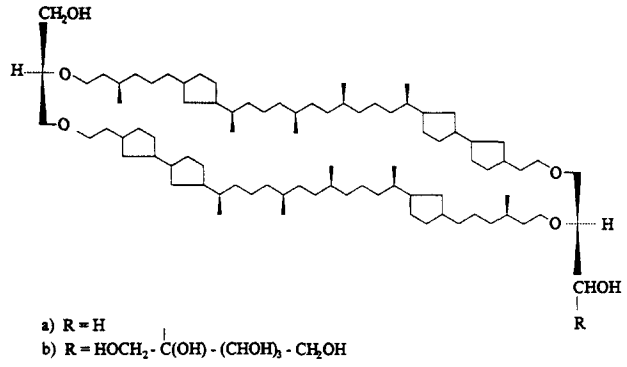

Fig. 1. Backbone of the archaeal tetraether lipids. (a) GDGT; (b) GDNT. The molecules may contain up to four rings per chain. The mean value is 2.3 rings per molecule.

temperatures. The destabilizing effect of molecules like Ca^{2+} or poly(ethylene glycol) in the external medium is also discussed.

2. Materials and methods

2.1. Materials

The polar lipid extract (PLE) was obtained from the membrane of *Sulfolobus solfataricus* by an overnight cold extraction in chloroform/methanol (1:1, v/v). PLE is a mixture of several bolaform compounds, as described in details elsewhere [6]. It has been shown by X-ray diffraction that in spite of its complexity PLE behaves as a single component [13]. Its average molecular weight is 1780. PLE was dissolved in a mixture of chloroform (Aristar, purity 99.4%, BDH, UK), methanol (purity 99.8%, Inalco, Milan, Italy) and water (65:25:4 by vol.). Egg PC (M_r 760) was obtained from Avanti Polar Lipids (Alabaster, AL, USA) and was dissolved in chloroform. Calcein and poly(ethylene glycol) (PEG, average M_r 3350) were purchased from Sigma (Milan, Italy). Sephadex G-50 was obtained from Pharmacia (Milan, Italy). All other chemicals were analytical grade. Water was purified by means of a Millipore Milli-Q system including a terminal 0.22 μm filter.

2.2. Vesicle preparation

Egg PC and PLE were dissolved in organic solvents as described in the previous section. After thorough mixing of the desired lipid composition, the solvent was evaporated under a stream of nitrogen. The last traces of solvent were removed under vacuum (1 mmHg) for 30 min. The lipid was then hydrated using a buffer containing 175 mM calcein, 2 mM Tes, 2 mM histidine, adjusted to pH 7.4. The lipid concentration was 2.5 mg/ml. The sample was sonicated in a bath-type sonicator for about 15 min in order to get a homogeneous liposomal dispersion. Unilamellar vesicles were then obtained using a probe-type sonicator (Ultrasonics Ltd., UK). Liposomes were sonicated for 15 min at 60°C while for pure egg PC an ice-bath was employed. To avoid overheating, the sonication was interrupted every 2 min for 1 min. The vesicle preparation was then centrifuged for 10 min at 11 500 × g in a Sorvall RC-SB Superspeed Centrifuge (Du Pont Instruments, CT, USA) to remove large lipid aggregates and titanium impurities possibly coming from the tip. Just before each experiment vesicles were separated from nonencapsulated calcein by using the minicolumn chromatographic centrifugation technique [14] on Sephadex G-50 columns. Vesicle diameter was measured by light scattering using a Brookhaven Instrument BI-90 Particle Sizer (Brookhaven Instruments, Holtsville, NY, USA). Measurements were performed at room temperature on calcein-free samples.

2.3. Release of vesicle contents

Calcein leakage was measured with an Aminco-Bowman spectrofluorometer. The lipid concentration in the cuvette was 25 nM. Measurements were performed in a final volume of 1.5 ml buffer containing 100 mM NaCl, 2 mM Tes, 2 mM histidine and 0.1 M EDTA (pH 7.4). When the release, R, in the presence of destabilizing agents was studied, the above medium contained also 15 mM $CaCl_2$ or 20% (w/v) PEG. Calcein fluorescence was continuously monitored for 1 h. The samples were excited at 490 nm and emission at 520 nm was continuously recorded as a function of time. The percentage of fluorophore release was estimated from the ratio $(F_t - F_0)/(F_{max} - F_0)(\%)$, where F_t and F_0 are the fluorescence at time t and 0, respectively, and F_{max} is the maximum fluorescence, which was determined by lysing the vesicles with sodium cholate (final conc. 0.3%, w/v) at the end of the experiment [15]. Half times were determined from the initial slope of the semilogarithmic plot of the latency (defined as $L = 100 - R$) as a function of time [14]. Each experiment was performed at least in duplicate on two different vesicle preparations. The experimental points shown in the graphics are the mean value of the release over a period of 1 min.

2.4. Pressure–area isotherms

Pressure–area isotherms of monolayers at the air/water interface were obtained by using a circular Teflon multicompartment trough (RCM2-T Monofilmmeter, Mayer Feintechnik, Göttingen, Germany). Monolayers were formed by spreading on purified water (pH 5.5) 40 μl of a 1 mM lipid solution with the aid of a Hamilton microsyringe. In the case of mixed monolayers, the two lipid components were carefully mixed at the required ratio before spreading. At least 10 min was allowed for the spreading solvent to evaporate. The monolayer was com-

pressed at a barrier speed of 0.1 Å^2/molecule per s. The temperature of the trough was kept at 25°C.

The excess surface free energy of mixing was evaluated by means of the equation [14]:

$$\Delta G^{\pi} = \int_{\pi^*}^{\pi} (A_{12} - N_1 A_1 - N_2 A_2) \mathrm{d}\pi \qquad (1)$$

where A_1 and A_2 are the areas per molecule in the pure films, A_{12} is the mean area per molecule in the mixed film, N_1 and N_2 are the molar fractions of components 1 and 2, respectively. The lower limit of integration π^* was chosen equal to 1 mN/m, in order to avoid possible fluctuations around $\pi = 0$.

3. Results

The experiments described below are aimed at gathering information on vesicle permeability, which, in turn, is a test for integrity and stability of membranes comprised of bolaform lipids and their mixtures with bilayer-forming lipids. Vesicle size was dependent on the membrane composition. Light scattering measurements indicated a linear decrease in vesicle diameter with increasing PC content from 90 ± 5 nm (for pure PLE) to 28 ± 5 nm (for pure egg PC).

Permeability was detected by dequenching of the released calcein. Fig. 2 shows the time dependent calcein release of PLE vesicles at 60° and 25°C. The results indicate that leakage is very low, even at high temperatures. The effect of destabilizing agents like Ca^{2+} and PEG is also shown in Fig. 2a. Previous studies have shown that Ca^{2+} is able to induce aggregation and fusion of vesicles only above 60°C [6], while PEG can induce fusion even at 25°C (Relini et al., unpublished data). As expected, the extent of release increases; however, only in the presence of PEG at 60°C the organization of membrane lipids and/or the change of the nature of the aqueous phase causes the membrane to become very leaky. Under the same experimental conditions leakage is always appreciably lower in bipolar lipid than in egg PC vesicles. An example is illustrated in Fig. 2a, which shows data on egg PC vesicles in the presence of PEG at 60°C.

Fig. 2b shows that vesicles formed by PLE and egg PC mixed at 1:2 molar ratio display an unusual stability and an extent of leakage even smaller than pure PLE vesicles. By contrast, results of Fig. 2b indicate that calcein release in the presence of PEG or Ca^{2+} is much higher. This behaviour indicates that although monolayer- and bilayer-forming lipids display favourable interactions which contribute to a tight and highly impermeable structure, destabilizing agents like Ca^{2+} and PEG cause a much larger perturbation to the system. In other words, the presence of monopolar lipids favours a more drastic change in the organization leading to a much higher calcein release. This behaviour looks quite interesting and suggested us to

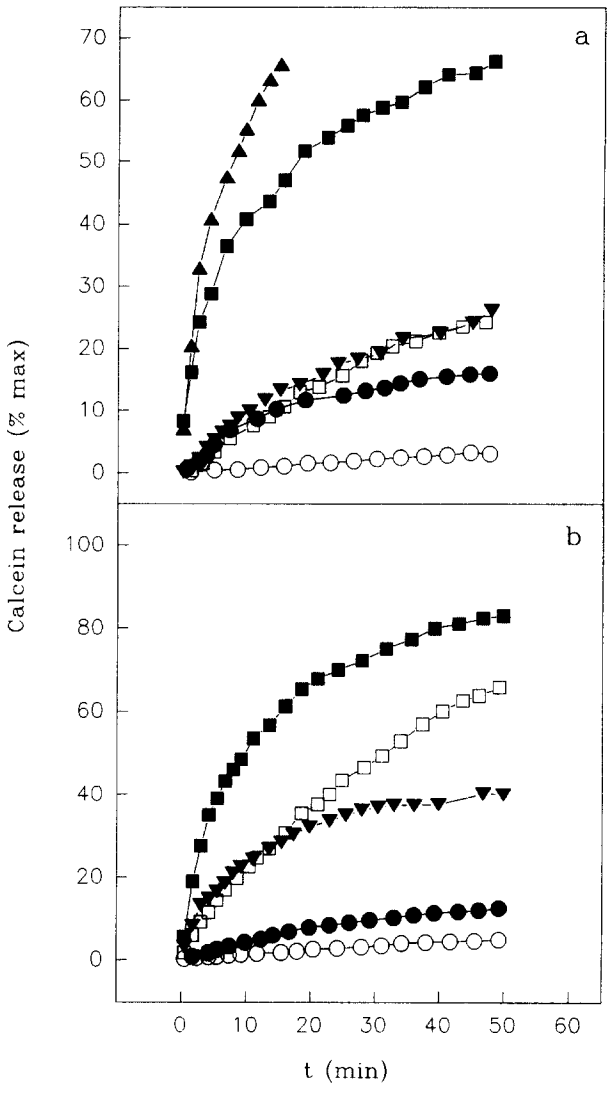

Fig. 2. Calcein release (% max) as a function of time. Empty symbols, $T = 25$°C; filled symbols, $T = 60$°C. (a) PLE and PC vesicles at the indicated physical conditions. ▲, egg PC in the presence of PEG; □, ■, PLE in the presence of PEG; ▼, PLE in the presence of Ca^{2+}; ○, ●, PLE in the absence of destabilizing agents. (b) mixed PLE/PC vesicles (1:2 m.r.). □, ■, in the presence of PEG; ▼, in the presence of Ca^{2+}; ○, ●, in the absence of destabilizing agents. PEG concentration: 20% (w/v); Ca^{2+} concentration: 15 mM.

explore permeability properties at various molar ratios. Fig. 3a plots the half time of calcein release as a function of the molar ratio. At both temperatures a maximum is found close to 1:2 m.r. Under the assumption that the vesicle permeability does not depend substantially on curvature, this indicates an optimal interaction of lipid components at that particular molar ratio. The maximum disappears in the presence of destabilizing agents, like Ca^{2+} at 60°C or PEG, a result which is compared with the behaviour in the absence of destabilizing agents at 25°C (Fig. 3b).

The occurrence of a minimum in the leakage is indicative of an optimal interaction between the two classes of

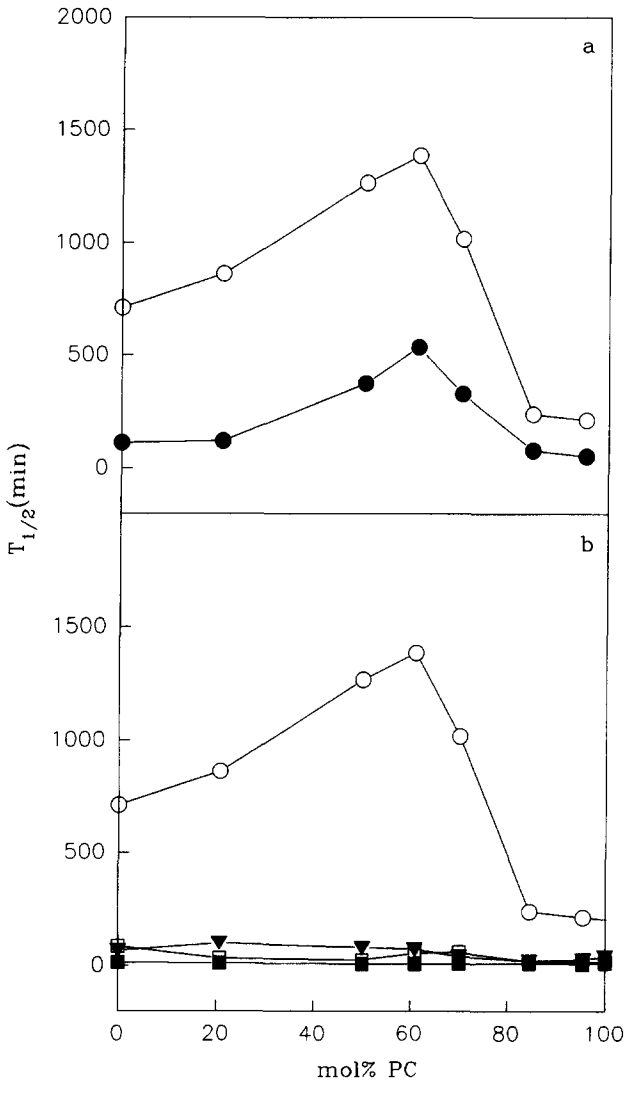

Fig. 3. Half-times of calcein release of vesicles prepared from PLE/egg PC mixtures at the indicated lipid compositions. (a) ○, $T = 25°C$; ●, $T = 60°C$. (b) Empty symbols, $T = 25°C$; filled symbols, $T = 60°C$. ○, in the absence of destabilizing agents; ▼, in the presence of 15 mM Ca^{2+}; □, ■, in the presence of 20% (w/v) PEG. Error bars are within the point dimensions.

lipid components. In order to elucidate further this point, studies have been performed on mixed PLE/PC monolayers at the air/water interface. The pressure-area isotherms, shown in Fig. 4, indicate that increasing the amount of PC the collapse pressure of the film gradually increases. This behaviour is typical of miscible compounds [16]. Further information on molecular interactions can be gathered by calculating the excess free energy of mixing, defined in the previous section. Fig. 5 shows ΔG as a function of the film composition. The different curves correspond to different values of the upper limit of integration (from 3 mN/m up to 25 mN/m). The values of ΔG are always negative, indicating attractive interactions between molecules [16]. Optimal interaction occurs at 70 mol% PC.

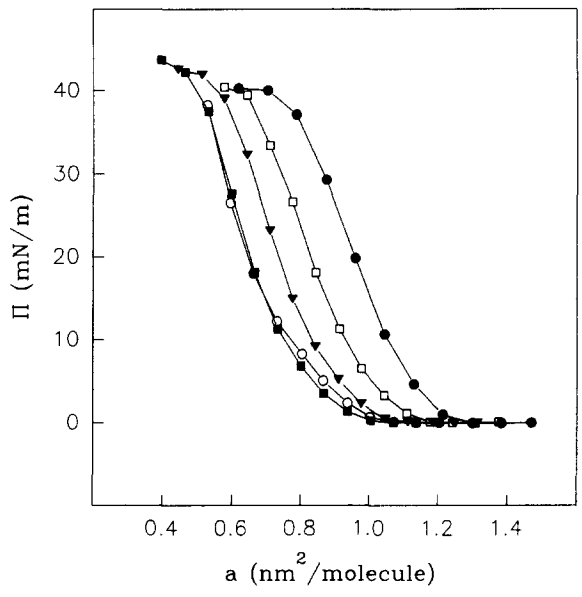

Fig. 4. Pressure–area isotherms of mixed PLE/PC monolayers at different molar ratios. ●, PLE; □, PLE/PC 2:1; ▼, PLE/PC 1:1; ■, PLE/PC 1:2; ○, PC. $T = 25°C$.

This value is quite close to the lipid composition at which vesicle permeability is minimal (Fig. 3a). However, the agreement can be only semiquantitative due to the different molecular organization and therefore different interactions which are found in a monolayer at the air/water interface and in a membrane between two aqueous solutions.

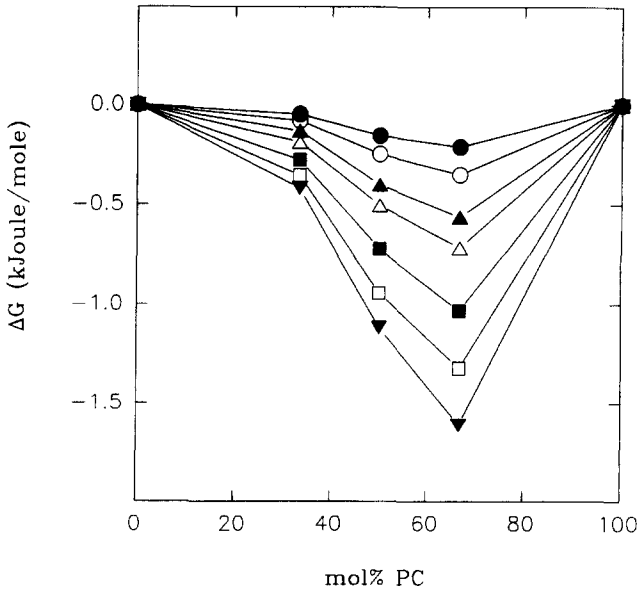

Fig. 5. Excess free energy of mixing in PLE/PC monolayers as a function of lipid composition. The different curves correspond to different values of the upper limit of integration defined in Eq. (1). ●, 3 mN/m; ○, 5 mN/m; ▲, 8 mN/m; △, 10 mN/m; ■, 15 mN/m; □, 20 mN/m; ▼, 25 mN/m.

4. Discussion

The main concern of this work is related to the stability and unusually low permeability of vesicles formed from bipolar lipids and from bipolar-monopolar lipid mixtures. It has been shown that vesicles from a polar lipid extract of *S. acidocaldarius* are stable up to $\simeq 100°C$ [8] and exhibit an extremely low proton permeability [9]. The thermal stability is mainly related to the bipolar structure of lipids, while the low proton permeability seems related to the presence of branching in the chains. In fact, an extremely low proton permeability is shown to occur in ether phytanyl liposomes [17]. The important finding of this work is that permeability is further lowered when a certain percentage of monopolar lipids is added. In particular, around PLE/PC 1:2 m.r. $T_{1/2}$ is maximum. Miscibility experiments on monolayers indicate that this ratio corresponds to a minimum in the free energy of mixing where molecular interactions are optimized. We suggest that a complex is formed at this molar ratio and the lipid mixtures at higher concentrations of egg PC can be considered as ideal cosolutions of this complex with egg PC. A similar result has been obtained by DSC measurements on a mixture of GDNT/DPPC [12]. This system shows a complex thermotropic behaviour, although the DPPC peak is never observed, indicating that phase separation does not occur. At low concentration DPPC acts merely as a spacer, accumulating on the nonitol side. Only near 1:2 m.r. a unique peak is observed and increasing the DPPC content, the transition temperature shifts towards that of pure DPPC.

In principle, sonicated vesicles are unlikely to represent thermodynamic equilibrium, because of the high curvature constraints which are present in the membrane. In spite of this limitation the permeability properties are fairly well correlated with miscibility data. In addition, an approach based on equilibrium thermodynamics allows to evaluate the vesicle radius in good agreement with experiments [18]. Therefore, we may conclude that curvature effects do not play a crucial role in determining the observed behaviour of the system.

It might be surprising that a monolayer at the air/water interface displays properties related to molecular interactions which are so similar to those in vesicles. However, geometrical considerations indicate that monopolar lipids are preferentially located on the outer side of the membrane and therefore interact mainly with one half of the bipolar molecules [18]. This disposition, which has been also proposed for vesicles comprised of GDNT/PC [19], accounts for the similarities between monolayers and vesicles. Structurally, egg PC per se does not appear to contribute to membrane impermeability. However, adding common lipids may relax physical constraints imposed by the curvature of vesicles and bipolarity of the molecules [18]. At the same time, the presence of bipolar molecules seems essential to chemical and thermal stability.

The permeability barrier is drastically affected by destabilizing agents like PEG or Ca^{2+} at 60°C. It has been shown that only PLE vesicles really fuse in the presence of Ca^{2+} at 60°C [6]. When egg PC is added, only lipid mixing occurs [6]. By contrast, PEG induces fusion even at 25°C (Relini et al., in preparation). This finding is in line with the fact, shown in Fig. 3b, that for mixed vesicles leakage in the presence of PEG is higher than in the presence of Ca^{2+}. This behaviour indicates that external agents can change membrane permeability, although the modulation induced by the monopolar/bipolar lipid ratio is lost. In the case of PEG this may be due to a change in the lipid molecular geometry due to dehydration and/or to a phase separation, as observed in other lipid systems [20].

These vesicles may offer a further advantage based on a new approach to the drug delivery problem. This approach will be the object of a future work and it is based on differential hydrolysis of the lipid polar heads in the mixed vesicles. In fact, since the two classes of lipids are differently affected by external agents like phospholipases, which hydrolyze the ester, but not the ether bonds [21], the permeability barrier can be reduced through hydrolysis of part of the polar heads. Differential hydrolysis of the polar heads can lead to an increased release at a later stage, when the target has been reached. Therefore, vesicles displaying long term stability, very low permeability and biochemically gated release, could constitute a first example of a new generation of drug loaded liposomes.

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