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Biochimica et Biophysica Acta 1194 (1994) 17–24

BBA Biochimica et Biophysica Acta

Calcium-induced interaction and fusion of archaeobacterial lipid vesicles: a fluorescence study

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Received 24 December 1993; revised 6 April 1994

Abstract

The lipids extracted from the membrane of the thermophilic archaeobacterium *Sulfolobus solfataricus* have an unusual bipolar structure. Each molecule is formed by two isoprenoid chains (with up to four cyclopentane groups per chain) ether-linked at both ends to glycerol or nonitol groups. These groups can be variably substituted, mainly with complex sugars. Fluorescence resonance energy transfer, aqueous contents mixing and calcein release assays were employed to assess whether bipolar lipid vesicles were able to undergo a calcium-induced fusion process. The possibility of getting fusion depends strongly on the phase behaviour of the lipids. With vesicles formed by the natural polar lipid extract (PLE), a mixture showing a complex polymorphic behaviour, the fusion process was observed above the temperature $T \simeq 60°C$ at 15 mM Ca^{2+}. By contrast, no fusion was observed in vesicles of P2, a fraction displaying only the lamellar phase. A dramatic change of the fusion process was observed when egg PC or P2 was added to PLE. In this case only lipid mixing, but not a real fusion process occurred at $T \geq 60°C$. The dependence of such a process on ionic conditions has also been studied. Additional experiments involving surface tension measurements on monolayers have been performed to assess the importance of a surface tension increase to get fusion. In contrast to other monopolar lipid systems, no detectable change in surface tension has been observed in our bipolar lipids even in cases in which the fusion process is present.

Key words: Lipid vesicle; Vesicle fusion; Lipid mixing; (Archaeobacterium)

1. Introduction

Sulfolobus solfataricus is an extreme thermophilic archaeobacterium whose plasma membrane is organized in a simple monolayer [1–4]. This is possible because the lipid fraction is comprised of a polar headgroup at each end of a double C_{40} hydrocarbon chain. Several fractions of these bipolar lipids have been isolated and characterized [5,6]. Physicochemical studies including X-ray diffraction and microcalorimetry [7–10] have demonstrated the polymorphic behaviour of these lipids.

Physiological processes which occur in archaeobacteria, such as cell duplication, imply a fusion-like process. While fusion has been widely studied in membranes composed of monopolar lipids, no studies have been published so far on bipolar lipid systems. To this purpose, we prepared vesicles from the natural polar lipid extract (PLE) of the plasma membrane of *Sulfolobus solfataricus*. An interesting question which arises is whether these vesicles are able to undergo fusion. To answer this question three different fluorescence techniques were employed: the resonance energy transfer (RET) [11] to get information on the lipid mixing, the Tb/DPA [12] assay, to monitor the aque-

Abbreviations: DPA, dipicolinic acid (pyridine-2,6-dicarboxylic acid); GDGT, glycerol dialkyl glycerol tetraether; GDNT, glycerol dialkyl nonitol tetraether; PC, phosphatidylcholine; PLE, polar lipid extract; PS, phosphatidylserine; RET, resonance energy transfer; Tes, 2-tris(hydroxymethyl)methylamino-1-ethanesulphonic acid.

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SSDI 0005-2736(94)00110-B

ous contents mixing, and calcein release [13], to test vesicle leakage. The parallel use of these techniques indicates that only above the temperature $T \simeq 60°C$ and in the presence of Ca^{2+} a real fusion process occurs. Several works on lipid vesicles have indicated that in some monopolar lipid systems the polymorphic behaviour is of crucial importance for fusion to occur [14,15]. In the case of PLE a transition from the lamellar to the cubic phase is observed in bulk at $T \simeq 80°C$ [7]. In order to test whether this transition is related to fusion, additional experiments were performed on vesicles obtained by mixing PLE either with egg phosphatidylcholine (PC) at several molar ratios, or with the bipolar lipid fraction P2 (Fig. 1), which shows only the lamellar phase. A series of experiments was also performed on vesicles comprised only of the latter fraction.

It has been proposed that for some lipid systems the surface tension increase in the membrane, due to calcium binding, induces vesicle fusion [16,17]. To test this hypothesis surface tension measurements were performed on monolayers comprised of PLE or mixtures of PLE with other bipolar and monopolar lipids. The results are compared with those obtained in phosphatidylserine (PS) monolayers.

2. Materials and methods

2.1. Materials

Bipolar lipids were extracted from the membrane of the thermophilic archaeobacterium *Sulfolobus solfataricus*. PLE was obtained by an overnight cold extraction

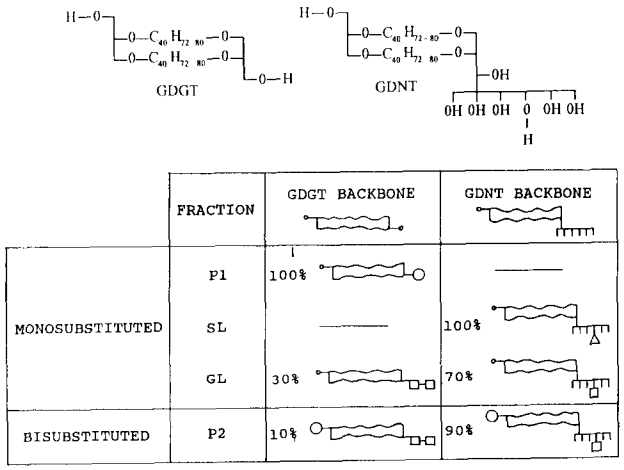

Fig. 1. Schematic structure of the 'backbone' molecules GDGT and GDNT and of their derivatives constituting the polar lipid extract (PLE) of the membrane of *Sulfolobus solfataricus*. Wriggles: hydrocarbon chains; small circles: unsubstituted glycerol OH; comb: nonitol; large circles: phosphomyoinositol; square: β-D-glucopyranose; double square: β-D-glucopyranosyl-β-D-galactopyranose; triangle: β-D-glucopyranosyl sulphate.

in chloroform/methanol (1 : 1, v/v). PLE is composed of different fractions [1], which were separated on a silica gel column. These fractions are labeled P1, GL, SL and P2 (see Fig. 1). The first three are designated 'monosubstituted' because a hydroxyl hydrogen on one of the heads of the backbone molecule is replaced by a different group. P2 is designated 'bisubstituted' because the hydroxyl hydrogen is substituted at both ends of the molecule. P1 has a backbone of glycerol dialkyl glycerol tetraether (GDGT) with one phosphomyoinositol headgroup. SL has a backbone of glycerol dialkyl nonitol tetraether (GDNT) with a sulphur-containing group on one polar head. GL is a mixture of molecules containing one sugar head, but 30% have a GDGT backbone and 70% have a GDNT backbone. Finally, P2 is a bisubstituted mixture of 10% GDGT and 90% GDNT both with one phosphomyoinositol and one sugar headgroup. The mean weight composition of PLE is 10% P1, 30% GL, 7% SL, 48% P2. The monopolar lipid diphytanyl glycerol was also present as a minor component (approx. 4%).

Egg PC, bovine brain PS and the fluorescent lipid analogues *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (*N*-NBD-PE) and *N*-lissamine-rhodamine B sulphonyl diacyl phosphatidylethanolamine (*N*-Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Bovine brain PS, purchased from Sigma (St. Louis, MO, USA), was employed for surface tension measurements on monolayers.

Terbium(III) chloride hexahydrate (99.9% pure) was obtained from Aldrich (Milano, Italy); dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) and calcein were purchased from Sigma. 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

Unilamellar vesicles (diameter approx. 90 nm) were prepared by sonication. The dry lipid film was hydrated with the required buffer to form multilamellar liposomes. To allow lipid hydration the sample was sonicated in a bath-type sonicator in order to get a homogeneous liposomal dispersion. The preparation was sonicated with a probe-type sonicator (Ultrasonics, UK) at 60°C for 10 min. Then it was centrifuged for 10 min at $11\,500 \times g$ in a Sorvall RC-SB Superspeed Centrifuge (Du Pont Instruments, Newtown, CT, USA) to remove large lipid aggregates and titanium impurities possibly coming from the tip [18].

2.3. Fluorescence and turbidity measurements

Fluorescence and turbidity measurements were performed using an Aminco Bowman spectrofluorometer.

Temperature control was achieved by water bath operated circulation around the jacketted cuvette. The temperature on the outside wall of the cuvette was continuously monitored by means of a temperature sensor (LM35DZ, National Semiconductors, RS Components, Milan, Italy). Control measurements of the temperature of the sample in the cuvette, performed with a thermocouple thermometer, showed that at equilibrium there is no difference between the temperature inside and on the walls of the cuvette. The solution in the cuvette was continuously stirred. To eliminate contribution to the signal from light scattering, in some experiments a long pass filter (GG495, Oriel, Milan, Italy) was employed. In addition, for RET measurements the spectrofluorometer was equipped with crossed polarizers [11]. Spectra were corrected subtracting the background contribution by appropriate baseline correction.

For turbidity measurements, the sample was excited at 500 nm in RET and Tb/DPA assays, at 400 nm in the calcein assay, and the scattered light was collected at 90°.

2.4. Resonance energy transfer assay

The calcium-induced lipid mixing of unilamellar vesicles was followed by RET employing the fluorescent probes NBD (photon donor, absorption at 470 nm, emission at 530 nm) and rhodamine (photon acceptor, absorption at 540 nm, emission at 585 nm) [11]. Unprobed lipids, *N*-NBD-PE and *N*-Rh-PE were mixed in the molar ratio 96.8:2:1.2 to form unilamellar vesicles. The excitation wavelength was chosen to be 20 nm below the absorption maximum of NBD to allow better resolution between the scattered light peak and the NBD emission peak; as well, less direct excitation of rhodamine is expected. All the RET experiments were performed in the same aqueous medium used for vesicle preparation. The total volume in the cuvette was 2 ml; the concentration of probed vesicles was 250 μg/ml, and the concentration of unprobed vesicles was 3 mg/ml. The low ratio of probed/unprobed vesicles (1:12) was chosen to increase the probability of fusing a probed vesicle with one devoid of the probes [11]. The data are expressed as efficiency of energy transfer, E, which is defined by the relationship [11]:

$$E = 1 - F/F_0$$

where F is the *N*-NBD-PE fluorescence in the presence of *N*-Rh-PE and F_0 is the *N*-NBD-PE fluorescence at maximal dequenching. F_0 was measured after disrupting the vesicles with Triton X-100 (1% final concentration). In some experiments, F_0 was also determined preparing a population of vesicles whose composition mimicked complete intermixing of lipids. The two calibration methods were found to be in good agreement.

2.5. Aqueous contents mixing assay

The Tb/DPA method [12] was employed to follow vesicle contents mixing subsequent to fusion. Two vesicle populations were prepared in either 15 mM $TbCl_3$ and 150 mM sodium citrate or 150 mM DPA. In addition, both media contained 2 mM histidine and 2 mM 2-tris(hydroxymethyl)methylamino-1-ethanesulphonic acid (Tes) adjusted to a final pH of 7.4. Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-50 medium. The columns (1.0 × 10 cm size; Pierce, USA) were filled with the gel and centrifuged twice for 1 min at 3000 × g in an ALC VISMARA 4225 rotor [19] to remove excess buffer (2 mM histidine, 2 mM Tes, 100 mM NaCl, 1.0 mM EDTA (pH 7.4)). In each column 100 μl of the vesicle preparation were filtered by centrifuging at 3500 × g for 1 min [19]. For purposes of calibration, part of the Tb vesicles were filtered without EDTA in the column buffer.

Measurements were performed in a final volume of 1.5 ml of 100 mM NaCl, 2 mM histidine, 2 mM Tes, 0.1 mM EDTA (pH 7.4). The total lipid concentration for a 1:1 mixture of Tb and DPA vesicles was 0.05 mM. The Tb/DPA complex was excited at 276 nm and the fluorescence emission was measured at 545 nm [12]. The data are expressed as percentage of the maximum fluorescence. This was determined by adding 0.5% (w/v) sodium cholate, in the presence of 20 μM external DPA, to Tb vesicles filtered without EDTA [12]. Vesicle concentration was equal to that used in the fusion assay.

2.6. Contents release assay

Calcein release [13] was employed to follow vesicle leakage. Vesicles were prepared in 175 mM calcein, 2 mM histidine, 2 mM Tes, adjusted to pH 7.4. Vesicles were separated from nonencapsulated material by gel filtration, as described in the contents mixing assay section. Measurements were performed in a final volume of 1.5 ml of the same buffer used in the Tb/DPA assay. The lipid concentration was 0.05 mM. The sample was excited at 490 nm and fluorescence was recorded at 520 nm. The data are expressed as percentage of the maximum fluorescence, determined by adding 0.5% (w/v) sodium cholate to the sample at the end of the experiment [13].

2.7. Surface tension measurements

The surface tension of the monolayers, γ, was measured with the Wilhelmy method [20] which employed

a microscope cover glass ($1 \times 2.2 \times 0.015$ cm^3) for the slide and a Cahn electrobalance to measure the weight (1000 mg full scale). The sensitivity of the measurement of γ by this apparatus was 0.1 dyn/cm.

The system was made entirely of glass to allow thorough cleaning. A triangular hanger of thin glass rod was fused to the top of each slide for suspension from a glass hook connected to the balance. The Pyrex dish chosen for the monolayer (6.9 cm diameter, 1.75 cm deep) was immersed in a water bath to control the temperature. To reduce evaporation when working at 60°C a similar dish, heated by a copper wire which covered its entire outside surface, was used upside down as a cover. Two holes (3 mm diameter) were provided in the cover to allow passage of the suspension rod and the addition of solutions. The aqueous subphase was stirred gently using a small magnetic bar.

The slide and dish were cleaned by washing in detergent, rinsing and boiling in distilled water, heating to 80°C in chromic/sulphuric acid, then again rinsing and boiling twice in distilled water. The cover was washed in detergent and repeatedly boiled in distilled water. Without these precautions, the surface tension of distilled water indicated contamination.

PS monolayers were formed at room temperature on an aqueous subphase solution containing 2 mM Tes, 2 mM histidine, 100 mM NaCl, 0.1 mM EDTA, titrated to pH 7.4. Bipolar lipid monolayers were formed at 60°C on the surface of distilled water. Before adding the lipid, the surface tension γ_0 of water or buffer was used as a check against contamination. Next the lipid was deposited on the liquid surface with a Hamilton syringe, then allowed to equilibrate, often 0.5 h or more. Finally, the concentrated calcium solution was added to the subphase with a long very fine flexible glass tube fastened to a Gilson pipette; in this way the mechanical disturbance of the addition was reduced but not eliminated. For both PS and bipolar lipid monolayers, measurements have been performed at an initial value of surface pressure ($\Pi = \gamma_0 - \gamma$) ranging from 15 to 30 dyn/cm in different experiments. At equilibrium the surface pressure was sufficiently constant to easily detect whether or not there was a change in its value upon the addition of calcium. The change in surface tension of the monolayer during calcium additions was determined from the trace of the electrobalance voltage on a chart recorder.

3. Results

3.1. Fluorescence measurements

The RET and the Tb/DPA assays were tested in a well studied system composed of pure PS vesicles. At 1.5 mM the time scale for fusion was seconds and the maximum extent of contents mixing was 45%, in agreement with previous reports [12]. Further control experiments were performed on PC/*N*-NBD-PE vesicles (98:2 molar ratio) to test the effect on NBD fluorescence of the high Ca^{2+} and Na^+ concentrations used in RET experiments involving bipolar lipids. It has been found that fluorescence is very little affected by high osmolarities.

In this work we succeeded in obtaining for the first time vesicles made of pure P2. Moreover, it was possible to form pure PLE vesicles; no addition of egg PC or increase in the amount of P2 [21] was necessary.

The RET assay was used to monitor the calcium-induced lipid mixing in sonicated vesicles composed of PLE, P2, PLE/PC and pure PC. PC vesicles were chosen as a control since they are known to be unable to undergo a fusion process in the presence of Ca^{2+} [22,23]. At room temperature, bipolar lipid vesicles did not show any change in transfer efficiency at calcium concentrations as high as 60 mM. On the other hand, raising the temperature to 60°C induced lipid mixing. Fig. 2a shows the kinetic behaviour of the efficiency of energy transfer in the presence of 15 mM Ca^{2+} at 60°C. Control experiments were also performed in the absence of calcium, in order to test the possibility of probe exchange due to vesicle collision and/or temperature-induced lipid mixing (Fig. 2b). It is worth noting that the difference in the initial level of transfer efficiency, obtained for bipolar and monopolar lipids, is mainly due to the different dilution of the probes [11]. In fact, the molar ratio between probed and unprobed lipids was held constant, but a bipolar molecule is equivalent to two monopolar molecules.

In the case of PLE and P2, the kinetic behaviour is quite different in the presence and in the absence of Ca^{2+}. In the presence of Ca^{2+} the transfer efficiency decreases until a saturation is reached, indicating that calcium-induced lipid mixing has occurred. On the other hand, without calcium a slow drift is observed, which can be interpreted as due to temperature induced membrane interaction. Lipid mixing in the presence of Ca^{2+} occurs also for PLE/PC, while in the absence of Ca^{2+} almost no effect is observed. In both cases no effect is observed in egg PC; the very slight change is probably due to probe exchange caused by vesicle collision. Comparing PLE and PLE/PC vesicles, it can be observed that the extent of lipid mixing decreases by increasing the PC content in the vesicles. This inhibitory effect of egg PC has already been observed in other monopolar systems [22]. Experiments performed increasing the ratio between probed and unprobed vesicles showed a similar kinetic behaviour, although the change in efficiency was smaller, as expected and already observed in other vesicle systems [11].

In the case of PLE/PC 1:2 vesicles the effect of the

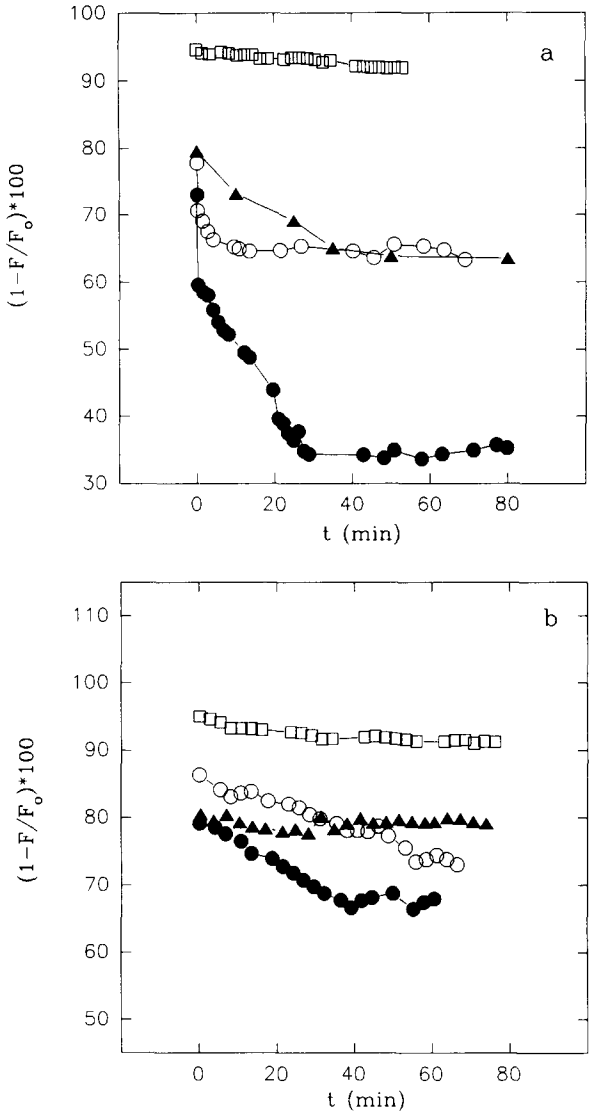

Fig. 2. Efficiency of energy transfer as a function of time at 60°C: (a) in the presence of 15 mM Ca^{2+} and (b) in the absence of Ca^{2+}. Vesicles were made of □, egg PC; ▲, PLE/PC 1:2 m.r.; ○, P2; ●, PLE. The error bars are not indicated since they are smaller than the point dimensions. In all figures the solid line has been drawn only to make it easier to read the graph.

ionic strength of the buffer on lipid mixing was also studied. For vesicles in distilled water, the efficiency of energy transfer as a function of calcium concentration reached a limiting value at approx. 5 mM Ca^{2+}, while for vesicles in buffer containing 150 mM NaCl this value was reached at approx. 10 mM Ca^{2+} (results not shown).

To test the effect of high osmolarities on vesicle size and stability, absorbance measurements were performed according to the method reported in Ref. [24]. The results showed that osmotic shocks caused by osmolarities in the typical range of fusion and release experiments had no effect on vesicle turbidity and therefore it is safe to work in this concentration range.

Once it has been established that calcium is able to induce lipid mixing at 60°C in vesicles comprised of P2, PLE and mixtures of PLE with lamellar-phase lipids (PLE/PC, PLE/P2), the second goal has been to test whether in these conditions not only lipid mixing, but also vesicle fusion with mixing of aqueous contents occurred. To this purpose the Tb/DPA assay has been employed. We found that in the presence of 15 mM Ca^{2+} no aqueous contents mixing occurs in vesicles made of PLE/PC, PLE/P2 and P2 even at temperatures as high as 85°C. On the other hand, in the same conditions PLE vesicles undergo a real fusion process with contents mixing. In fact, the fluorescence peaks at 490 nm and 545 nm of the $Tb(DPA)_3^{3-}$ complex [12]

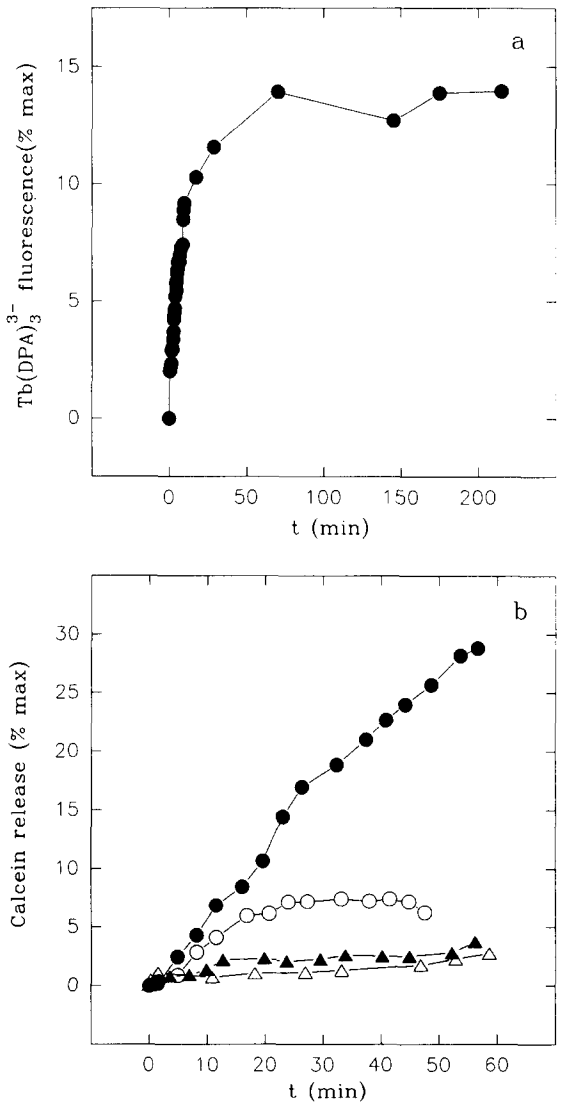

Fig. 3. (a) Fluorescence of the complex $Tb(DPA)_3^{3-}$ (% of max) as a function of time at 60°C after the addition of 15 mM Ca^{2+} to a 1:1 mixture of PLE vesicles containing, respectively, Tb and DPA. (b) Calcein release (% of max) as a function of time at 60°C for vesicles made of ○, ●, PLE; △, ▲, P2, in the absence (empty points) and in the presence (filled points) of 15 mM Ca^{2+}.

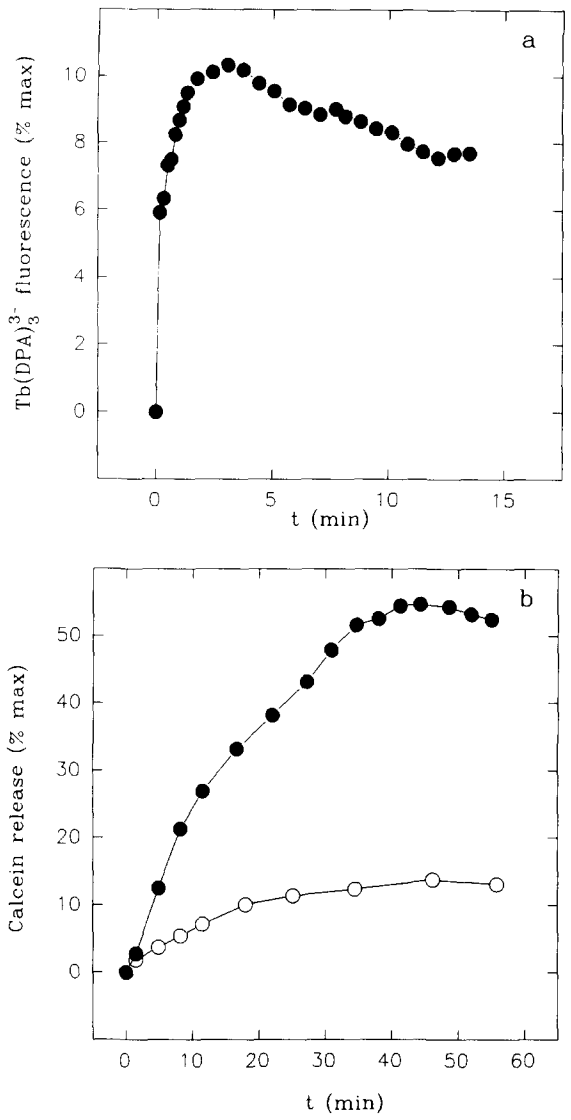

Fig. 4. (a) Fluorescence of the complex $Tb(DPA)_3^{3-}$ (% of max) as a function of time at 70°C after the addition of 15 mM Ca^{2+} to a 1:1 mixture of PLE vesicles containing, respectively, Tb and DPA. (b) Calcein release (% of max) as a function of time at 70°C for PLE vesicles ○, in the absence and ●, in the presence of 15 mM Ca^{2+}.

appear subsequent to 15 mM calcium addition. The kinetic behaviour of this fusion process at $T = 60$°C and $T = 70$°C is shown in Figs. 3a and 4a. From a comparison of these figures it appears immediately that at 70°C the kinetics of the process is much faster. The extent of fusion is on the same order of magnitude; in both cases, however, it is nearly four times smaller than in PS. In the absence of Ca^{2+} no contents mixing was observed under the same temperature conditions.

Calcein release for PLE vesicles in the presence as well as in the absence of calcium is included (Figs. 3b and 4b) in order to test vesicle leakage. The results show a much larger increase in calcein release in the presence of calcium. A comparison between the kinetics of fusion and release at 70°C indicates that while the first process is completed in less than 5 min, release occurs for a much longer time. Therefore, release is not only due to the fusion process, but also to a change in membrane stability or integrity after Ca^{2+} addition. On the other hand, vesicles undergoing lipid mixing but not fusion, such as P2 and PLE/PC, show an extremely low amount of leakage, around 3%, both in the presence and in the absence of calcium (see Fig. 3b for P2).

3.2. Surface tension measurements

Before measuring the surface tension in the bipolar lipid systems we repeated measurements of surface tension of monolayers of PS as a function of calcium concentration. The agreement with literature data [16,17] is satisfactory.

The surface tension of monolayers of PLE was measured as a function of calcium additions in various concentrations up to 50 mM. The effect of the calcium additions on the surface tension was below 0.5 dyn/cm, the noise level of the system. This level, considerably larger than the accuracy of the measurement, was mostly due to the insertion of the syringe through the monolayer surface for the calcium additions: adding distilled water, or even inserting and withdrawing the empty needle had an effect comparable to a calcium addition.

We conclude that the addition of calcium in concentrations up to 50 mM and temperatures of 60°C had no measurable effect on the surface tension of PLE monolayers.

4. Discussion

It has been proposed that membrane fusion is driven by an increase in the surface free energy of the membrane [16,17]. In divalent cation-induced fusion, this increase is due to cation binding to the negatively charged lipid polar headgroups, which become dehydrated and bridged together, with the consequent exposure of a portion of the hydrocarbon core to the water phase. To have fusion it is necessary that the interaction energy of the apposed membranes is sufficient to provide the work required to remove the bound water from their surfaces. This implies an increase in γ which, from theoretical calculations, has to be around 20 dyn/cm [16]. The smaller changes observed experimentally in phosphatidylserine monolayers (8 dyn/cm at the threshold cation concentration which induces vesicle fusion) are explained by the lack of membrane curvature, which has an effect in increasing the surface tension in vesicles [16].

Our results show that upon calcium addition no increase in surface tension has been obtained in mono-

layers made of PLE. In principle, this might be due to the fact that the binding sites for calcium are inaccessible from the water phase; for example, this could occur if the disposition of most of the phosphorous groups were on the air side of the monolayer. The limiting area just before collapse indicates that in the case of PLE most of the molecules stand upright, with one polar group on the water side and the other one on the air side [25]. The chemical structure of the lipids suggests that calcium binds to the phosphomyoinositol groups which are present in fractions P1 and P2 of PLE (Fig. 1). The P1 fraction, which is a monosubstituted component, has phosphomyoinositol on the water side because of the difference in polarity between the two ends of the molecule. Experiments designed to greatly increase the probability of having phosphomyoinositol on the water side by forming monolayers with P1-enriched PLE, still gave no significant change in γ. Therefore we can conclude that in our system surface tension changes do not play a significant role in the fusion mechanism. This result is consistent with previous findings which show that for long chain saturated lipids the surface area is already minimal and therefore Ca^{2+} does not induce a change in surface tension [26].

The resonance energy transfer results indicate that Ca^{2+} promotes lipid mixing in vesicles comprised of PLE, P2, PLE/PC (Fig. 2a) and PLE/P2 (results not shown). However, the change in transfer efficiency is much larger in PLE with respect to the other three systems. Light scattering measurements show a similar behaviour: in fact, subsequent to Ca^{2+} addition, a 10-fold increase in scattered light is observed in PLE vesicles, while a 4-fold increase occurs for P2. All these findings are fully explained by the experiments on aqueous contents mixing, which showed that only PLE vesicles are able to undergo a real fusion process triggered by Ca^{2+}.

From the point of view of phase behaviour there is a noticeable difference between P2 and PLE; in fact, P2 displays always the lamellar phase, while PLE shows a more complex phase diagram. X-ray diffraction data indicate that at high degree of hydration a transition between the lamellar (L_α) and the cubic (Q) phase occurs around 80°C [7]. Therefore, the presence of a L_α–Q transition seems to be a crucial factor to get fusion induced by Ca^{2+}. In fact, although such transition is observed for PLE in bulk at 80°C, in vesicles the molecules could have local nonlamellar arrangements at temperatures as low as 60°C. Indeed, much faster fusion kinetics are observed at 70°C (Fig. 4a). The importance of polymorphism is stressed by the results obtained on mixtures containing egg PC or P2. In fact, it is known that addition of PC to a lipid mixture stabilizes the lamellar phase [27]; a similar effect could occur in PLE/PC vesicles as well as in PLE/P2 vesicles and this would explain the lack of fusion in these systems. Therefore, whatever the molecular mechanism of fusion may be, we may conclude that vesicles made of a bipolar lipid assuming only the lamellar phase, such as P2, are unable to fuse; furthermore, the presence of a component which favours the lamellar phase (as in PLE/PC or PLE/P2) hinders the process, as already observed in monopolar systems [22]. A comparison of release in PLE and P2 vesicles (Fig. 3b) in the absence of Ca^{2+} indicates that the permeability to calcein is higher in PLE. This fact may be surprising since X-ray diffraction experiments have shown that in the lamellar phase the hydrocarbon layer is thicker in PLE than in P2 [8]. However, the higher constraints on P2 molecules, which are anchored to both aqueous interfaces, make the membrane more rigid. Therefore, packing of molecules rather than membrane thickness is the main factor determining membrane permeability in bipolar lipid vesicles.

In monopolar lipid systems the role of surface charge in regulating intervesicle contact has been widely elucidated [28]. A comparison of experiments performed on P2 and PC vesicles shows that lipid mixing occurs only in P2; this is because of the presence in P2 of negative charges able to bind calcium. A further indication of the importance of negative surface charges is provided by experiments performed at different ionic strengths. The results indicate that competition between monovalent and divalent ions clearly affects the extent of lipid mixing in PLE/PC vesicles at a fixed calcium concentration. For instance, at 5 mM Ca^{2+} the transfer efficiency is 1.5-times higher in the presence than in the absence of 100 mM NaCl in the buffer (results not shown).

Lipid mixing is also strongly affected by temperature. Experiments performed on P2 and PLE/PC vesicles have shown that Ca^{2+}-induced lipid mixing occurs only at temperatures higher than 50°C. In addition, when lipid mixing occurs, the amount of leakage monitored by calcein release is extremely low (approx. 3% of the maximum at 60°C), and it is comparable to that observed in the absence of calcium. Therefore, the aggregates which are formed retain separate aqueous contents, although vesicles stick together mixing their membrane lipids. This kind of structure seems to be rather unusual, although already observed in monopolar lipid systems, such as PG vesicles in the presence of calcium [29] and PA/PC vesicles in the presence of magnesium [30]. A better understanding of the nature of the structures formed in all these systems can be given by freeze-fracture experiments, which will be the object of a future work. Preliminary experiments using poly(ethylene glycol) indicate that with this strong fusogenic molecule [31], the fusion process can occur even at room temperature in most bipolar systems (Cassinadri et al., in preparation).

It could be difficult to visualize on a molecular scale

both the processes of bipolar lipid mixing and fusion of membranes formed entirely by bipolar lipids. To this purpose, it might be useful to recall that monosubstituted molecules can behave similarly to monopolar lipids: in fact, the unsubstituted glycerol can partition in the apolar core [7], a behaviour which confers a higher degree of plasticity to the membrane. The fact that only PLE undergoes a real fusion process (with mixing of aqueous contents) is a very intriguing result, since this is the native mixture present in the membrane of *Sulfolobus solfataricus*, and might have important physiological implications in vivo.

Acknowledgments

The authors express their thanks to G. Menestrina and G. Belmonte for advice in the filtration procedure and to E. Pagnotta and A. Ottombrino for technical help in the production and purification of bipolar lipids. Thanks are also due to G. Gaggero for mechanical improvement of the fluorescence apparatus. This work has been supported by the Italian Ministry of University and Scientific Research (MURST) 60% and 40% grants and by CNR grant Progetto Finalizzato Chimica Fine II.

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