Biochimica et Biophysica Acta, 732 (1983) 714–718
Elsevier

BBA Report

BBA 70110

VESICLES FROM MIXTURES OF BIPOLAR ARCHAEBACTERIAL LIPIDS WITH EGG PHOSPHATIDYLCHOLINE

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(Received April 18th, 1983)

Key words: Liposome; Archaebacterial lipid; Bipolar lipid; Phosphatidylcholine; Asymmetric distribution; (Caldariella acidophila)

Bipolar lipids from the membranes of archaebacterium *Caldariella acidophila* can form small unilamellar liposomes, when sonicated from lipid mixtures containing at least 25 mol% egg phosphatidylcholine. With increasing contents of archaebacterial lipid the inner radius of highly sonicated vesicles increases (from approx. 90 Å to approx. 160 Å) concomitant with an enhanced asymmetric distribution of the phosphatidylcholine molecules towards the outer face of the lipid bilayer membranes.

The lipids isolated from the membranes of the thermophilic archaebacterium *Caldariella acidophila* are bipolar and consist of two non-equivalent polar heads linked by two C_{40} alkylic chains [1,2]. The unusual bipolar structure of these archaebacterial lipids, which are essentially macrocyclic tetraethers, impose constraints on the architecture of the membranes which they form. If they do not bend and their two polar groups are located on the two opposing sides of the membrane they are bound to resist curving and formation of closed vesicles. Addition of unipolar lipids is expected to restore the ability to form closed vesicles with their size and their radius of curvature depending on the molar ratio of the lipids in the mixture.

In order to investigate the dependence of these structural constraints on the lipid composition we tried to prepare sonicated vesicles from different mixtures or archaebacterial lipid and egg phosphatidylcholine (PC) following standard procedures [3]. Archaebacterial lipids and PC were mixed in the desired molar ratios in chloroform/methanol (1 : 1, v/v), the organic solvent was removed under N_2 and aqueous buffer (150 mM NaCl/10 mM Tris, pH 7.5) was added to the dried lipid films, to yield liposome suspensions of 1 mM. Large multilamellar liposomes, obtained after vigorous vortex-mixing at 50–60°C, were transformed into small unilamellar vesicles, either by sonication of the liposome suspension for 15 min at 50–60°C, under a continuous N_2 stream in a Branson Type 12 tip sonifier equipped with a microtip, or by sonication in bath sonifier (Laboratory Supplies, Hicksville, NY) for 10 min. Contaminations from the titanium tip (in the first case) and larger, non-sonicated liposomes or liposome aggregates were removed by centrifuging the small unilamellar vesicle suspension for 5 min in an Eppendorf microfuge at $10000 \times g$. Following this last centrifugation step (lipid recovery at least 95%), the small unilamellar vesicle suspensions were sealed under N_2 and left for at least 1 h at 60°C, to facilitate annealing processes [4]. To determine the entrapped volume of these liposomes,

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Abbreviations: PC, phosphatidylcholine; ANS, 8-anilino-1-naphthalenesulfonic acid.

we used radiotracers, [^{14}C]dipalmitoylphosphatidylcholine (0.1 μCi) and [^{3}H]inulin (2 μCi) (both obtained from New England Nuclear, Boston, MA, U.S.A.) as markers for the lipid phase and the aqueous space respectively. The labelled liposomes with their entrapped contents were separated from the non-entrapped inulin by passing 0.3 ml liposome suspensions over a Sephadex G-75 column (0.5 × 18 cm). The radioactivity of the eluant was analyzed in a PLD Tricarb scintillation counter (Packard Instr. Co.) using Triton-Toluene as scintillation fluid. The entrapped volume was expressed either per mole lipid or alternately per mole equivalent taking a mole archaebacterial lipids as 2 mole equivalents. The latter approach seems to be more appropriate, since the molecular weight of archaebacterial lipids is 7% less and the molecular volume is about 4% more than twice the molecular weight or volume, respectively, of PC. The entrapped volume per mole lipid or mole equivalent is presented in Fig. 1 as a function of lipid composition. It is evident from this figure that the entrapped volume of tip sonicated lipid vesicles increases gradually from about 0.35 l/mole for pure PC vesicles till about 1.35 l/mol or 0.77 l/mol equiv. of lipid mixture containing 75% archaebacterial lipids, 25% PC. The entrapped volume of the bath sonicated vesicles varies between 0.55 l/mol for pure PC and about 1.25 l/mol or 0.83 l/mol equiv. for vesicles prepared from 50% archaebacterial lipids and 50% PC. At lower molar percentages of PC, no closed vesicles seem to be obtained even by prolonged tip-sonication, since no entrapped inulin could be observed eluting in the void volumes.

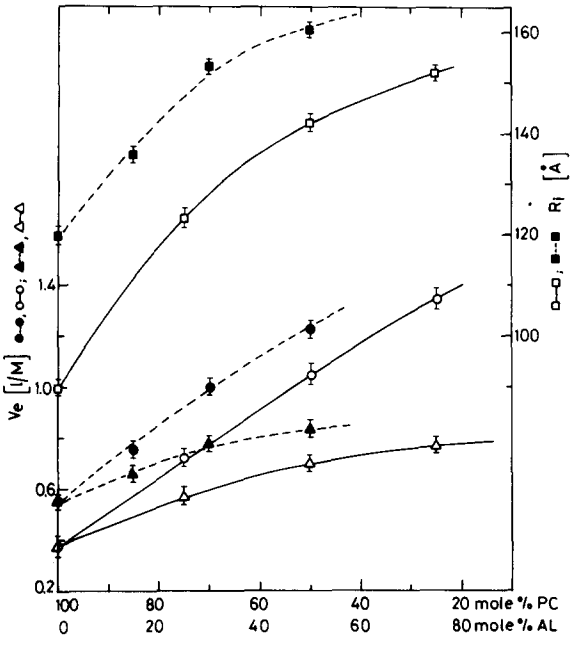

Fig. 1. Entrapped volumes (V_e) and inner radii (R_i) of sonicated unilamellar vesicles prepared from mixtures of archaebacterial lipid (AL) and egg phosphatidylcholine (PC) as function of the lipid composition. The data for V_e are plotted for the tip-sonicated (empty symbols) and for the bath-sonicated (filled symbols) vesicles. The entrapped volumes are expressed as liter per mole lipid (circles) or as liter per mole-equivalent lipids (triangles). The inner radii, R_i, as calculated from Eqn. 1, are plotted for the tip-sonicated vesicles (empty squares) and for the bath-sonicated vesicles (filled squares). Mean values ± experimental error from two independent preparations.

To obtain the size of the vesicles from their entrapped volume one has to know, besides the molar volumes, also the thickness of the bilayer and the thickness of the hydration layer which does not dissolve inulin. According to Mason and Huang [5], the thickness of the bilayer of a PC vesicle is 37 Å and the thickness of the hydration layer is between 5 Å and 6 Å. The entrapped volume per lipid equivalent V_e is then

$$V_e = \bar{v}_{eq.}(R_i - 6)^3/(R_i + 37)^3 - R_i^3 \qquad (1)$$

The values of the inner radii, R_i, as calculated by Eqn. 1 with $\bar{v}_{eq} = 0.81$ are presented in the upper curves of Fig. 1. In the case of tip-sonicated vesicles they vary between 90 Å for pure PC to 152 Å for vesicles containing 75 mol% or 85 mol equiv.% archaebacterial lipids. In the case of bath sonicated vesicles, R_i increased from 120 Å for pure PC to 161 Å for vesicles containing 50 mol% or 66.7 mol equiv.% of archaebacterial lipids.

Addition of at least 25 mol% PC will allow curvature of the bilayer in order to form closed vesicles by sonication. An important aspect of the membrane structure is whether or not the PC molecules are distributed equally between the inner and the outer monolayer of the bilayer membrane. The transbilayer symmetry of PC in membranes with various archaebacterial lipid contents, was investigated fluorimetrically. The fluorescence intensity of an aqueous solution of 8-anilino-1-

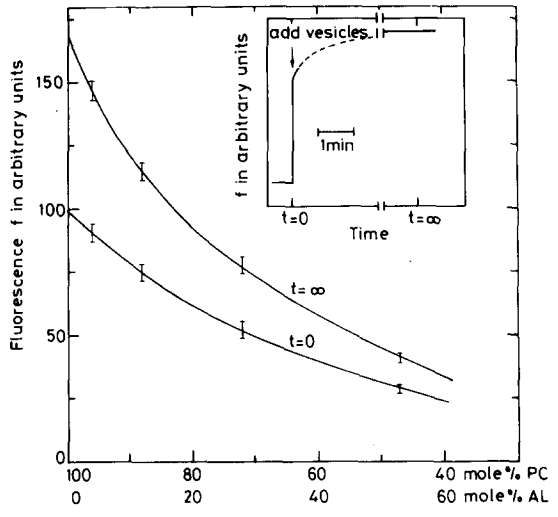

Fig. 2. Fluorescence, in arbitrary units, of 4 μM 8-anilino-1-naphthalenesulfonic acid (ANS) in the presence of bath sonicated vesicles containing different mixtures of PC and archaebacterial lipids (total lipid concentration 130 μM), $t = 0$, initial fluorescence upon addition of the vesicles, $t = \infty$, fluorescence after prolonged equilibration (2 h) of the dye (cf. inset and text). ANS fluorescence was measured at $t = 22°C$ in a Perkin-Elmer 1000 M fluorimeter, using a 367 nm bandpass filter for excitation and a 450 nm cut-off filter for observing the emitted light. The curves are eye-fitted through the averaged results ± S.D. from three independent experiments performed at least in duplicate.

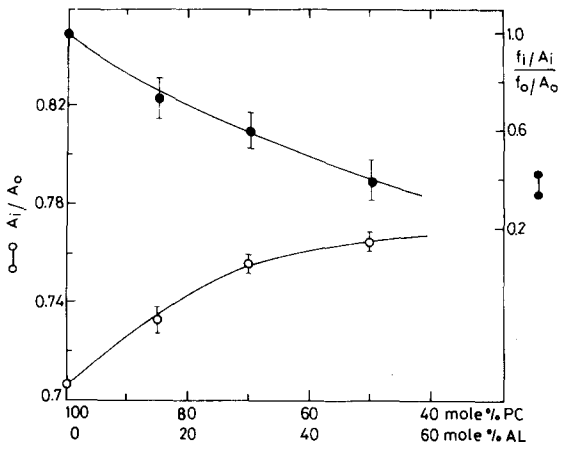

Fig. 3. Inner/outer area ratio, A_i/A_o, of bath-sonicated vesicles prepared from various mixtures of PC and archaebacterial lipids (AL) (○). The ratio of the PC concentrations in the inner and the outer monolayers of the membranes is proportional to $(f_i/A_i)/(f_o/A_o)$ (●). f_i and f_o denote the ANS fluorescence originating from the inner and the outer monolayers, as obtained from Fig. 2.

naphthalenesulfonic acid (ANS) is strongly enhanced upon addition of PC [6], but not of archaebacterial lipids. The fluorescence of ANS in the presence of archaebacterial lipids is by two orders of magnitude lower than in the presence of the same amount of PC. Plotted in Fig. 2 is the ANS fluorescence in the presence of vesicles containing increasing amounts of archaebacterial lipids. As seen from Fig. 2, the ANS fluorescence decreases strongly with the archaebacterial lipids content in the liposomes. The instantaneous fluorescence increase upon adding the liposomal vesicles to the stirred ANS solution ($t = 0$) was considered to result from the interaction of the ANS with the outer bilayer (lower curve in Fig. 2). The fluorescence increases slowly due to ANS penetration into the inner bilayer (see inset). The upper curve in Fig. 2 gives the fluorescence after a time (over 2 h) ($t = \infty$) long enough to complete penetration.

In the case of pure PC, the fluorescence ratio from the inner and the outer layer is considered to equal the inner/outer area ratio, A_i/A_o, at the planes where the chromophores are located. These lanes do not coincide with the spherical shells of the radii R_i and $R_o = R_i + 37$ (Eqn. 1). For $R_i =$ 120 Å, the radii of the spherical planes of sites of the chromophores have to be about 127 Å and 150 Å, respectively, to render $A_o/A_i = 0.715$. This indicates that the chromophores are buried in the low dielectric domain near the polar boundary which is in line with previous findings, using other model membranes [6]. We calculated the A_i/A_o ratios in the mixed lipid vesicles from the R_i values assuming that the distance of 23–24 Å between the two planes of the chromophore sites is preserved (Fig. 3). The ratio $(f_i/A_i)/(f_o/A_o)$, which is a function of the PC concentration ratio inside to outside, is presented in the same Fig. 3 for different PC concentrations in the liposomes. Taking the dependence of the ANS fluorescence on the PC concentration in the lipid mixtures from Fig. 2, one can estimate the PC concentrations at the inner and the outer layer. Their ratios are about 0.8 for 85% PC, about 0.65 for 70% PC and about 0.50 for 50% PC. As the total concentration of PC in the

lipid mixture decreases it accumulates more preferentially in the outer layer.

Another approach for determination of the internal-volume-to-area ratio of the vesicles obtained from the different lipid mixtures was based on the half-time determination of carboxyfluorescein leakage from the vesicles. The fluorescence of liposome contained carboxyfluorescein entrapped at high concentrations is fully quenched and it increased proportionally with the release into the solution [7]. Under the assumption that the bilayer permeability of carboxyfluorescein does not substantially depend on lipid composition and on the curvature, the half-time of carboxyfluorescein release should be proportional to the volume to area ratio or to the inner radius of the vesicles. Tip-sonified carboxyfluorescein-containing liposomes were prepared essentially as described above. The aqueous buffer (10 mM Tris, pH 7.5) added to the dried lipids contained 100 mM carboxyfluorescein, purified as described elsewhere by one-step elution on Sephadex LH-20 columns [8]; Liposome-entrapped carboxyfluorescein separated from free dye upon eluting 200 μl aliquots of the liposome suspension over a Sephadex G-50 columns (0.5 × 10 cm) with 150 mM NaCl/10 mM Tris (pH 7.5) as eluant and collecting the liposomes eluted in the void volume. The measurement of carboxyfluorescein leakage from the liposomes and determination of the half-times for carboxyfluorescein release were done as previously described [8,9]. The results presented in Fig. 4 show that the half-time of the release from vesicles formed from a lipid mixture containing 25% PC is about 3-times the half-time of the release from pure PC vesicles. In agreement with the radiotracer experiments, we find that: (1) no entrapped carboxyfluorescein is found in lipid mixtures containing less than 25 mol% PC; and (2) the total fluorescence of liposome entrapped carboxyfluorescein obtained after detergent disrupture of the liposomes is approx. 3-fold higher for vesicles containing 25 mol% PC than for pure PC vesicles (data not shown). An additional parameter, besides the increase in vesicle size, which could slow down the rate of carboxyfluorescein leakage from PC-poor liposomes, is the decrease in membrane fluidity in archaebacterial lipid-rich liposomes. The microviscosity of the lipid bilayers, as determined by the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene [10] or of ANS [6] increases initially with increasing molar percentage of archaebacterial lipids and reaches a constant value at

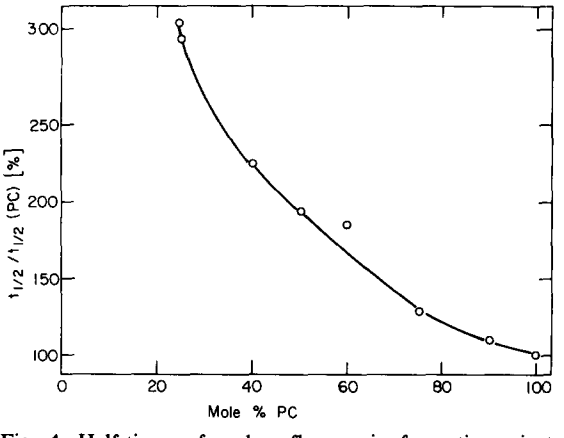

Fig. 4. Half-times of carboxyfluorescein from tip-sonicated liposomes prepared from mixtures of archaebacterial lipid and egg PC. To facilitate comparison between four independent experiments, the $t_{1/2}$ values are normalized to the release of carboxyfluorescein from pure PC vesicles, which was determined as internal control in each experiment. $t = 25°C$.

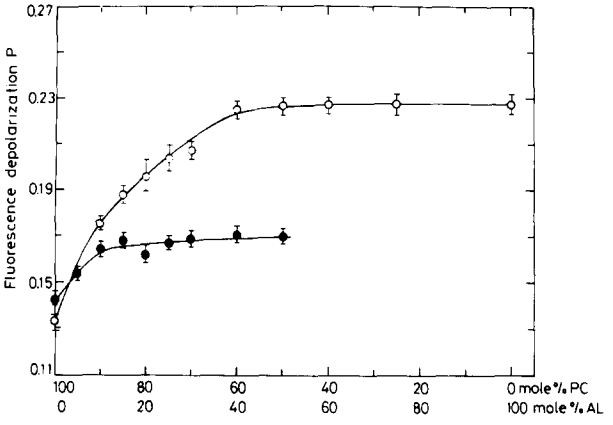

Fig. 5. Fluorescence depolarization, P, of bath-sonicated vesicles as a function of the lipid composition. P values, measured in an Elscint MV 1 microviscosimeter at $t = 21°C$, were obtained upon labelling the vesicles with either ANS (●) or diphenylhexatriene (○). The diphenylhexatriene experiments were performed on original preparations and on aliquots eluting in the void volume of a G-75 column (see text) without any detectable differences in the P values of the samples. Data: mean ± S.D. of three independent experiments.

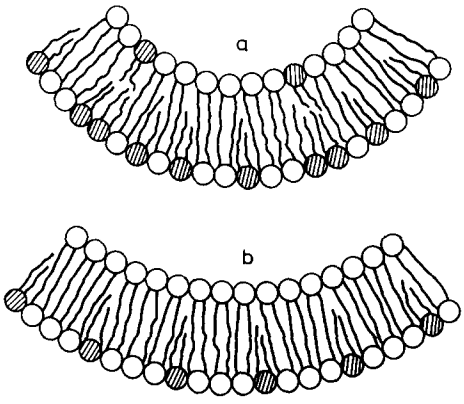

Fig. 6. Model of the bilayer structure of vesicles composed of bipolar archaeolipid-poor (a) and archaeolipid-rich (b) mixtures with egg PC. With increasing archaebacterial lipids content, the unipolar PC molecules are oriented towards the outer face of the lipid membrane.

35% and 15% archaebacterial lipids and reaches a constant value at 35% and 15% archaebacterial lipids respectively (Fig. 5). As in this region the variation in $t_{1/2}$ is small (cf. Fig. 4), permeability changes with fluidity do not seem to be an important factor. Fluorescence polarization of diphenylhexatriene is an excellent tool for determining lipid composition between 0 and 40% archaebacterial lipids. By this criterion, there was no difference in lipid composition of the vesicles as obtained immediately after preparation or after elution in the void volume of the G-75 column.

It is evident from these results that addition of monopolar lipids is required to obtain closed vesicles from the bipolar lipids. An apparently similar situation is observed upon formation of closed bilayer vesicles from lipid mixtures containing up to 75% phosphatidylethanolamine, which by itself does not arrange to form liposomes, but rather hexagonal structures [11]. However, the structural constraints are probably different in the two cases. For archaebacterial lipids, the minimal amount of PC required to obtain closed vesicles is approx. 25 mol%. The PC molecules have to be preferentially on the outside of the vesicle to perform the task. If the area per PC is the same as that of the bipolar lipid, the minimal possible value for R_i is obtained when all the PC molecules are on the outside, as illustrated in Fig. 6. In the presence of 25% PC, this minimal value of R_i would be $(R_i)_{min} = \delta/[(1 + n_{PC}/n_{AL})^{\frac{1}{2}} - 1] \approx 240$ Å for $\delta = 37$ Å. This value is by more than 50% larger than the largest values calculated from the entrapped volume data. This difficulty can be resolved if (a) the cross-sectional area of PC is larger than that of the bipolar lipid and (b) if the asymmetric bipolar lipid is oriented with its larger headgroup toward the outside, as occurs in the plasma membranes of the archaebacterium [11]. In any event, it is clear that the molecular structure of the lipid components influence and may even determine the architecture of the membrane superstructure.

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