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POLYMORPHIC PHASE BEHAVIOUR OF CARDIOLIPIN FROM BOVINE HEART AND FROM *BACILLUS SUBTILIS* AS DETECTED BY ^{31}P-NMR AND FREEZE-FRACTURE TECHNIQUES

EFFECTS OF Ca^{2+}, Mg^{2+}, Ba^{2+} AND TEMPERATURE

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The structures formed by aqueous dispersions of cardiolipin isolated from bovine heart and *B. subtilis* have been studied by ^{31}P-NMR and freeze-fracture electron microscopy. The sodium salts of both cardiolipins form bilayers. The Ca^{2+}, Mg^{2+} and Ba^{2+} salts undergo well-defined bilayer → hexagonal (H_{II}) transitions, the temperature of which is dependent on the cation involved and the fatty acid composition of the cardiolipin.

It is well-established that a large part of the lipids in biological membranes are arranged in a lipid bilayer. Each membrane, however, contains substantial amounts of lipids which prefer non-bilayer configurations of which the hexagonal H_{II} phase is the most common one [1]. There is now considerable evidence to suggest that non-bilayer lipids and the structures they form can play important functional roles in membrane processes such as fusion and transbilayer transport of lipids [1]. Of particular importance in this respect are lipids which can undergo isothermally bilayer → non-bilayer transitions. Cardiolipin found in the inner membrane of beef heart mitochondria is an example of such a lipid. In the absence of divalent cations the bilayer phase is preferred, whereas in the presence of Ca^{2+}, at physiological temperatures, this lipid is organized in the hexagonal H_{II} phase [2,3]. Cardiolipin can also be found in large quantities in the membrane of Gram-positive bacteria [4]. This cardiolipin which is synthesized via a different pathway [5] has a much more saturated fatty acid composition [4]. It has been suggested that the bacterial cardiolipin might play a more structural role [6] as compared to the mitochondrial cardiolipin which appears to play important functional roles in some membrane processes [7] and which is tightly bound to [8,9] and regulates the activity of various mitochondrial enzymes [8–10].

Studies with different phosphatidyl ethanolamines have revealed that the temperature of the bilayer → H_{II} transitions is strongly dependent on the fatty acid composition of the lipid in such a way that the temperature is increased with decreasing unsaturation [11]. As the possible differences in the functional roles of the two types of cardiolipin might be related to differences in phase behaviour of these lipids, we have investigated in

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this study by ^{31}P-NMR and freeze-fracturing the polymorphic phase behaviour of these cardiolipins in particular as a function of the type of cation present. It will be shown that the Ca^{2+}, Mg^{2+} and Ba^{2+} salts undergo well-defined bilayer $\rightarrow H_{II}$ transitions which occur at a much higher temperature for the bacterial cardiolipin.

Total lipids were isolated from bovine heart or logarithmically grown *B. subtilis* cells (grown as described in Ref. 4) according to Bligh and Dyer [12] or as described in Ref. 4, respectively. Neutral lipids and some residual protein present in the bovine heart lipid extract were removed by acetone and ethanol precipitation, respectively. The negatively charged phospholipids were precipitated by barium acetate, whereafter the barium salts of cardiolipin were obtained in a pure form by quantitative HPLC on silicagel. Cardiolipin was converted into its sodium salt by treatment with Na_2SO_4. Details of this isolation procedure will be published elsewhere. The lipid purity was over 99% as determined by HPTLC. The cation composition was measured by röntgen microanalysis by STEM (scanning transmission electron microscopy), and was found to be $>98\%$ Na^+. The fatty acid composition of the lipids was determined by GC of the fatty acid methyl esters and was (in mol%); bovine heart cardiolipin: 16:0 * (1%), 16:1 (1%), 18:1 (5.7%), 18:2 (88.5%) and 18:3 (3.8%); *B. subtilis* cardiolipin: 14:0 (1.6%), $15:0_{i+a}$ (49.9%), $16:0_i$ (5.4%), 16:0 (6.5%), 17:0 (16%), $17:0_a$ (15.1%), 18:0 (2.4%), 18:1 (0.2%), 18:2 (1.0%), and other minor branched chain and saturated fatty acids (1.9%). Thus, the bovine heart cardiolipin contains 98% unsaturated fatty acids, whereas the bacterial cardiolipin contains 98.8% saturated and branched chain fatty acids.

Lipid samples for ^{31}P-NMR were prepared by dispersing 50 μmol phospholipid, dried as a thin film from chloroform in 0.8 ml of 10% ^2H_2O containing 100 mM NaCl, 10 mM Tris-HCl buffer (pH 7.0) as described before [11]. Divalent cations were added as aliquots of a 100 mM solution. In some cases glycerol (30%, v/v) was added to the samples to prevent freezing. Freeze-fracture electron microscopy was performed as described before [13], 30% (v/v) of glycerol was added to the samples to prevent freeze-damage. DSC measurements were performed on a Perkin Elmer DSC 2, using 17 μl sample pans and a 5 K/min scan rate on 30% glycerol containing dispersions of the lipid in buffer as described elsewhere [18]. High power proton noise-decoupled ^{31}P-NMR spectra were obtained at 36.4 MHz as described before [11]. To enhance the signal to noise ratio, the free induction decays were multiplied by an exponential function resulting in a 50 Hz line-broadening.

^{31}P-NMR is a convenient method to discriminate between bilayer and hexagonal type of structures. Lipids organized in extended bilayers give rise to broad asymmetrical spectra with a low-field shoulder and a high-field peak [1]. Figs. 1 A and E, show the ^{31}P-NMR spectra of aqueous

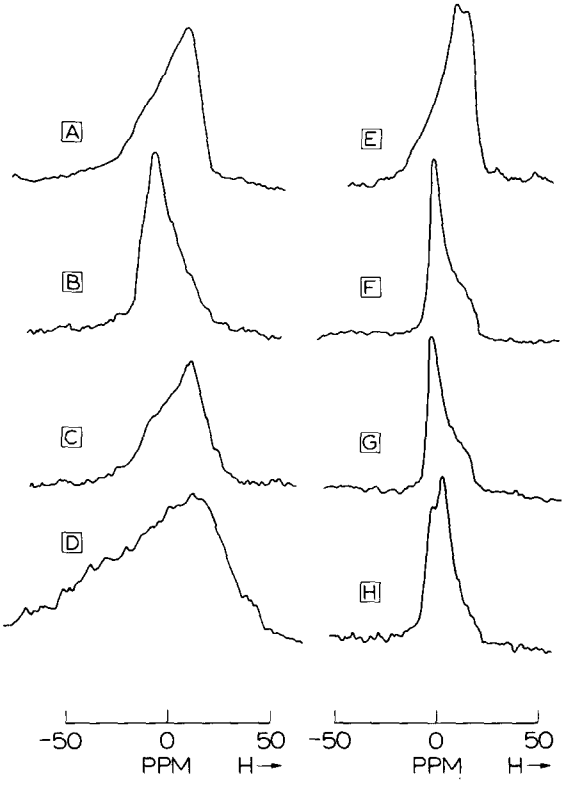

Fig. 1. 36.4 MHz ^{31}P-NMR spectra of aqueous dispersions of cardiolipin isolated from *B. subtilis* (A–D) and bovine heart (E–H) in the absence of Ca^{2+} at 25°C (A, E) or in the presence of 100 μl 1 M $CaCl_2$ at 50°C (B, F), 37°C (C, G) and −10°C (D, H). In experiments D, H 30% glycerol (v/v) was added. This amount of glycerol did not affect the bilayer to hexagonal H_{II} transition of the bovine heart cardiolipin-Ba^{2+} salt.

* Fatty acids are coded by chain length: number of double bonds, or presence of methyl branching (i=*iso*, a=*ante-iso*).

dispersions of the sodium salts of both cardiolipin species at 25°C. Both spectra have predominantly this 'bilayer' type of lineshape. The relatively large line-width of the spectrum of the bacterial cardiolipin most likely is due to the presence of some gel state cardiolipin as by calorimetry a broad reversible endothermic transition is observed from 10 to 30°C.

The proton decoupling power we use is insufficient to completely remove the dipolar ^{2}H-^{31}P coupling for lipids in the gel state. Below 25°C this line broadening is even much more pronounced (data not shown). In the ^{31}P-NMR spectrum of the bovine heart cardiolipin, a small isotropic component is present which might arise from some smaller vesicles in the preparation.

Addition of excess Ca^{2+} results in visual precipitation of the Ca^{2+} salts of both lipids. In agreement with previous data [2,3] the Ca^{2+} salt of the bovine heart cardiolipin above 0°C is organized in the hexagonal H_{II} phase. This can be inferred from the characteristic ^{31}P-NMR spectrum (Figs. 1 F–G), with reduced width and a reversed asymmetry which is caused by fast lateral diffusion of the lipid molecules around the pipes of the hexagonal phase [1]. Below 0°C an 'isotropic' component

Fig. '3. Freeze-fracture electron microscopy of the Ca^{2+}-salt of bovine heart cardiolipin quenched from −15°C. Final magnification: 100000×.

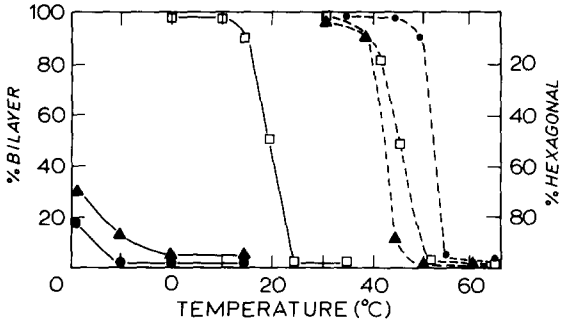

Fig. 2. Temperature dependency of the bilayer→hexagonal H_{II} transition of various cardiolipin salts. The salts were formed by addition of 100 μl of a 1 M solution of the metal chloride. ▲——▲, Ca^{2+} salt; ●——●, Mg^{2+} salt; and □——□, Ba^{2+} salt of bovine heart cardiolipin. ▲------▲, Ca^{2+} salt; ●------●, Mg^{2+} salt; and □------□, Ba^{2+} salt of *B. subtilis* cardiolipin. The fraction of lipid in the bilayer or hexagonal H_{II} phase was estimated from the ^{31}P-NMR spectrum by cutting and weighing of the different peaks. Except for the Ca^{2+} salt of bovine heart cardiolipin below 0°C the ^{31}P-NMR spectrum of which contained a small isotropic component (Fig. 1H) only 'bilayer' or 'hexagonal' type of ^{31}P-NMR spectra were observed.

appears in the ^{31}P-NMR spectrum (Fig. 1H), demonstrating rapid isotropic motion of part of the lipid molecules. As has been observed in many other non-bilayer lipid containing systems [1], the presence of this isotropic peak is associated with the occurrence of lipidic particles in freeze-fracture micrographs of this preparation (Fig. 3).

The Ca^{2+} salt of the bacterial cardiolipin above 50°C is also organized in the hexagonal H_{II} phase as can be inferred from the overall line-shape and chemical shift position of the dominant spectral feature (Fig. 1B). The reason for the larger line-width of this spectrum as compared to the spectrum of the Ca^{2+} salt of the bovine heart cardiolipin is not understood, but possibly could be due to a decreased lateral diffusion rate of the bacterial lipid. At lower temperatures, however, the bilayer phase is preferred, such that at the growth temperature of 37°C the lipid is almost completely organized in a bilayer configuration

(Fig. 1C). At even lower temperatures a strong line broadening is observed (see for instance Fig. 1D), which most likely is caused by a gel → liquid crystalline phase transition as a broad endotherm ranging from 10 to 35°C was observed by DSC. The full temperature dependence of the phase behaviour of the Ca^{2+}, Mg^{2+} and Ba^{2+} salts of both cardiolipins is shown in Fig. 2. In agreement with X-ray [2] and recent freeze-fracture data [15] also the Mg^{2+} and Ba^{2+} salts of bovine heart cardiolipin adopt hexagonal H_{II} phases. Transitions to lamellar structures occur below 25°C and −10°C, respectively. The Mg^{2+} and Ba^{2+} salts of the bacterial cardiolipin also undergo well-defined bilayer → hexagonal transitions, which occur, however, at much higher temperatures. The bilayer to hexagonal H_{II} transitions of the divalent cation-cardiolipin salts were reversible and showed less than 5°C hysteresis.

The preference of a lipid to adopt a particular phase can be understood in terms of the molecular shape of the lipid [1]. Molecules with an overall cylindrical shape will prefer a bilayer organization whereas cone-shaped molecules (polar head group at the smaller end of the cone) prefer inverted phases suchas the H_{II} phase or inverted micellar structures [1]. This molecular shape concept can also be used to rationalize our present findings. In aqueous dispersions of the sodium salt of cardiolipin the lipid is organized in a bilayer configuration which must be stabilized by electrostatic repulsion between the two charged phosphate groups in the molecule thereby maximizing the cross sectional area of the head group. In the presence of the divalent cation a complex is formed in which the phosphates are pulled together to form the divalent cation-cardiolipin complex which has a decreased head group area thus favouring hexagonal phase formation. This probably is accompanied by polar head group dehydration, analogous to the phosphatidylserine-Ca^{2+} complex [16]. At lower temperature due to an increased chain order the cross sectional area of the hydrocarbon chains decreases such that now the bilayer phase is preferred. Also the higher bilayer → H_{II} transition temperature of the more saturated *B. subtilis* divalent cation-cardiolipin complexes can be understood in those terms. The interplay between head group interactions and hydrocarbon chain packing is also apparent in the phase behaviour of the different cation-cardiolipin complexes. In the case of the bovine heart cardiolipin the barium salt has a much higher bilayer → H_{II} transition temperature than the Ca^{2+} and Mg^{2+} salts which most likely results from the large ionic size of Ba^{2+} (ion diameter Ca^{2+} = 1.98, Mg^{2+} = 1.32 and Ba^{2+} = 2.68 Å) leading to a relatively large head group, thereby stabilizing the bilayer configuration up to higher temperatures. For the bacterial cardiolipin apparently the hydrocarbon chain packing dominates the overall shape of the molecule as the differences in bilayer → H_{II} transition temperatures of the various salts are relatively small. That fully saturated cardiolipins prefer bilayer organization has been demonstrated for synthetic cardiolipins and their divalent cations salts by Rainier et al. [17].

Another interesting feature revealed by these experiments is that only in the case of the bovine heart Ca^{2+}-cardiolipin salt at low temperatures not the hexagonal H_{II} a bilayer phase but an 'isotropic' phase with associated lipidic particles is observed. This 'isotropic' phase is in mixed lipid systems found as a common intermediate between the bilayer and hexagonal H_{II} phase [1]. In this light it is also relevant to mention that the bilayer hexagonal H_{II} transition induced by increasing amounts of Ca^{2+} in bovine heart cardiolipin also proceeds via this intermediate phase [3]. Apparently the non-bilayer preference of the Ca^{2+}-cardiolipin salt can also be more easily expressed inthe formation of inverted micellar structures.

Finally, it should be noted that the bacterial cardiolipin under 'physiological' conditions prefers a bilayer organization supporting the hypothesis [6] that this lipid in bacterial systems might play a structural role.

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