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Biochimica et Biophysica Acta 1189 (1994) 225–232

BBA Biochimica et Biophysica Acta

Effect of divalent cations on lipid organization of cardiolipin isolated from *Escherichia coli* strain AH930

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(Received 20 July 1993)

Abstract

Escherichia coli strain AH930 is a lipid biosynthetic mutant, which is unable to synthesize phosphatidylethanolamine. Instead it produces large amounts of phosphatidylglycerol and cardiolipin and has an absolute requirement for certain divalent cations. Cardiolipin was isolated from this mutant strain and its interaction with divalent cations was studied by various biophysical techniques. Monolayer measurements showed that the cations decrease the molecular surface area of cardiolipin in the order $Ca^{2+} \approx Mg^{2+} > Sr^{2+} > Ba^{2+}$. ^{31}P-NMR and X-ray diffraction measurements demonstrated a comparable sequence for the ability of the cations to promote H_{II} phase formation in dispersions of the *E. coli* cardiolipin: Ca^{2+} and Mg^{2+} induced H_{II} phase formation at 50°C, Sr^{2+} at 75°C, while Ba^{2+} was found to be unable to promote H_{II} phase formation in the temperature range measured. Furthermore, all divalent cations were found to increase the temperature at which the transition to the liquid-crystalline phase takes place, which was below 5°C for the lipid in the absence of divalent cations. In the presence of Sr^{2+}, Mg^{2+} and Ba^{2+} and at 25°C two lamellar phases were observed, one corresponding to a liquid-crystalline phase, the other to either a gel or a crystalline phase. In the presence of Ca^{2+} at 25°C and even at 45°C no evidence for a liquid-crystalline phase was obtained and only a crystalline phase could be observed. The ability of the different cations to promote H_{II} phase formation in the isolated *E. coli* cardiolipin was found to correlate with their ability to support growth of the mutant strain (De Chavigny, A., Heacock, P.N., Dowhan, W. (1991) J. Biol. Chem. 266, 5323–5332), suggesting that cardiolipin with divalent cations can replace the role of phosphatidylethanolamine in the mutant strain, and that this role involves the preference of these lipids for organization in non-bilayer lipid structures.

Key words: Cardiolipin; Lipid polymorphism; Acyl chain packing; NMR; Small angle X-ray diffraction; Wide angle X-ray diffraction; (*E. coli*)

1. Introduction

Cardiolipins are a class of tetraacylphospholipids found mostly in mitochondrial membranes and in membranes of some bacteria (for reviews, see Refs. [1] and [2]). They have been of interest because of their involvement in functional membrane processes [3]. They are tightly bound to various enzymes [4–7] and regulate the activity of a number of proteins involved in transport processes across the membrane [6–9]. Furthermore, they display a strong polymorphic behavior and can undergo a transition from a lamellar phase to organization in the hexagonal H_{II} phase in the presence of certain divalent cations [10–12]. This ability of lipids to adopt non-bilayer structures is believed to be important for membrane functioning [13], which is supported by the observation that several organisms regulate their lipid composition to maintain a careful bal-

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Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; SAXD, small angle X-ray diffraction; WAXD, wide angle X-ray diffraction.

0005-2736/94/$07.00
SSDI 0005-2736(93)E0318-5

ance between the ratio of bilayer and non-bilayer lipids [14-16].

Studies on the phase behaviour of cardiolipin isolated from bovine heart, which is highly unsaturated [17], showed that a temperature dependent transition from a bilayer to a hexagonal H_{II} phase can be induced by divalent cations, with Ca^{2+} and Mg^{2+} being the most efficient, followed by Ba^{2+} [10]. This cation dependence is dependent on the fatty acid composition of the cardiolipin, because in cardiolipin isolated from *Bacillus subtilis*, which is much more saturated and contains branched acylchains, the transition to an H_{II} phase occurred at much higher temperatures, and at nearly the same temperature for each of the cations [10].

Nothing is known about the effect of divalent cations on the phase behaviour of cardiolipin from *Escherichia coli*. Yet this is particularly relevant in view of the proposed functional role of cardiolipin in the lipid biosynthetic mutant strain AH930 [18]. This strain is unable to synthesize phosphatidylethanolamine (PE), which is a typical non-bilayer preferring lipid and normally accounts for about 70–80% of the phospholipids in the *E. coli* membrane [19]. Instead the strain synthesizes large amounts of cardiolipin and phosphatidylglycerol (PG) and develops an absolute requirement for milimolar amounts of certain divalent cations [18]. This raised the intriguing possibility that cardiolipin with divalent cations can replace the role of PE in the mutant strain, and that it is the H_{II} phase promoting activity of the various cations which is important for their ability to support growth.

To gain insight into this possibility we first isolated and purified cardiolipin from the *E. coli* strain AH930. It will be shown that this strain offers a convenient source for *E. coli* cardiolipin, because of the large amount of cardiolipin produced and because of its relatively simple lipid composition, which facilitates purification. Next we investigated the interaction of this phospholipid with different divalent cations using monolayer techniques, ^{31}P-NMR, small angle X-ray diffraction (SAXD) and wide angle X-ray diffraction (WAXD). The results will be discussed in relation to the interaction of divalent cations with other cardiolipins and with negatively charged lipids in general, and next in relation to the reported [16,18] growth promoting properties of the different cations in strain AH930.

2. Materials and methods

2.1. Growth of bacteria

Escherichia coli cardiolipin was isolated from the mutant strain AH930 (*pss93::kan* [18]). Startcultures were prepared in 10 ml LB medium (10 g Bacto-tryptone (Sigma), 5 g yeast extract (Sigma) and 10 g NaCl per liter of culture), supplemented with 50 mM $MgCl_2$ at pH 7.4, by inocculation with fresh colonies from LB agar, containing 50 mM $MgCl_2$. After 24 h growth at 37°C these cultures were diluted 1/40 into fresh medium and grown for 16 h (final $OD_{660\ nm} \sim 0.8$). Cells (2.8 g wet wt/l culture) were harvested by low-speed centrifugation at 4°C and washed twice with ice-cold 0.9% (w/v) NaCl solution.

2.2. Isolation and purification of lipids

After resuspension of the cells in 0.9% (w/v) NaCl solution, the lipids were extracted according to Bligh and Dyer [20] and converted to their Ca^{2+}-salt form by using a modified Bligh and Dyer protocol, in which the aqueous phase contained 100 mM $CaCl_2$. The lipid composition was analyzed by TLC, using precoated TLC plates (Merck) impregnated with 1.2% boric acid in ethanol/water (1:1, v/v) and developed in chloroform/ methanol/ water/ ammonium hydroxide (120:75:6:2, by vol.) [21]. The main lipids in the extract of the AH930 cells were cardiolipin and PG. PE, which is normally the dominant lipid in *E. coli* was clearly absent. The extract furthermore contained a small percentage of PA and some unidentified front-material. The yield was about 40 mg of total lipid extract per liter of culture.

The lipid extract was then dissolved in a small volume of chloroform and applied to a silica (Polygosil 63–100 μm) column, which was first eluted with chloroform to remove neutral lipids and next phospholipids were eluted with chloroform/ methanol (1:1, v/v). The composition of the phospholipids was: 49 mole% PG, 49 mole% cardiolipin and 2 mole% PA, as determined by two-dimensional HPTLC on silica plates (Kieselgel 60, Merck) with chloroform/ methanol/ water/ ammonia (68:28:2:2, by vol.) as the first, and chloroform/ methanol/ acetic acid (65:25:10, by vol.) as the second eluens. The phosphate content in each spot was determined according to Rouser et al. [22].

The total phospholipid extract was next dissolved in a small volume of chloroform and applied to a silica column, preequilibrated with chloroform. Upon chromatography with chloroform/ methanol/ water (65:25:4, by vol.) cardiolipin eluted first from the column, followed by PG, contaminated with some cardiolipin. The cardiolipin fraction was found by 2D-TLC analysis to contain a minor amount of PA (< 0.4% on phosphate basis) as well as some other minor impurities, which appeared at the front upon elution on TLC plates. To remove these impurities the lipid was first converted back to its Na^+-salt by using a modified Bligh and Dyer protocol in which the aqueous phase contained 0.1 M EDTA, 0.1 M NaCl, 0.05 M Tris at pH 8.2 [17]. Next the lipid was dissolved in methanol

(10 mg/ml) and precipitation was carried out by dropwise addition of a $CaCl_2$ solution, as described by Smaal et al. [17]. As a final step the cardiolipin was again converted to its Na^+-salt as descibed above. The precipitation step effectively removed the remaining front-material. The lipid was estimated to be 99.5% pure, based on 2D TLC analysis. The purity was confirmed by running TLC plates in different eluents. In all cases the cardiolipin eluted as one spot. The final yield was 16 mg of *E. coli* cardiolipin per liter of culture.

2.3. Fatty acid analysis

The fatty acid composition of the purified cardiolipin was determined after preparation of the fatty acid methyl esters, by incubation of the lipid in sulphuric acid/methanol (5:95, v/v) for 2 h at 70°C. The methyl esters were identified and quantified with a set of standard fatty acid methyl esters on a Perkin Elmer (8500) gas–liquid chromatograph, using a fused silica capillary column. The fatty acid composition was found to be: 5.3% myristic acid (14:0), 50.0% palmitic acid (16:0), 14.6% palmitoleic acid ($16:1_c$), 12.6% *cis*-9,10-methylenehexadecanoic acid ($17:\Delta$), 1.1% stearic acid (18:0), 8.6% *cis*-vaccenic acid ($18:1_c$), 5.1% *cis*-7,8-methylenetetradecanoic acid ($19:\Delta$) and 1.8% unidentified.

2.4. Sample preparation

To approx. 20 μmoles of dry cardiolipin 1 ml buffer (100 mM NaCl, 10 mM Pipes, pH 7.4) was added and the lipids were allowed to hydrate for 1 hour. After several cycles of freeze-thawing to obtain a homogeneous dispersion, 1 ml of the same buffer was added, but now containing an excess of 40 mM of either $CaCl_2$, $MgCl_2$, $SrCl_2$ or $BaCl_2$. For control measurements buffer without divalent cations was added. Next the samples were spun down for 15 min at 4°C at 8000 × *g* and the pellets were used for NMR and X-ray measurements.

2.5. ^{31}P-NMR measurements

^{31}P-NMR spectra of the dispersions were recorded at 121.5 MHz on a Bruker MSL 300 spectrometer, with a 17 μs 90° pulse, a 2–3 s interpulse time and using gated proton-noise decoupling. 1000 Free induction decays were accumulated to which an exponential multiplication was applied, resulting in a 100 Hz linebroadening after Fourier transformation. All spectra are scaled to the same height, except the spectrum of the sample of *E. coli* cardiolipin with excess Ca^{2+} at 35°C, of which the intensity is plotted relative to that of the spectrum at 50°C. Relative percentages of H_{II} phase present were determined in each spectrum by subtraction of the estimated lineshapes of the pure components, followed by integration of the peak areas before and after subtraction.

2.6. X-ray diffraction

SAXD measurements were performed on a Kratky camera as described previously [23], using a CuKα beam (1.54 Å) and a distance between sample and detector of 32.0 cm. Samples were mounted in steel sample holders between two sheets of cellophane and measured at different temperatures after 10 min equilibration time at each temperature.

WAXD patterns were measured at Station 7.2 of the Synchrotron Radiation Source in Daresbury at a wavelength of 1.488 Å. The sample to film distance was set to 8.57 cm. A detailed description of the experiments and Station 7.2 is given elsewhere [24].

2.7. Monolayer experiments

Monolayer measurements were performed at 22°C in a thermostatically controlled box [25] with a 10 mM Tris-buffer at pH 7.4 as subphase, containing either 20 mM NaCl, $CaCl_2$, $MgCl_2$, $SrCl_2$ or $BaCl_2$. A teflon trough of dimensions 32.2 × 17.3 cm was used. After spreading 50 nmoles of cardiolipin from a chloroform/methanol (80:20, v/v) solution, the monolayer was compressed at a rate of 0.28 nm^2/min, while the change in surface pressure was recorded with a Cahn electrobalance.

3. Results

The interaction of *E. coli* cardiolipin with various divalent cations was first investigated by monolayer techniques. Pressure-area curves (not shown) were recorded as described in the Materials and methods section by compressing a monolayer of cardiolipin on a subphase containing the different cations. Table 1 shows the area per molecule, calculated from these pressure/area curves, at 30 mN/m. The results are the first evidence that the various cations influence the properties of the *E. coli* cardiolipin each in a charac-

Table 1
Molecular area of *E. coli* cardiolipin in the presence of different cations (20 mM) at a surface pressure of 30 mN/m

Cation present	Molecular area (Å^2)
Na^+	115.0
Ba^{2+}	113.2
Sr^{2+}	108.4
Mg^{2+}	105.0
Ca^{2+}	105.0

teristic way. In the absence of divalent cations a molecular area of 115 mN/m is calculated, while the presence of divalent cations clearly causes a contraction of the area per molecule. The sequence in which this contraction occurs is $Ca^{2+} \approx Mg^{2+} > Sr^{2+} > Ba^{2+}$.

Next the interaction of *E. coli* cardiolipin with divalent cations was investigated using lipid dispersions. ^{31}P-NMR spectra of cardiolipin in the absence and presence of different divalent cations were recorded at intervals of 5 C° from 0°C till 75°C. A large cation dependence of this phase behaviour was observed, as illustrated in Fig. 1 for some selected temperatures. In the absence of divalent cations at all temperatures the ^{31}P-NMR spectra are characterized by a low-field shoulder and a high-field peak, typical for lipids organized in a liquid-crystalline bilayer [26,27]. Similar spectra are obtained for cardiolipin in the presence of excess Ba^{2+}, except that the lines are broadened, demonstrating an interaction between the cation and the cardiolipin headgroups. When Sr^{2+} is added to the dispersion the lipids again are present in a liquid-crystalline bilayer at lower temperatures of 35°C and 50°C. However, at 75°C a second spectral component becomes visible, characterized by a low-field peak and a high-field shoulder, and characteristic for lipids in an inverted hexagonal (II) phase [26,27]. In the presence of Mg^{2+} such an H_{II} phase characteristic second spectral component can be observed already at 50°C, while in the presence of Ca^{2+} at this temperature all lipids appear to be organized in the H_{II} phase. Fig. 2 shows the relative percentage of lipids in an H_{II} phase as a function of temperature for each of the cations added. From this figure it is concluded that the order of H_{II} phase promotion for the different cations is $Ca^{2+} > Mg^{2+} \gg Sr^{2+}$, whereas Ba^{2+} and Na^{+} do not induce an H_{II} phase in the temperature range measured.

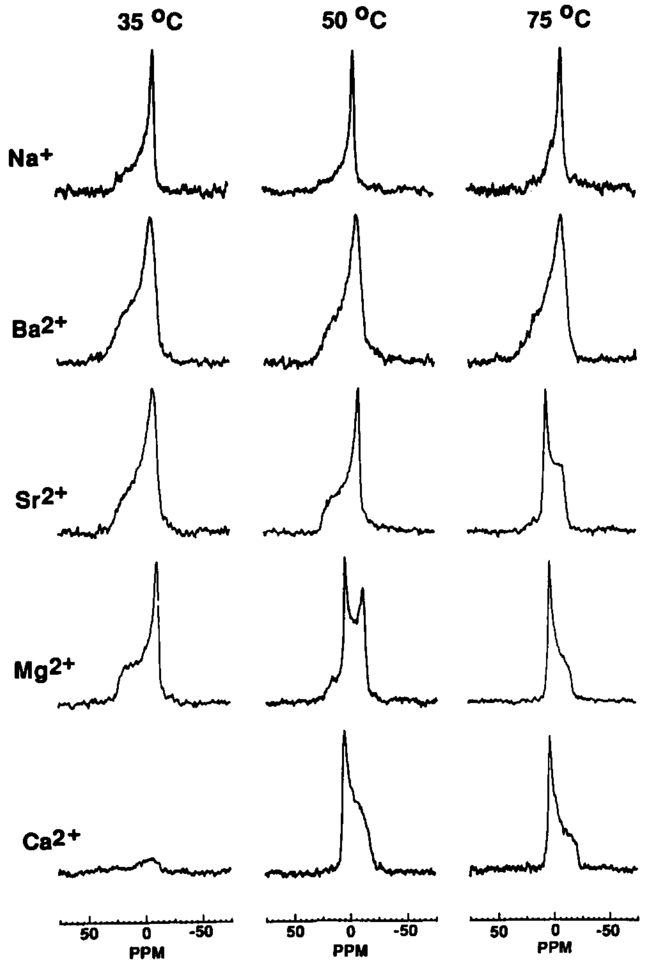

Fig. 1. Proton-noise decoupled 121.4 MHz ^{31}P-NMR spectra of dispersions of *E. coli* cardiolipin at various temperatures, and in the absence and presence of excess different divalent cations, as indicated in the figure.

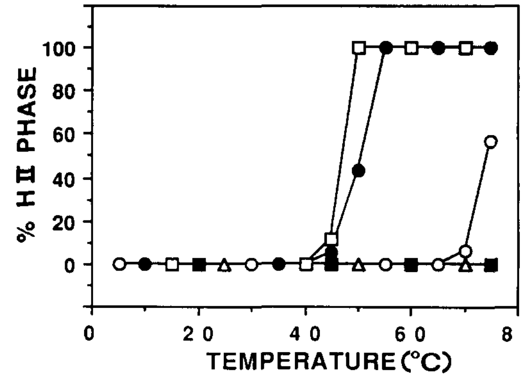

Fig. 2. Relative percentage of hexagonal H_{II} phase formed by dispersions of *E. coli* cardiolipin as a function of temperature in the presence of excess Na^{+} (△), Ba^{2+} (■), Sr^{2+} (○), Mg^{2+} (●) and Ca^{2+} (□).

Upon comparison of the ^{31}P-NMR spectra at 25°C in Fig. 1, another feature is immediately evident, i.e. the loss of intensity in the presence of Ca^{2+}. This low intensity was observed up till about 45°C (not shown), after which a direct transition to the H_{II} phase took place, as evident from the ^{31}P-NMR spectrum at 50°C (Fig. 1). Also in the presence of the other divalent cations a loss of intensity was observed, but occurring gradually and below about 25°C (not shown). In the absence of divalent cations at all temperatures ^{31}P-NMR spectra typical for a liquid-crystalline bilayer were observed (not shown).

The phase behaviour of the *E. coli* cardiolipin was further characterized by small and wide angle X-ray diffraction. In the absence of divalent cations the SAXD patterns at all temperatures showed a broad scattering profile, in agreement with previous reports on dispersions of negatively charged lipids [28,29]. However, sharp peaks were observed in the presence of the various divalent cations. Table 2 shows the first-order repeat distances observed for cardiolipin in

Table 2
First-order repeat distances (Å) in the crystalline (L_c) or gel (L_β) phase, in the liquid-crystalline (L_α) phase, and the tube diameter in the H_{II} phase of *E. coli* cardiolipin in the presence of different divalent cations

Cation present	Temperature	Interbilayer repeat distance (Å)		Tube diameter (Å)
		L_β/L_c	L_α	H_{II}
Ba^{2+}	25°C	55.4	49.3	–
Sr^{2+}		57.0	50.4	–
Mg^{2+}		55.7	50.0	–
Ca^{2+}		49.2	–	–
Ba^{2+}	45°C	–	48.2	–
Sr^{2+}		–	48.7	–
Mg^{2+}		–	48.4	–
Ca^{2+}		49.3	–	–
Ba^{2+}	75°C	–	46.0	–
Sr^{2+}		–	46.6	64.7 [a]
Mg^{2+}		–	–	60.0 [a]
Ca^{2+}		–	–	50.6 [a]

[a] Tube diameter, corresponding to $(2/\sqrt{3})\times$(1st-order repeat distance).

the presence of these cations at three different temperatures. At 25°C and in the presence of Ba^{2+}, Sr^{2+} and Mg^{2+} a peak with high intensity was observed at a repeat distance of about 50 Å, and either a shoulder or a peak with low intensity was visible at a slightly larger repeat distance (not shown). The ^{31}P-NMR results suggest that these originate respectively from lipids present in a liquid-crystalline phase and from a small fraction of lipids present in a gel phase. In agreement with this interpretation the WAXD pattern at 25°C of *E. coli* cardiolipin in the presence of Sr^{2+} (Fig. 3A) exhibits a sharp band at 4.2 Å (outer ring), indicative for a gel (L_β) phase with a hexagonal packing of the acyl chains, as well as a diffuse band at about 4.6 Å, indicative for lipids in the liquid-crystalline (L_α) phase. The reflections at larger distances (inner rings) correspond to higher order reflections of two different lamellar repeat distances, identical to those observed from SAXD measurements, thus clearly confirming the coexistence of two lamellar phases. Interestingly, in the

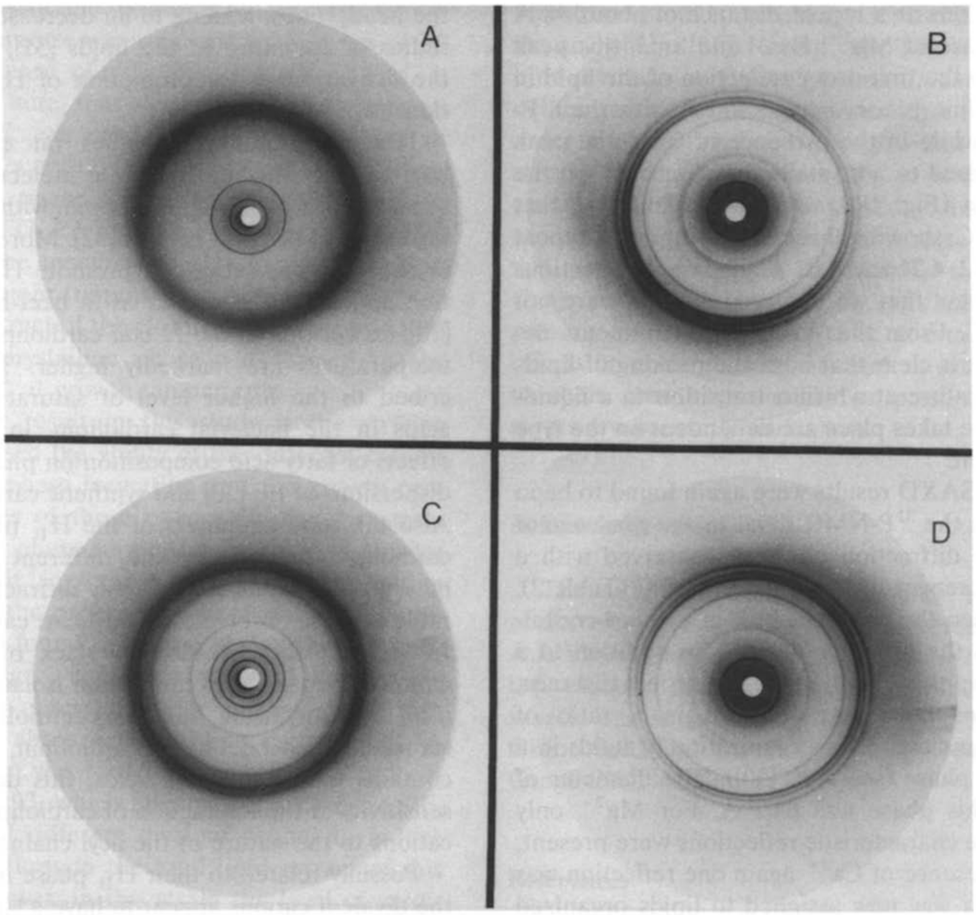

Fig. 3. Wide angle X-ray diffraction pattern of dispersions of *E. coli* cardiolipin in the presence of excess Sr^{2+}(A), Ca^{2+} (B) and Mg^{2+} (C) at 25°C, and in the presence of excess Ca^{2+} at 45°C (D).

presence of Mg^{2+} a different WAXD pattern is obtained (Fig. 3C). The sharp band at 4.2 Å is missing and three outer rings are observed at repeat distances of 4.93, 4.37 and 4.08 Å, indicative for the presence of lipids in a crystalline phase, with a highly ordered packing of the acyl chains. Also in this case lipids are present in a liquid-crystalline L_α phase, as evident from the diffuse band at about 4.6 Å, which is visible between the two outer rings. In constrast, no evidence for a liquid-crystalline phase was found in the presence of Ca^{2+}. SAXD measurements exhibited a single peak with a repeat distance of 49 Å (Table 2), while the WAXD results showed three sharp outer rings, corresponding to repeat distances of 4.41, 4.23 and 4.01 Å, respectively (Fig. 3B). The reflections at larger repeat distances (inner rings) were found to correspond to higher order reflections of a lamellar phase with a similar repeat distance as found by the SAXD measurements (Table 2). In addition some weak reflections were present at 11.5, 8.4 and 6.6 Å, of which we do not know the origin. These results demonstrate that in the presence of Ca^{2+} cardiolipin adopts a crystalline phase, different from the one observed in the presence of Mg^{2+}.

At 45°C the SAXD patterns exhibited one peak for all divalent cations at a repeat distance of about 48 Å (Table 2). In case of Mg^{2+}, Ba^{2+} and Sr^{2+} this peak was assigned to the first-order reflection of the lipid in a liquid-crystalline phase, in agreement with the ^{31}P-NMR results, while in the presence of Ca^{2+} the peak was again assigned to a crystalline state, based on the WAXD pattern (Fig. 4D), which was similar to that obtained at 25°C, showing three outer rings with repeat distances of 4.42, 4.24 and 4.02 Å. The weak reflections of unknown origin that we observed at 25°C were not present at 45°C. From the WAXD measurements described above it is clear that both the packing of lipids and the temperature at which a transition to a liquid-crystalline phase takes place are dependent on the type of cation present.

At 75°C the SAXD results were again found to be in agreement with the ^{31}P-NMR data. In the presence of Ba^{2+} only one diffraction peak was observed with a slightly smaller repeat distance than at 45°C (Table 2), as expected when the lipids are still in a liquid-crystalline bilayer. In the presence of Sr^{2+} in addition to a sharp peak at approximately the same repeat distance, weak reflections could be observed in a ratio of $1:1/\sqrt{3}:1/2$, indicative for organization of lipids in a hexagonal H_{II} phase (see Ref. [30]). The diameter of the tubes in this phase was 64.7 Å. For Mg^{2+} only these H_{II} phase characteristic reflections were present, while in the presence of Ca^{2+} again one reflection was observed, which was now assigned to lipids organized in an H_{II} phase. The tube diameters were calculated to be 60.0 Å and 50.6 Å, respectively.

4. Discussion

In this study we isolated cardiolipin from *E. coli* strain AH930 and investigated its interaction with divalent cations by various biophysical techniques. Here we will first discuss this interaction in relation to the effects of divalent cations on lipid organization of other cardiolipins and of negatively charged lipids in general. Next we will discuss the interaction of this *E. coli* cardiolipin with divalent cations in relation to their growth promoting properties in the strain AH930.

The results clearly show that divalent cations can induce H_{II} phase formation in dispersions of the *E. coli* cardiolipin, and that the H_{II} phase promoting activity is dependent on the type of cation present. The order of H_{II} phase promotion was $Ca^{2+} > Mg^{2+} \gg Sr^{2+}$, whereas Ba^{2+} and Na^{+} did not induce an H_{II} phase in the temperature range measured. Interestingly, the monolayer data showed that divalent cations induce a decrease of surface area and that the sequence in which the contraction occurs is $Ca^{2+} \approx Mg^{2+} > Sr^{2+} > Ba^{2+}$, which is rather similar as the sequence for promotion of H_{II} phase formation. This suggests that the decrease in surface area is induced by a cation-induced decrease of the cross-sectional area of the headgroups, leading to an decrease of the intrinsic radius of curvature of the lipids [31], which could be the driving force for promotion of H_{II} phase formation.

Previous monolayer studies on cardiolipin from bovine heart showed the same molecular area as observed here for *E. coli* cardiolipin, with also a contracting effect of divalent cations [32]. Moreover, the ability of the different cations to promote H_{II} phase formation appears to be similar as in beef-heart cardiolipin [10], except that in the *E. coli* cardiolipin the transition temperatures are markedly higher. This can be ascribed to the higher level of saturation of the fatty acids in the bacterial cardiolipin, in analogy to the effects of fatty acid composition on phase behaviour in dispersions of PE [30] and synthetic cardiolipins [33,34]. Also the tube diameters of the H_{II} phases of *E. coli* cardiolipin induced by the different cations and as measured by small angle X-ray diffraction are comparable to those observed in beef-heart cardiolipin [11,12]. In view of these results the lack of a pronounced cation-dependence in cardiolipin isolated from *B. subtilis* [10] is surprising. Since this cardiolipin is also more saturated than beef-heart cardiolipin, but in addition contains branched fattys acids, this demonstrates the sensitivity of the interaction of cardiolipin with divalent cations to the nature of the acyl chains.

Possibly related to their H_{II} phase inducing activity, the divalent cations appear to have a strong interaction with the headgroups, which in ^{31}P-NMR may lead to a loss of signal intensity, as most clearly evident in the presence of Ca^{2+}. Such a loss of intensity had been observed previously upon addition of Ca^{2+} to model membranes of PG [35–37], PS [38] and PA [39], and is ascribed to immobilization of the lipid headgroups due to dehydration of the interface. Here it is the first time that this effect is reported for cardiolipin. That indeed dehydration occurs is supported by the relatively small repeat distances that were observed by small angle X-ray diffraction in dispersions of *E. coli* cardiolipin with Ca^{2+} at 25°C and at 45°C, and by electron micrographs of these samples (not shown), which showed that the bilayers are very closely stacked.

The interaction of the ions with the lipid headgroup clearly results in an increase in the gel to liquid-crystalline phase transition temperature. While in the absence of divalent cations the lipids are still present in a liquid-crystalline state at 5°C, in the presence of Ca^{2+} even at 45°C there was no trace of a liquid-crystalline phase, whereas for the other divalent cations a coexistence of gel or crystalline and liquid-crystalline phases was found at 25°C. Whether a gel or crystalline phase is formed at low temperatures, and how the acyl chains are packed in the crystalline phase, depends on the type of cations present and possibly also on the exact temperature. A similar combination of cation-induced formation of crystalline phases and an increase in transition temperature was reported for bilayers of PS [40] in the presence of different divalent cations. It should be noted here, that although at growth temperature purified *E. coli* cardiolipin in the presence of Ca^{2+} thus forms a crystalline phase, there is no indication that such a phase occurs in vivo, where next to cardiolipin also PG, PA and large amounts of proteins are present in the membrane. Also in the total lipid extract of this strain (unpublished observations) or in the total lipid extract of the closely related strain AD93 [16] no gel or crystalline phase was formed in the presence of Ca^{2+} at growth temperature.

An important result in this study is the striking correlation between the ability of the different cations to promote H_{II} phase formation and the growth promoting properties of these cations in vivo for strain AH930 [18]. H_{II} phase formation occurs in the order $Ca^{2+} > Mg^{2+} \gg Sr^{2+}$, whereas no H_{II} phase formation was observed in the presence of Ba^{2+} and Na^{+}. Similarly, growth was found to be maximal in the presence of Ca^{2+} and Mg^{2+}, followed by Sr^{2+}, whereas no growth could be observed in the presence of either Ba^{2+} or in the absence of divalent cations [18]. A similar correlation was found between growth properties of strain AD93, which also cannot synthesize PE, and the ability of different divalent cations to induce non-bilayer structures in the total lipid extract of this strain [16]. Moreover, in this strain the level of cardiolipin appeared to be regulated to compensate for differences in H_{II} phase inducing activitiy of different cations [16]. Together these data strongly support the hypothesis that cardiolipin with divalent cations can replace PE in membrane functioning in *E. coli* and that the importance of these lipids for membrane functioning is related to their ability to organize in non-bilayer lipid structures. Although for pure cardiolipin the H_{II} phase itself is adopted only at temperatures above the growth temperature, it is possible that in the intact membrane related non-bilayer structures are formed at lower temperatures. These non-bilayer structures could be involved as intermediates in functional membrane processes during which the bilayer organization must be locally and temporarily disrupted, e.g., in processes like cell division [41], which indeed was found to be closely coupled to PE synthesis [42], the formation of adhesion sites between outer and inner membrane [43] or possibly during translocation of proteins [44].

An alternative or perhaps additional possibility for the mechanism of polymorphic regulation of lipid composition, is that the presence of non-bilayer preferring (type II) lipids is important for lipid packing. Consistent with this are the results from the present study, which show that the divalent cations can have a large effect on acyl chain packing. In general the presence of type II lipids in a bilayer organization leads to a "frustration" and increased order or lateral pressure in the acyl chains [31]. Recent ^{2}H-NMR measurements in lipid extracts of *Acholeplasma laidlawii*, grown on deuterated fatty acids, suggested that indeed the lipid order appears regulated to stay within certain boundaries [45]. Similar experiments, in which the *E. coli* mutant AH930 or AD93 is used to incorporate deuterium labelled fatty acids in the bacterial membrane, will provide a unique opportunity to measure acyl chain packing in derived *E. coli* lipid extracts and possibly even in the intact cells, and to investigate the effects on chain packing of divalent cations in relation to their growth promoting properties. Such experiments are presently under way.

Acknowledgements

The authors would like to thank Dr. R.A. Demel for performing the monolayer measurements, and Drs. A.J. Verkleij and J. Bijvelt for carrying out freeze-fracture electron microscopy experiments. Part of this research was supported by U.S. Public Health Service Grant GM20487 (to W.D.) and by NATO Research Grant CRG901006.

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