Biochimica et Biophysica Acta, 693 (1982) 1–12
Elsevier Biomedical Press

BBA 71412

FURTHER ASPECTS OF THE Ca^{2+}-DEPENDENT POLYMORPHISM OF BOVINE HEART CARDIOLIPIN

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(Received July 21st, 1982)

Key words: Ca^{2+} dependence; Cardiolipin polymorphism; Cubic phase; ^{31}P-NMR

The influence of cations on the structure of aqueous dispersions of the sodium salt of bovine heart cardiolipin was investigated using binding experiments, ^{31}P-NMR, freeze-fracture electron microscopy, small angle X-ray diffraction and batch calorimetry techniques. In the 1–3 mM concentration range, Ca^2 induces a bilayer → hexagonal H_{II} transition for the lipid. During this transition there is a marked increase in Ca^{2+} binding from a maximum of 0.35 Ca/cardiolipin in the bilayer to 1.0 Ca/cardiolipin in the hexagonal H_{II} phase. Only when the cardiolipin liposomes are exposed to locally high Ca^{2+} concentrations is the bilayer → hexagonal H_{II} transition accompanied by the appearance of an intermediate 'isotropic' structure characterized by an isotropic ^{31}P-NMR signal and lipidic particles. In contrast, in mixed dioleoylphosphatidylcholine/cardiolipin (1:1) liposomes, Ca^{2+} concentrations as low as 100 μM will induce an 'isotropic' structure under conditions where no locally high Ca^{2+} concentrations can occur. In this system at higher Ca^{2+} concentrations (above 5 mM) the hexagonal H_{II} phase formation occurs. At least 80% of the phosphatidylcholine can be incorporated into this phase. The Ca^{2+}-induced bilayer to hexagonal transition is an endothermic reaction with a ΔH of approx. 1.8 kcal/mol. Removal of Ca^{2+} from the hexagonally organized calcium-cardiolipin (1:1) complex by dialysis is an extremely slow process with a half-time in excess of 80 h. After 23 h of dialysis at a Ca/cardiolipin ratio of 0.86 an 'isotropic' structure is observed, characterized by an isotropic ^{31}P-NMR signal and the presence of lipidic particles. After 70 h of dialysis (Ca/cardiolipin = 0.7) a new phase is observed. This phase which is optically isotropic and highly viscous separates from a lipid-free aqueous phase and contains 111 mM cardiolipin (15.5% by weight). The phospholipid molecules undergo rapid isotropic motion and the freeze-fracture morphology indicates the presence of a highly curved interconnected bilayer network separating various aqueous compartments. No defined X-ray diffraction bands can be observed for this phase. These characteristics are typical for cubic phases. This phase is metastable as mechanical agitation immediately induces the formation of large bilayer vesicles.

Introduction

Cardiolipin, present in eukaryotic cells, is in many respects a unique membrane lipid. Chemically, it can be defined as a 'double' phospholipid, containing four fatty acids and, at neutral pH, two charged phosphates. It is synthesized [1] and found exclusively in the inner mitochondrial membrane, where it is the only major (20 mol% of the total lipids, thus 33% by weight) negatively charged phospholipid [2].

In general, cardiolipins isolated from mammalian sources are highly unsaturated and specifically enriched in linoleic acid [3]. Several studies

have indicated that this lipid is involved in different inner mitochondrial membrane functions. The best documented is the absolute requirement of the activity of the cytochrome-*c* oxidase for cardiolipin [4,5]. Also, the activity of other mitochondrial enzymes is strongly affected by the presence of this lipid [6]. Furthermore, cardiolipin might be involved in different transport processes, in particular that of Ca^{2+} [7] and it plays an important structural role in this membrane [6]. These functional abilities must be a reflection of specific and unique structural features of the cardiolipin molecule.

One of the most remarkable properties of cardiolipin is the polymorphism of aqueous dispersions of this lipid. In excess buffer, the sodium salt of bovine heart cardiolipin is organized in extended bilayers [8–10]. Under similar conditions the Ca, Mg, Mn and Ba salts, dependent upon the temperature, can form the lamellar and hexagonal H_{II} phase [8,11,12]. At physiological temperatures the hexagonal H_{II} phase is preferred. This isothermal regulation of the macroscopic structure of cardiolipin by divalent cations and also by mitochondrial inner membrane proteins such as cytochrome *c* [13] forms the basis for the hypothesis [7] that non-bilayer lipid structures formed by cardiolipin account for the functional properties of this lipid. Conceptually, it is difficult to reconcile the presence of extended areas of hexagonal H_{II} phase with the barrier function of the membrane. Therefore, this hypothesis is strengthened by the observation that in mixtures of cardiolipin with bilayer forming lipids a new non-bilayer lipid structure, the 'lipidic particle' has been observed [14,15]. This structure has been interpreted by us as representing intrabilayer inverted micelles [14–17]. Although other models have been proposed [18–20] recent evidence [21] strongly supports the inverted micellar model. The presence of lipidic particles in cardiolipin containing model membranes markedly affects the dynamical properties of the system as evidenced by fast lipid flip-flop and divalent cation transport [22]. Furthermore, these structures are associated with vesicle fusion [16,23].

Despite the wealth of information on the polymorphism of bovine heart cardiolipin still several basic questions are unanswered. Such questions include: at which Ca^{2+} concentration is the bilayer to hexagonal H_{II} transition induced? How much Ca^{2+} is bound to cardiolipin in the lamellar and hexagonal phase and what are the thermodynamics of the transition? Is the absolute Ca^{2+} concentration or the concentration difference across the bilayer determining the phase change, in particular in view of the mechanism of vesicle fusion and the occurrence of an 'isotropic' structure with associated lipidic particles upon the addition of limiting amounts of Ca^{2+} to cardiolipin liposomes [9]? Furthermore, is the bilayer → hexagonal transition reversible, are there structural intermediates and what are the kinetics of the reversed reaction? With these and related questions in mind we report in this paper ^{31}P-NMR, freeze-fracture electron microscopy, small angle X-ray diffraction, batch calorimetry and $^{45}Ca^{2+}$ binding experiments on aqueous dispersions of bovine heart cardiolipin.

Materials and Methods

Lipids

The sodium salt of bovine heart cardiolipin was either purchased from Avanti (Birmingham, AL, U.S.A.) or isolated from bovine heart. In the latter case the tissue was extracted with chloroform and methanol followed by acetone and ethanol precipitations whereafter the barium salts of the negatively charged phospholipids were precipitated by the addition of barium acetate. The barium salt of cardiolipin was obtained in a pure form by quantitative high performance liquid chromatography (HPLC) on silica gel. The sodium salt was obtained by treatment with Na_2SO_4. Details of this isolation procedure will be published elsewhere. Both cardiolipin species were over 99% pure as determined by high performance thin-layer chromatography, had a cation composition of > 98% Na^+ as determined by scanning transmission electron microscopy and gave identical results in our experiments. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (dioleoylphosphatidylcholine) was synthesized as described before [24].

Chemicals

$^{45}Ca^{2+}$ was obtained from the Radiochemical Centre (Amersham, U.K.) as the chloride in water

(2 mCi/ml) with a specific activity of 1.08m Ci/mg Ca. The Ca^{2+} ionophore A23187 was obtained from Boehringer (Mannheim, F.R.G.). Calcium and sodium chloride were ultrapure. All other chemicals were of analytical grade.

Preparation of model membranes

Dry phospholipid firms were prepared by evaporation from chloroform in a round-bottom flask of approx. 50 μmol of lipid followed by storage under high vacuum. In some cases the chloroform solution of the lipids was directly evaporated in a 10 mm NMR tube under a stream of N_2 followed by storage overnight under high vacuum. The lipids were then dispersed by vortexing in 1.0–5.0 ml 100 mM NaCl, 10 mM Tris-HCl, pH 7.0 buffer in some cases containing 20% (v/v) ^2H_2O. When the Ca^{2+} ionophore A-23187 was used it was added as a 1 mg/ml solution in ethanol to the chloroform solution of the lipids (100 μg ionophore per 50 μmol of cardiolipin) prior to evaporation.

$^{45}Ca^{2+}$ binding experiments

In order to determine both the phospholipid structure by ^{31}P-NMR and the $^{45}Ca^{2+}$ binding to cardiolipin, three methods were used.

a. Addition method. In this method small aliquots of a concentrated $CaCl_2$ solution are added to a concentrated lipid dispersion in the NMR tube. Typically, 50 μmol of cardiolipin were dispersed in 1.2 ml buffer containing 20% ^2H_2O. To these liposomes 2 μl $^{45}CaCl_2$ solution (4 μCi of ^{45}Ca) and aliquots of a 1 M $CaCl_2$ solution were added after which the ^{31}P-NMR spectrum was recorded. After each $CaCl_2$ addition a 50-μl sample was taken which was centrifuged for 30 min at 100 000 × *g* in a Beckman airfuge. The amount of bound Ca was determined by measuring the ^{45}Ca content of the liposome-free supernatant. This method has the disadvantage that both the Ca^{2+}/cardiolipin ratio and the total Ca^{2+} concentration are changing after each Ca^{2+} addition. Furthermore, with this method, liposomes are locally exposed to high Ca^{2+} concentrations.

b. Dialysis method. 50 μmol of cardiolipin were dispersed in 5.0 ml buffer, whereafter the liposomes were pelleted by centrifugation at 4°C for 20 min at 16 000 × *g*. The liposomal pellet was resuspended in buffer and brought to a final volume of 0.8 ml. This suspension was transferred to a microcollodion bag (Sartorius, Göttingen, F.R.G.), which was fitted on to a 9 mm outer diameter glass tube. The collodion bag was then placed at room temperature in 500 ml 100 mM NaCl, 10 mM Tris-HCl, pH 7.0 stirred buffer containing 10% ^2H_2O. 10 μl $^{45}CaCl_2$ solution (20 μCi ^{45}Ca) was added to the dialysis buffer and the desired amount of $CaCl_2$ (from a 1 M stock solution). After 3 h of equilibration the bag was placed in a 10 mm outer diameter NMR tube containing 0.2 ml of the buffer in which the equilibration was carried out. After the ^{31}P-NMR spectrum was recorded a 50-μl aliquot of the liposomal suspension was removed from the bag. 2 μl were used for lipid phosphate analysis [25], 5 μl for LSC and the remainder of the liposomes was pelleted by centrifugation at room temperature for 20 min at 100 000 × *g* in a Beckman airfuge. 5 μl of the lipid-free supernatant was collected for liquid scintillation counting (LSC). Subsequently the Ca^{2+} concentration in the 500 ml buffer was increased and the whole procedure was repeated until a complete tritration curve was obtained. Control experiments revealed that no significant binding of $^{45}Ca^{2+}$ to the dialysis bag occurred and that the half-time of $^{45}Ca^{2+}$ equilibration across the bag was 50 min. Furthermore, no chemical degradation of cardiolipin could be detected at the end of the experiment. With this procedure it is possible to determine both the ^{31}P-NMR spectrum of the cardiolipin liposomes and the $^{45}Ca^{2+}$ binding under conditions where there is always a molar excess of Ca^{2+} present. The dissociation constant K_{Ca}, defined as:

$$K_{Ca} = \frac{[\text{cardiolipin}]_{free}[Ca^{2+}]_{free}}{[\text{cardiolipin-Ca}]_{complex}}$$

was determined using a non-linear regression analysis of the experimental data points.

c. Immersion method. This method is similar to the dialysis method (b) except that the collodion bag containing the 50 μmol of cardiolipin (made in the presence of the Ca^{2+} ionophore) was immersed in 100 μl 1 M $CaCl_2$ (containing 4 μCi ^{45}Ca) present in a 10 mm NMR tube. The ^{31}P-NMR spectrum and Ca^{2+} binding was determined

as a function of time. In this way a large molar excess of ca^{2+} is present in a small volume (like in the addition method (a)), but due to the rate limiting diffusion of Ca^{2+} through the semipermeable membrane of the bag, no Ca^{2+} concentration gradients are induced across the liposomal bilayers.

Reversibility of the Ca^{2+}-cardiolipin complex formation

85 μmol of cardiolipin and the Ca^{2+} ionophore were dispersed in 0.6 ml buffer. The liposomes were transferred to a microcollodion bag connected to a 9 mm tube. 100 μl 1 M $CaCl_2$ and 2 μl (4 μCi) $^{45}CaCl_2$ was added. The bag was placed in a 10 mm NMR tube and the ^{31}P-NMR spectrum was recorded. Subsequently the bag was placed in 500 ml stirred buffer at room temperature whereafter as a function of time the $^{45}Ca^{2+}$ concentration in the dialysate and the ^{31}P-NMR spectrum of the cardiolipin dispersion were determined.

Techniques

36.4 MHz proton decoupled (input power 18 watt) ^{31}P-NMR spectra of aqueous dispersions were recorded at 30°C as described before [26]. Signal intensities of the various spectral components were obtained by computer subtraction methods using reference spectra consisting of only one spectral component. The estimated error in the signal intensities is 10%. For freeze-fracturing the samples were quenched by jet freezing [27] without the use of cryoprotectants and subsequently worked up in a Balzer freeze-etch machine according to standard procedures. Small angle X-ray diffraction was carried out at room temperature using a Kratky camera with a 10×0.2 mm Cu-Kα beam (40 kV, 23 mA) and exposure times of 1–3 h. The aqueous sample was mounted in a slit ($12 \times 2 \times 2$ mm) in between two sheets of cellophane. Radioactivity was determined by LSC using standard techniques. The isothermal enthalpy of reaction between Ca^{2+} and cardiolipin was measured at 25.0 ± 0.1°C using an LKB 2107 batch microcalorimeter equipped with gold cells. In each experiment 4.0 ml of 3 mM cardiolipin in buffer was mixed with 2.0 ml of buffer containing increasing amount of Ca^{2+}. In the reference cell 4.0 ml of buffer and 2.0 ml of Ca^{2+}-containing buffer were added. The heats of reaction were corrected for frictional heats. For each experiment two friction runs and usually one electrical calibration were done. Electrical calibration was adjusted to produce a heat effect within ±20% of that observed for the reaction mixture. The electrical calibration heaters were checked by measuring the heat of dilution of sucrose [28].

Results

Liposomes prepared from the sodium salt of cardiolipin are organized in extended bilayers as evidenced by the characteristic asymmetrical ^{31}P-NMR lineshape with a low-field shoulder and a high-field peak [29] separated by approx. 32 ppm (Fig. 1A). As noted before [12] a small (approx. 5%) isotropic component is present in the spectrum which most likely originates from some smaller vesicles present in the preparation as can be detected by freeze-fracturing. Addition of aliquots of a concentrated $CaCl_2$ solution (cf. addition method) induces the formation of an iso-

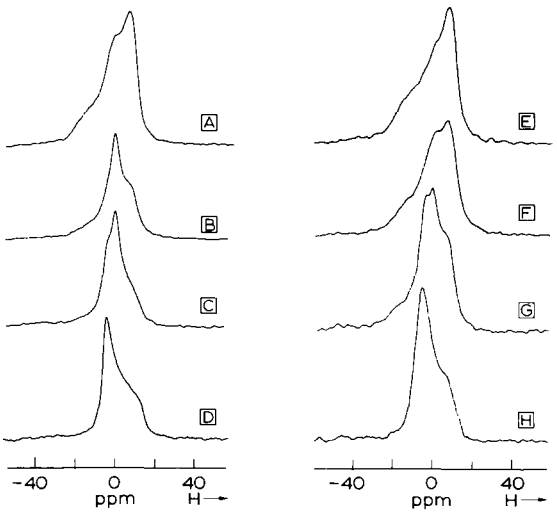

Fig. 1. 36.4 MHz ^{31}P-NMR spectra of cardiolipin liposomes before (A, E) and after the addition of Ca^{2+} via the addition (B, C, D) or the dialysis method (F, G, H). Spectra B, C, D were obtained from liposomes (50 μmol cardiolipin in 1.2 ml buffer) to which was added 15, 25 and 35 μl 1 M $CaCl_2$, respectively. The free Ca^{2+} concentrations were determined to be 0.80, 1.49 and 5.3 mM, respectively. In spectra F, G, H the liposomes were dialyzed against 0.8, 1.5 and 3.0 mM Ca^{2+}, respectively.

tropic spectral component (Figs. 1B and 1C). In the presence of a molar excess of Ca^{2+} the lineshape typical of lipids in the hexagonal H_{II} phase [29] with a reversed asymmetry and reduced width is observed (Fig. 1D). These and additional data are plotted in a quantitative manner in Fig. 2. In the insert the macroscopic structure of cardiolipin is plotted versus $(Ca^{2+}/\text{cardiolipin})_{total}$. The intermediate structure(s) giving rise to an isotropic signal are formed at the lowest amount of Ca^{2+} added. No further change in the spectrum is observed above a $(Ca^{2+}/\text{cardiolipin})_{total}$ of approximately 1. Due to Ca^{2+} binding to cardiolipin in this experiment we are simultaneously changing $(Ca/\text{cardiolipin})_{total}$ and $[Ca^{2+}]_{free}$. Since only the free Ca^{2+} concentration will determine the Ca^{2+} binding to and the structure of cardiolipin, the structural data are plotted in Fig. 2 as a function of $[Ca^{2+}]_{free}$. In the 1–5 mM $[Ca^{2+}]_{free}$ range the hexagonal phase is formed. The 'isotropic structure' already is found at a free Ca^{2+} concentration in excess of 200 μM. Furthermore, Fig. 2 shows that increasingly more Ca^{2+} is bound to cardiolipin until a limiting 1 : 1 binding is observed in the hexagonal H_{II} phase. Freeze-fracturing of a sample in which $(Ca^{2+}/\text{cardiolipin})_{total} = 0.5$ revealed the presence of bilayers with associated lipidic particles and areas of hexagonal H_{II} phase (Figs. 3A and 3B). At $(Ca^{2+}/\text{cardiolipin})_{total} > 1$ only the pure H_{II} was observed. Inclusion of 100 μg of the Ca^{2+}-ionophore A 23187 in the liposomes did not significantly affect the ^{31}P-NMR and Ca^{2+}-binding characteristics of the system under the experimental conditions described in the legends of Figs. 1 and 2.

Since in the addition method the induction of

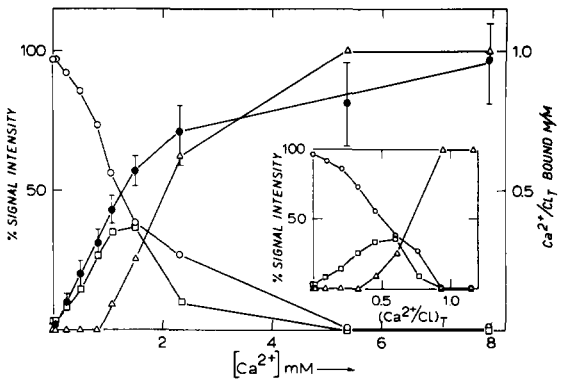

Fig. 2. Ca^{2+} binding (●———●) to and structure of cardiolipin liposomes: addition method. The amount of signal originating from lipid organized in extended bilayers (○———○), isotropic structures (□———□) or hexagonal H_{II} phase (△———△) were determined from 36.4 MHz ^{31}P-NMR spectra of the liposomes after the addition of aliquots of a 1 M $CaCl_2$ solution and are plotted as a function of the free Ca^{2+} concentration. The insert shows the structural data plotted versus the ratio of total amount of Ca^{2+} and cardiolipin present in the sample. The error bars represent the S.D. in the amount of Ca^{2+} bound.

the 'isotropic' structure with associated lipidic particles might be the result of an exposure of cardiolipin to local high Ca^{2+} concentrations, the experiment was repeated with cardiolipin liposomes containing the Ca^{2+} ionophore, using the dialysis method. In this method the liposomes are incubated in a controlled way in a large volume of Ca^{2+} containing buffer, such that the liposomes are never exposed to local high Ca^{2+} concentrations and the $(Ca/\text{cardiolipin})_{total}$ ratio always exceeds 1. Figs. 1 E–H and 4 show that upon increasing the Ca^{2+} concentration from 1 to 3 mM a transition from a lamellar to a hexagonal H_{II}

TABLE I

CALORIMETRY OF THE Ca-CARDIOLIPIN COMPLEX FORMATION

Heat and enthalpy of reaction are corrected for the heat of dilution of Ca^{2+} caused by Ca^{2+} binding to cardiolipin. Fo details see text.

Ca^{2+} added (mM)	Ca^{2+} total (mM)	$(Ca/\text{cardiolipin})_{total}$	Heat of reaction (mcal)	Enthalpy of reaction (kcal/mol)
3	1	0.5	2.8	0.2
15	5	2.5	15.5	1.3
100	33.3	16.7	21.4	1.8

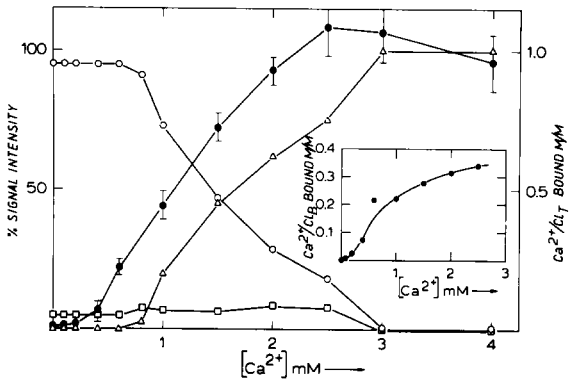

Fig. 4. Ca^{2+} binding (●———●) to and structure of cardiolipin liposomes: dialysis method. The amount of signal originating from lipids organized in extended bilayers (○———○), isotropic structures (□———□) or hexagonal H_{II} phase (△———△) were determined from 36.4 MHz ^{31}P-NMR spectra of the liposomes and are plotted versus the Ca^{2+} concentration of the dialysis medium. The insert shows the Ca^{2+} binding to cardiolipin in the lamellar phase (CL_B) versus the Ca^{2+} concentration of the dialysis medium. The data in the 1 to 2.5 mM Ca^{2+} range are corrected for the amount of Ca^{2+} bound to that part of the structures organized in the hexagonal H_{II} phase assuming a 1 : 1 binding. The error bars represent the S.D. in the amount of Ca^{2+} bound.

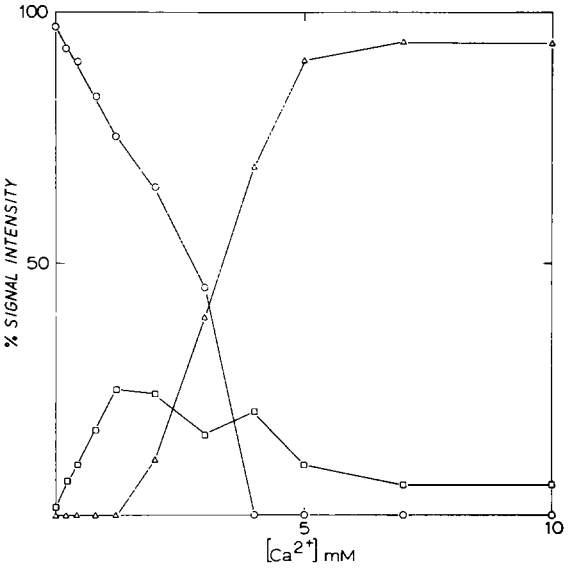

Fig. 5. Effect of Ca^{2+} on the structure of dioleoylphosphatidylcholine/cardiolipin (1 : 1) liposomes: dialysis method. The amount of signal originating from lipids in extended bilayers (○———○), isotropic structures (□———□) or hexagonal H_{II} phase (△———△) were determined from 36.4 MHz ^{31}P-NMR spectra of the liposomes (50 μmol cardiolipin, 50 μmol dioleoylphosphatidylcholine, 100 μg A23187 in 1.0 ml buffer) and are plotted versus The Ca^{2+} concentration of the dialysis medium.

structure is observed without the formation of a significant amount of the 'isotropic' structure. In the hexagonal H_{II} phase Ca is stoichiometrically bound to cardiolipin (Fig. 4). In the insert of this figure the Ca^{2+} binding to cardiolipin in the lamellar phase is shown to reach a limiting value of 0.35 Ca/cardiolipin at a free Ca^{2+} concentration of 2.5 mM, the highest concentration at which lamellar phase can still be detected. From such experimental data intrinsic Ca-cardiolipin binding constants are difficult to derive due to the complexity of non-specific ion-absorption processes [30]. Using a simple binding model and curve fitting procedures a dissociation constant K_{ca} of 1.5 ± 0.5 mM could be derived from these data.

When Ca^{2+} was added to the cardiolipin liposomes via the immersion method very similar results were obtained (data not shown), in that a time-dependent change from a bilayer to a hexagonal H_{II} phase was observed without the occurrence of the intermediate 'isotropic' structure.

Since Ca^{2+} addition from a concentrated solution to mixed phosphatidylcholine-cardiolipin systems also results in the appearance of an 'isotropic' structure in association with lipidic particles [15], we thought it of interest to see whether similar structures would be observed when the Ca^{2+} was added via the dialysis method. As shown in Fig. 5 for dioleoylphosphatidylcholine/cardiolipin (1 : 1) liposomes, in contrast to the pure cardiolipin system Ca^{2+} concentrations in excess of 100 μM already induces an isotropic ^{31}P-NMR signal. The 'isotropic' structure occurs as intermediate between the lamellar and the hexagonal H_{II} phase. This latter phase is formed in the 1–5 mM Ca^{2+} range. Since more than 90% of the total

Fig. 3. Freeze-fracture electron microscopy of various Ca-cardiolipin systems. Cardiolipin liposomes (50 μmol cardiolipin in 1.2 ml buffer) after the addition of 25 μl 1 M $CaCl_2$ (A, B). Ca-cardiolipin (1 : 1) complex after dialysis against Ca^{2+}-free buffer for 23 (C, D, E) and 70 h (F). Final magnification 80000×.

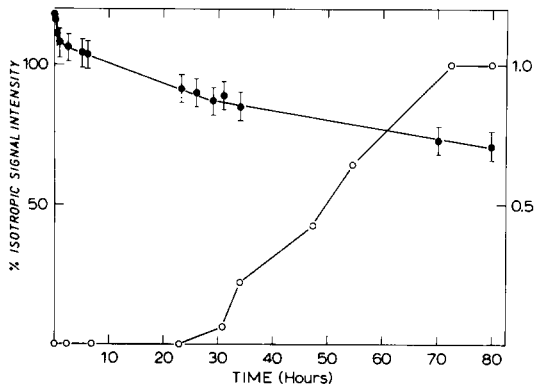

Fig. 6. Ca^{2+} binding to (●———●) and structure of (○———○, % isotropic signal) the Ca-cardiolipin complex as a function of the dialysis time against excess buffer. The error bars represent the S.D. in the amount of Ca^{2+} bound to the cardiolipin liposomes.

lipids are organized in the hexagonal H_{II} phase above this Ca^{2+} concentration, it can be calculated that at least 80% of the bilayer phase preferring dioleoylphosphatidylcholine is incorporated into the hexagonal H_{II} phase.

The heat of reaction between cardiolipin and Ca^{2+} was measured by batch calorimetry using an experimental procedure which is comparable to the addition method except that lower cardiolipin and Ca^{2+} concentrations are used. In the presence of a limiting amount of Ca^{2+} which in the addition method induces a bilayer → isotropic transition of 30% of the lipids and a bilayer → hexagonal

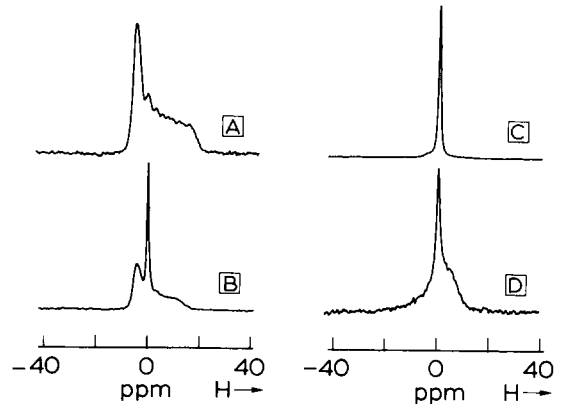

Fig. 7. Effect of Ca^{2+} removal from the Ca-cardiolipin (1 : 1) complex by dialysis on the 36.4 MHz ^{31}P-NMR spectra of cardiolipin. (A) Before dialysis, (B) and (C) after 23 and 70 h of dialysis, respectively. (D) As (C) after 2 min vortexing.

H_{II} transition of 10% of the lipids (Fig. 2), an endothermic reaction with a ΔH of 0.2 kcal/mol was observed (Table I). In the presence of a molar excess of Ca^{2+} the endothermic reaction has a heat content of 1.3 kcal/mol which further increased to 1.8 kcal/mol in the presence of a 16.7-fold molar excess of Ca^{2+}.

Dilution of the Ca-cardiolipin (1 : 1)-salt in a large excess of Ca^{2+}-free buffer did not affect the ^{31}P-NMR characteristics of the system for several hours. To get a more quantitative measure on the kinetics of the dissociation of the Ca-cardiolipin complex we dialyzed the salt against buffer and monitored both structure and Ca-binding as a function of time (Figs. 6 and 7). After an initial fast release of excess unbound Ca^{2+} the bound Ca^{2+} is exceedingly slowly released from the complex (Fig. 6). The half-time of the dissociation is in excess of 80 h. After approx. 23 h when still 86% of the Ca^{2+} is present a change in the ^{31}P-NMR from hexagonal H_{II} → 'isotropic' occurs (Fig. 7). In the next 50 h when an additional 12% of Ca^{2+} is removed (Fig. 6), only a very sharp (linewidth at half-weight 13 Hz) ^{31}P-NMR signal is observed (Figs. 6, 7). At this time the visual appearance of the sample has changed dramatically from a milky dispersion of lipid aggregates to a two-phase sys-

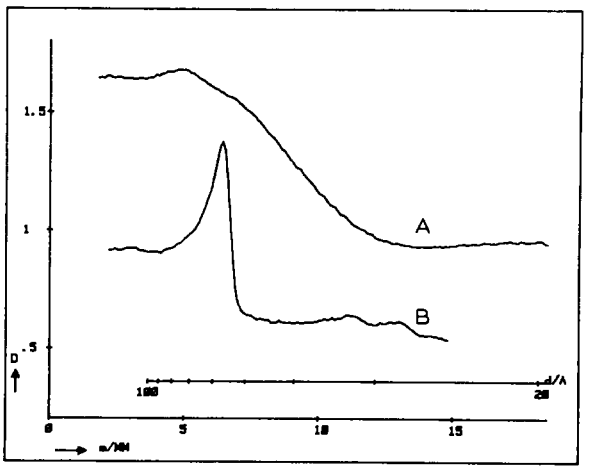

Fig. 8. Densitometer tracings of small angle X-ray diffraction patterns of the Ca-cardiolipin (1 : 1) complex before (B) and after (A) 70 h of dialysis against Ca^{2+}-free buffer. The intensity of the diffraction D (in arbitrary units) is plotted both versus the distance (in mm) on the film between the diffracted and the non-diffracted beam and the corresponding lattice value d (in Å).

tem consisting of a lipid-free aqueous supernatant and an optically clear, highly viscous lower phase. The isotropic phase contained 111 mM cardiolipin (15.5% by weight) which accounted for all the cardiolipin initially present. Freeze-fracturing revealed that after 23 h of dialysis the sample consisted of hexagonal H_{II} phase and lipidic particles containing bilayers (Figs. 3C, D, E). The optically clear isotropic phase consisted of an interwoven network of lipid and water fracture planes (Fig. 3F). The lipid-fracture planes appear to be interconnected with sizes of approx. 1000–2000 Å. Small angle X-ray diffraction measurements of the Ca-cardiolipin (1 : 1) complex revealed diffraction bands at d values of 57, 33 and 28 Å (Fig. 8). Such a diffraction pattern with a $1 : 1/\sqrt{3} : \frac{1}{2}$ relationship between the first and higher order diffraction bands, is characteristic for a hexagonally organized lipid phase. The distance between the tubes is 57 Å. In the optically clear isotropic phase no defined diffraction bands can be observed (Fig. 8). Instead, a broad (ranging from $d = 30$–90 Å) diffuse band, typical [31] of an X-ray scattering profile for a liquid-like concentrated 'solution' of small aggregates is observed. Another feature of this phase is its mechanical instability. Brief vortexing results in the formation of a milky turbid dispersion and a change in the ^{31}P-NMR spectrum (Fig. 6) indicating the formation of bilayer structures. This was further evidenced by freeze-fracturing, which revealed the presence of many bilayer vesicles of variable size in the preparation.

Discussion

An aqueous dispersion of the sodium salt of bovine heart cardiolipin undergoes a bilayer → hexagonal H_{II} phase transition when the Ca^{2+} concentration is increased through the 1–3 mM range, both when Ca^{2+} is added from a concentrated solution as well as when the liposomes are dialyzed against excess Ca^{2+} containing buffer. In the hexagonal H_{II} phase Ca^{2+} is stoichiometrically bound to cardiolipin. From the cation specificity of the induction of the phase change [8,11,12] as well as from monolayer data [32] a binding of Ca^{2+} to the two phosphates of one cardiolipin molecule seems most likely. From space filling models of cardiolipin it can be estimated that the two charged phosphates are separated by approx. 9 Å assuming an extended conformation of the connecting glycerol molecule. Such a configuration seems most likely in view of both charge repulsion arguments as well as from the similarity in the ^{31}P-NMR residual effective chemical shift anisotropy of the phosphates in cardiolipin and other phospholipids such as phosphatidylcholine [9]. For these lipids an extended conformation of the base part of the polar headgroup oriented parallel to the plane of the membrane has been documented (for review see Ref. 32).

Since in the hexagonal H_{II} phase of the Ca-cardiolipin (1 : 1) complex the headgroup appears to be dehydrated [8] and the diameter of the dehydrated Ca^{2+} ion is 1.98 Å it is most likely that the two phosphates are pulled together over a considerable distance thereby reducing the headgroup's size. Molecular models reveal that this can only be accomplished when the connecting glycerol is bended. Assuming a similar configuration of the phosphatidic acid moieties of cardiolipin and other phospholipids such that the glycerol is perpendicular to the plane of the bilayer and the acyl chain at the 2-position is bent at C_2 [34], this subsequently will have to result in an increased hydrocarbon chain area. Analogous with previous suggestions [26] we consider the change in molecular shape as the main driving force behind the bilayer → hexagonal H_{II} transition.

The heat of the Ca-cardiolipin complex formation levels off with higher Ca^{2+} concentrations at approximately 1.8 kcal/mol. The bilayer → hexagonal H_{II} transition thus is an endothermic reaction with an enthalpy ΔH of approx. 1.8 kcal/mol. For thermally induced bilayer → hexagonal H_{II} phase transitions in aqueous dispersions of phosphatidylethanolamine similar low energy endothermic transitions have been observed using differential scanning calorimetry [26]. The low heat content of these transitions must reflect the fluid character of both the bilayer and hexagonal H_{II} phases. The exact origin of the slight increase in ΔH at high Ca^{2+} concentrations is not understood and will be studied in more detail using flow calorimetry.

Prior to the induction of the hexagonal H_{II} phase Ca^{2+} binding to cardiolipin in the lamellar phase occurs. Although the present data do not

allow for a discrimination between 'adsorbed' and bound Ca^{2+} it is clear that maximally 0.35 Ca^{2+} per cardiolipin can be bound in the lamellar phase at a free Ca^{2+} concentration of 2.5 mM. Thus at the bilayer → hexagonal transition a large increase in Ca^{2+} binding occurs. It is of interest to compare these data with those obtained for bovine brain phosphatidylserine. It is also observed with this negatively charged phospholipid that above a threshold concentration of 1–2 mM Ca^{2+} an 1 : 2 Ca^{2+} phosphatidylserine complex is formed resulting in a large structural change [35,36]. However, the organization of the Ca-phosphatidylserine complex is markedly different from that of the Ca-cardiolipin complex, in that the phosphatidylserine is organized in a closely packed cochleated lamellar arrangement in which the acyl chains are in the gel state [35,36]. The Ca-phosphatidylserine complex formation is an exothermic reaction with a ΔH of -4.7 ± 0.5 kcal/mol [37,38] in agreement with a chain crystallization process. Thus, despite the similarities in effect of Ca^{2+} on phosphatidylserine and cardiolipin, the structural properties of the resulting complexes are very specific and emphasize the importance of polar headgroup Ca^{2+} interactions for membrane structure.

The formation of the intermediate 'isotropic' structure characterized by an isotropic ^{31}P-NMR signal with associated lipidic particles was only observed when intermediate amounts of Ca^{2+} were added as aliquots from a concentrated solution to the cardiolipin liposomes. The formation of this structure must be the result of the transient existence of local high Ca^{2+} concentrations as inclusion of the Ca^{2+} ionophore A23187 in the liposomes did not prevent its formation. Ca^{2+} introduction via dialysis methods only caused a bilayer → hexagonal H_{II} transition. Our interpretation is that due to locally high Ca^{2+} concentrations, Ca-cardiolipin (1 : 1) complexes are formed locally in the outer monolayer of the outer bilayer of the liposomes. In this complex the molecules favour hexagonal H_{II} phase formation. However, the bilayer → hexagonal H_{II} transition must be a highly cooperative event and can occur only when extended areas of molecules would like to undergo the same phase change. Instead, rapid fusion events between destabilized areas of the Ca-cardiolipin (1 : 1) complex in one or between different cardiolipin liposomes occur resulting in the formation of inverted micelles at the sites of fusion. In the dialysis experiments the gradual increase in Ca^{2+} concentration leads to increased Ca^{2+} binding and a gradual decrease in surface charge density inducing a close approximation of extended bilayer surfaces allowing a cooperative bilayer → hexagonal H_{II} transition to occur.

In mixed dioleoylphosphatidylcholine/cardiolipin bilayers addition of Ca^{2+} from a concentrated solution [15] or via the dialysis method (this study) even at Ca^{2+} concentrations as low as 0.2 mM induces the formation of the 'isotropic' structure. In agreement with observations made in other lipid mixtures [15] lipidic particle formation must be the result of the coexistence of both bilayer and hexagonal H_{II} phase preferring lipids. It could be suggested that the presence of phosphatidylcholine interferes with the formation of extended areas of the Ca-cardiolipin (1 : 1) complex thereby inhibiting hexagonal H_{II} phase formation. However, it should be noted that at higher Ca^{2+} concentrations substantial amounts (up to 80%) of dioleoylphosphatidylcholine can be incorporated into the hexagonal H_{II} phase of cardiolipin (Ref. 11, and this study). For a better understanding of the phase changes in this system the kinetics of the various processes will have to be investigated.

The bilayer → hexagonal H_{II} phase transition can be readily reversed by dialysis of the Ca-cardiolipin (1 : 1) complex against EDTA containing buffers resulting in the formation of large bilayer vesicles [9,10]. Our experiments show that in the absence of EDTA not only the rate of this process is extremely slow but that also several structural changes occur. Below a critical amount of Ca^{2+} bound (Ca/cardiolipin = 0.86) a structure is formed, characterized by an isotropic ^{31}P-NMR signal and lipidic particles thus resembling the structure induced by adding limiting amounts of Ca^{2+} to the cardiolipin liposomes. Upon further removal of Ca^{2+} (Ca/cardiolipin = 0.7) another structural change occurs, in that an optically clear, highly viscous cardiolipin-rich phase separates out of solution. The cardiolipin molecules undergo rapid isotropic motion in this phase. These characteristics are typical for cubic phases [39]. Since

this phase does not give a discrete X-ray diffraction pattern as often [39] encountered with cubic phases, the exact structure is unknown. Our freeze-fracturing data would be consistent with a cubic phase related to the 'Schwarz's primitive cubic minimal surface' structure in which the lipids are organized in a continuous highly curved bilayer which separates two independent three-dimensional systems of aqueous channels (see for instance Fig. 3 in Ref. 40). From the linewidth of the ^{31}P-NMR signal it can be estimated [41] that the average radius of bilayer curvature is 180 Å assuming similar lateral diffusion rates of cardiolipin and egg phosphatidylcholine. The curbic phase is metastable as mechanical agitation immediately results in the formation of large bilayer vesicles. The mechanism(s) of the phase changes observed upon the removal of Ca^{2+} from cardiolipin are obscure at the moment. Since thermally induced bilayer → hexagonal H_{II} transitions in general are fully reversible [29] the complex phase behaviour of this system must be the result of the way the hexagonal H_{II} triggering agent Ca^{2+} is released from the structure. As the aqueous channels in the hexagonal H_{II} phases appear to be open to the surrounding aqueous phase as evidenced by the observation that paramagnetic ions broaden the entire ^{31}P-NMR signal of hexagonally oriented phosphatidylethanolamines (van Echteld, C.J.A., unpublished observations) it can be expected that Ca^{2+} is relased from the ends of the tubes. At these sites cardiolipin would like to adopt a bilayer configuration but this is inhibited because these molecules are still connected to the remainder of the hexagonal H_{II} phase. In these transitional regions apparently lipidic particles are formed. After a possible reorganization the lipidic particles dissociate and the cubic phase results.

The relationship between the lipidic particles and cubic phases is of particular interest since both structures have now been observed in different mixtures of hydrated membrane lipids and both structures have been reported as intermediates between lamellar and hexagonal H_{II} phases. For instance, cubic phases have been reported for aqueous dispersions of glycolipids from *Acholeplasma laidlawii* [42], whereas lipidic particles have been observed in many other lipid systems [29]. The present study demonstrates that one type of lipid can adopt both structures. The exact interrelationship between lipidic particles and cubic phases as well as the biological significance of this latter phase can only be speculated upon. However, it is noteworthy that the morphology of the prolamellar body [43] which is involved in the formation of thylakoid membranes of chloroplasts and which contains substantial amounts of the hexagonal H_{II} phase preferring monogalactosyl diacylglycerol [21] greatly resembles the structure of some of the cubic phases observed in model membranes [40].

Acknowledgements

Dr. M.R. Egmond is thanked for the calculation of the dissociation constants and Dr. T.F. Taraschi for correcting the English. The excellent technical assistance of W.S.M. Geurts van Kessel and Ms. M. Tieman in the synthesis and isolation of the lipids is greatfully acknowledged.

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