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Intramolecular hydrogen bonding in cardiolipin *

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Fourier transform infrared (FT-IR) spectroscopy was used to determine whether intramolecular hydrogen bonding between the C-OH and P-OH groups exists in beef heart cardiolipin (CL) or in hydrogenated beef heart cardiolipin (18:0-CL) as compared to the synthetic 2′-deoxy analogue of cardiolipin (16:0-dCL). Such intramolecular hydrogen bonding would provide a structural basis for proton conduction on the molecular level. In aqueous dispersions at 20°C, both 18:0-CL and 16:0-dCL exist in the gel phase as bilayers with gel to liquid-crystalline transitions (T_m) at 61 and 56°C, respectively, whereas the unsaturated CL exists in the non-bilayer (hexagonal II) state. Evidence for intramolecular hydrogen bonding of the C-OH group in aqueous dispersions of 18:0-CL is provided by the large increase in T_m observed on changing the aqueous medium from H_2O to D_2O but specific hydrogen-bonded C-OH...PO_2^- species cannot be identified because water molecules also compete for the PO_2^- binding sites. However, C-OH...PO_2^- hydrogen bonds can be identified in dry films of the sodium salt of 18:0-CL or in CCl_4 solution. In contrast, such hydrogen bonds cannot be formed in the deoxy analogue (16:0-dCL) indicating that the central C-OH group in 18:0-CL could provide a structural basis for proton conduction, involving the phosphate groups.

Introduction

Submitochondrial particles capable of carrying out oxidative phosphorylation are known to contain phosphatidylcholine, phosphatidylethanolamine and cardiolipin as major membrane phospholipids [1]. The membrane bound multienzyme system, consisting of an oxidation chain and a 'coupling device' utilizes the energy of oxidation to generate ATP from ADP and P_i. Studies on the reconstitution of the 'coupling device' of oxidative phosphorylation from hydrophobic and soluble mitochondrial proteins (F_0-F_1-ATPase, catalysing $^{32}P_i$-ATP exchange and ATPase activity) in phospholipid vesicles have shown that optimal activities were obtained with mixtures of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin in the molar ratio 3:2:0.1, respectively [1]. The first two of these phospholipids provide a bilayer lipid matrix for ordering of the enzyme proteins, while the cardiolipin is required for optimal activity of the 'coupling device' [1].

The precise role of cardiolipin in this coupling process is not known, but it has been postulated that anionic lipids, including cardiolipin, may function as a proton-conducting chain to transport protons along the polar surfaces of mitochondrial and chloroplast membranes [2,3]. In extremely halophilic bacteria, another anionic phospholipid, the diphytanylether analogue of phosphatidylglycerophosphate (PGP), which has a similar structure to cardiolipin (see Scheme I), has recently been shown to be capable of participating in proton-conducting pathways in monolayer systems [4]. This phospholipid may have a similar function to cardiolipin in the purple and red membranes of extremely halophilic bacteria [5,6]. The structural basis for proton conduction by PGP has been shown to reside in the presence of the free central glycerol hydroxyl group,

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Abbreviations: CL, cardiolipin (diphosphatidylglycerol); dCL, 2′-deoxycardiolipin (diphosphatidyl-1,3-propanediol); 18:0-CL, hydrogenated beef heart cardiolipin; 16:0-dCL, synthetic 2′-deoxycardiolipin with palmitoyl groups; PGP, phosphatidylglycerophosphate; FT-IR, Fourier transform infrared.

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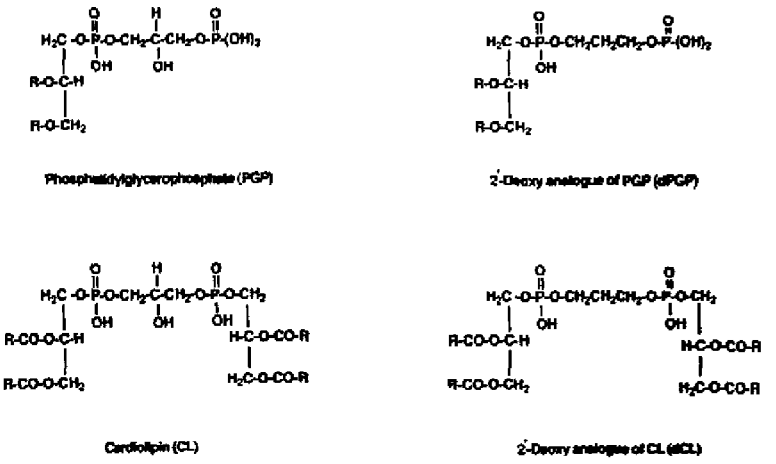
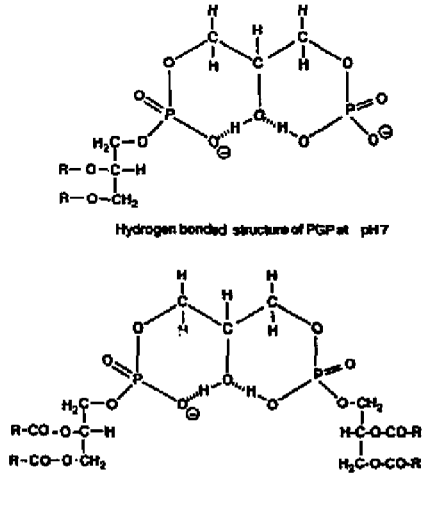


Scheme I. Non-ionized structures of phosphatidylglycerophosphate (PGP) and cardiolipin, and their deoxy analogues.

which forms intramolecular hydrogen bonds with the phosphate groups [5], resulting in an anomalously high pK (> 11) for one of the P-OH groups [6] and hence retention of a proton at physiological pH. This proton, together with protons from water could form the basis of a proton conducting pathway [4–6].

Examination of the structure of cardiolipin (Scheme I) reveals a striking similarity to PGP with respect to the presence of a central OH group on the polar glycerol moiety which could hydrogen bond with either of the two P-OH groups. Thus it appeared that cardiolipin has the potentiality, like PGP, of forming an intramolecular hydrogen bonded system that could result in the retention of a proton and hence could act as part of a proton conducting pathway, as suggested in Scheme II.



Scheme II. Comparison of intramolecularly hydrogen bonded structures of PGP and cardiolipin involving the C-OH and the phosphate groups.

In support of this supposition, we have recently found [7] that hydrogenated beef heart cardiolipin (18:0-CL) has only a single ionized phosphate group at pH $\leq$ 7, the second phosphate group being only titratable above pH 7; in contrast, the synthetic 2'-deoxy cardiolipin (16:0-dCL) which lacks the central glycerol OH group (Scheme I), titrated normally as a dibasic acid up to pH 7. To obtain further support for the proton conducting potential of cardiolipin, we have examined the natural beef heart cardiolipin (CL), the hydrogenated beef heart cardiolipin and the synthetic deoxy cardiolipin by Fourier transform infrared (FT-IR) spectroscopy to determine whether the expected intramolecular hydrogen bonded C-OH/P-OH system exists in CL but not in dCL. In the present study we also addressed the question of the physical structures of these lipids in aqueous dispersions and as dry films.

Experimental

Materials

Beef heart cardiolipin (Na salt) was purchased from Sigma Chemical Co. St. Louis, MO. Its fatty acid composition was: 16:0, 0.5%; 16:1, 0.4%; 18:0, 0.2%; 18:1, 6.7%; 18:2, 92.2%. Hydrogenated beef heart cardiolipin was prepared by catalytic hydrogenation with Adams' catalyst ($PtO_2 \cdot H_2O$) in methanol for 30 minutes at room temperature [8]. Its fatty acid composition was: 16:0, 9.3%; 18:0, 76.6% and 18:1(*trans*) 9.4%. Deoxy cardiolipin containing largely (95.5%) 16:0 acyl chains (16:0-dCL) was synthesized as described elsewhere [7]. Ammonium salts or free acid forms of these cardiolipins and analogues were prepared as described previously [7,8]; sodium salts were prepared by titration of the free acid with methanolic

NaOH to pH 7.5, followed by precipitation with acetone.

Methods

Infrared spectra. Infrared spectra were obtained on a Digilab FTS-60 Fourier transform spectrometer equipped with a mercury cadmium telluride detector. For cardiolipin samples dissolved in organic solvents (1–10 mg lipid/100 μl CCl_4) we used sealed 50 μm thick KBr cells. Cardiolipin dispersions in aqueous solution (3 mg/100 μl H_2O or D_2O) were investigated in demountable 50 μm thick CaF_2 cells. For temperature control a cell mount was used through which thermostatically controlled triethyleneglycol/water was circulated. Bath temperatures were stable to within 0.05 C°. The temperature in the cell was monitored by a platinum thermoelement. Measurements as a function of temperature were performed by increasing the bath temperature in steps of 2 C° with a waiting period of 15 min between consecutive temperature steps. The operation of regulating the temperature and recording individual spectra was completely under the control of the spectrometer computer [9].

Polarized attenuated total reflection (ATR) infrared experiments were performed with an overhead ATR unit (Wilmad Inc., New Jersey, Model 2FM) equipped with a parallelogram-shaped zinc selenide ATR plate (50 × 20 × 2 mm, face angle 45°). Thin cardiolipin films were prepared by spreading 50 μl of a 1 mM lipid solution in chloroform uniformly on one side of the zinc selenide crystal, according to a procedure described by Fringeli [10]. Linear polarized light was produced by placing a wire grid polarizer (Cambridge Instruments, model IGP 225) behind the ATR unit. A home-built device was used to adjust the polarizer from the keyboard of the computer, to obtain parallel (p) or perpendicularly (s) polarized light relative to the plane of incidence of the infrared beam. Single beam spectra of the blanc zinc selenide crystal recorded with both polarizer settings were used as references for the polarization measurements of the cardiolipin films. Typically, 500 interferograms were co-added, triangularly apodized and zero filled once to yield a final resolution of 2 cm^{-1} and an encoding interval of 1 cm^{-1}.

Data treatment. Infrared frequencies associated with particular vibrational modes were obtained from third order derivative spectra [11]. For the carbonyl stretching bands Fourier self deconvolution was applied as a method of band narrowing [12,13]. Dichroic ratios ($R = A_p/A_s$) were determined by measuring the integrated area under a specific infrared absorption band, or in some cases the peak heights, of spectra recorded with parallel (p) and perpendicular (s) polarized light. The R values were then used to calculate the mean angle θ between the direction of the oscillating dipole moment of a specific vibration and the normal to the ATR crystal. We applied the methodology for thin films on ATR crystals, described in detail by Fringeli [10,14]. The face angle of the crystal was 45° and the values used for the refractive indices were $n_1 = 2.4$ for zinc selenide, $n_2 = 1.55$ for the dry lipid films, and $n_3 = 1.0$ for air.

Results and Discussion

Cardiolipin in CCl_4 solution and as solid films

Fig. 1 shows the infrared spectra of the ammonium salts of 18:0-CL, beef heart CL and 16:0-dCL as dilute solutions in CCl_4 in the spectral ranges 3400–2800 cm^{-1} (panel A) and 1800–1650 cm^{-1} (panel B). The strong infrared bands at 2854 and 2924 cm^{-1} in 18:0-CL and 16:0-dCL are respectively due to the symmetric and antisymmetric CH_2 stretching vibrations of the acyl chains in these lipids. The frequency values are consistent with a high degree of *gauche* conformers in the methylene groups of the hydrocarbon chains [15]. In beef heart CL these IR absorption bands occur at somewhat higher frequencies (2857 and 2929 cm^{-1}) and are also broader, indicating a still higher degree of conformational and motional disorder in the cardiolipin with unsaturated acyl chains. The most obvious difference between the spectra of beef heart CL and the hydrogenated 18:0-CL is the absence of a sharp band at ≈ 3010 cm^{-1} in 18:0-CL. This vibration represents the olefinic =C-H stretching mode [16], and is caused by the large number of cis double bonds in the natural beef heart CL which contains > 90% 18:2 acyl chains. The absence of this band in 18:0-CL is evidence for the complete hydrogenation of the *cis* double bonds.

In the spectrum of 18:0-CL there are two broad

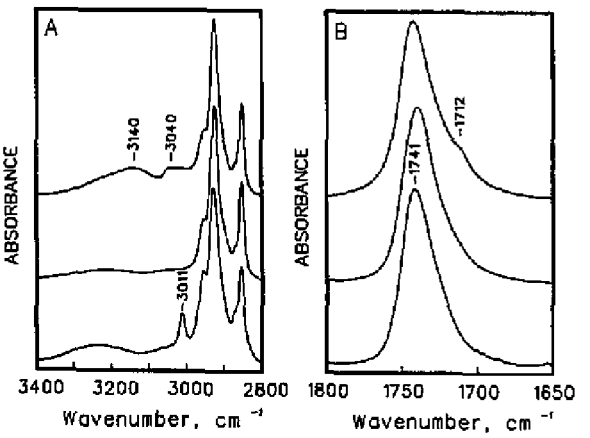


Fig. 1. FT-IR spectra of the ammonium salts of 18:0-CL (top), 16:0-dCL (middle) and beef heart-CL (bottom), as solutions in CCl_4 in the region of the C-H stretching (panel A) and ester C=O stretching bands (panel B).

absorption bands at ≈ 3140 and ≈ 3040 cm^{-1} which are not present in the spectra of beef heart CL and 16:0-dCL (see below). On treatment of the CCl_4 solutions with D_2O the bands shift to 2360 and 2250 cm^{-1} (data not shown). Therefore, they can be identified as arising from O-H stretching vibrations, such as the glycerol C-OH and/or the P-OH groups in 18:0-CL; 16:0-dCL lacks both of these bands, but this is to be expected since it does not contain a C-OH group and both P-OH groups are ionized in the NH_4^+ salt form. A free O-H stretching vibration is expected to absorb in the frequency range 3500–3600 cm^{-1}, with a decrease in frequency if the OH group is involved in hydrogen bonding. The fact that the position of these bands is not concentration dependent strongly suggests that the C-OH group is involved in intramolecular hydrogen bonding. Dry films of the NH_4^+ salts of 18:0-CL also exhibit two bands at ≈ 3150 and ≈ 3050 cm^{-1}, that shifted to ≈ 2350 and 2250 cm^{-1} when the films were exposed to D_2O vapor for 1 h and then flushed with dry nitrogen. Dry films of the ammonium salts of 16:0-dCL and beef heart CL do not show these bands. We surmise that a lamellar structure with ordered acyl chains (at least near the polar head group) is necessary for the formation of an intramolecular hydrogen bond; this is not possible with beef heart CL because of its high degree of unsaturation which does not allow the formation of bilayer structures. Instead it forms a hexagonal II structure in which the C-OH and PO_4^- groups are buried. This structure is retained by beef heart CL even in CCl_4 solution and would not be expected to show strong OH bands.

All three cardiolipin derivatives dissolved in CCl_4 exhibit a broad ester C = O stretching band with the maximum around 1741 cm^{-1} (see Fig. 1B) which is typical of non-hydrogen-bonded C = O groups [17]. However, a pronounced shoulder on the low frequency side is clearly visible at 1712 cm^{-1} in the spectrum of 18:0-CL, suggesting that in this lipid some of the ester C = O groups act as proton acceptors for the glycerol C-OH and/or phosphate P-OH groups. The absence of this band in the spectrum of beef heart CL in CCl_4 solution (Fig. 1B) may be attributed to the different spatial arrangement of C-OH/P-OH and C = O groups in the hexagonal II phase.

Aqueous dispersions of cardiolipin

CH_2 stretching region. Fig. 2 shows infrared spectra of 18:0-CL (at 20 and 75°C) and beef heart CL (at 20°C) in D_2O in the region of the C-H stretching bands. The spectrum of natural beef heart CL at room temperature is very similar to that of the hydrogenated 18:0-CL at 75°C, except that the olefinic =C-H stretching band at 3009 cm^{-1} is missing in the latter. The frequencies of the symmetric and antisymmetric CH_2 stretching bands of 18:0-CL at 75°C (2853 and 2924 cm^{-1}, respectively) and of beef heart CL at 20°C (2855 and 2926 cm^{-1}, respectively) are characteristic of disordered acyl chains of lipids in the liquid crystalline state or in the hexagonal-II phase [18]. At 20°C 18:0-CL exhibits CH_2 stretching bands at 2850 cm^{-1} and 2916 cm^{-1}, which indicates that at room temperature this phospholipid forms a multilamellar gel state with a high degree of acyl chain order [19,20]. In this respect the spectrum of 16:0-dCL (not shown for brevity), is similar to that of 18:0-CL.

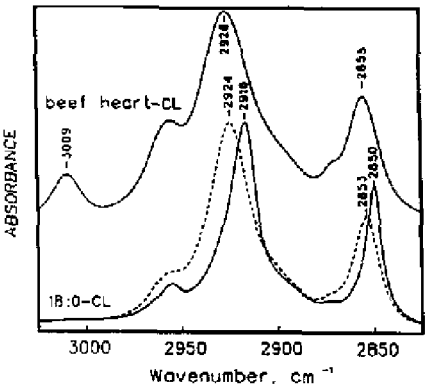


Fig. 2. FT-IR spectra of aqueous (D_2O) dispersions of cardiolipin ammonium salts in the region of the C-H stretching bands. Bottom: 18:0-CL at 20°C (solid line) and at 75°C (broken line). Top: beef heart-CL at 20°C.

The thermotropic phase behavior of 18:0-CL and 16:0-dCL can be studied by monitoring the frequency of the symmetric CH_2 stretching band as a function of temperature [15]. The frequency vs. temperature plots of the two lipids dispersed either in D_2O (Fig. 3A) or

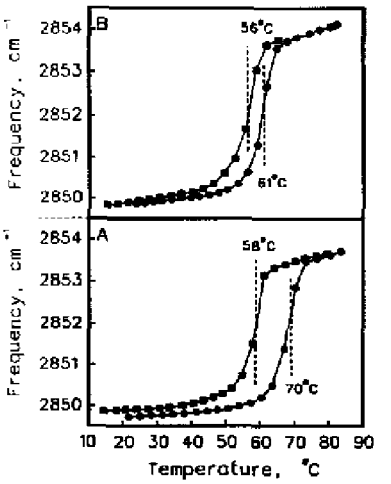


Fig. 3. Temperature profiles of the frequencies of the symmetric CH_2 stretching vibrations of 16:0-dCL (squares) and 18:0-CL (circles) dispersed either in D_2O (panel A) or in H_2O (panel B). Broken vertical lines indicate the phase transition temperatures.

in H_2O (Fig. 3B) show clearly that 16:0-dCL exhibits a gel to liquid-crystalline phase transition with a T_m of 56 °C in H_2O and 58 °C in D_2O. Thus, replacement of H_2O with D_2O has only a small effect on the absolute value of the phase transition temperature. However, with the 18:0-CL system, replacement of H_2O with D_2O leads to an increase in T_m by approx. 9 °C (from 61 to 70°C). A rationalization for these observations is that the free glycerol OH group in 18:0-CL is involved in intramolecular hydrogen bonding in the gel phase since it is well known that an exchange of OH by OD leads to a strengthening of hydrogen bonding and hence to an increase in T_m [21]. However, the 16:0d-CL, lacking the free OH group, would not be capable of showing such an increase in T_m in D_2O.

C = O stretching region. Since carbonyl groups act as proton acceptors one may expect differences in the frequency and bandshape of the C = O stretching vibrations of 18:0-CL and 16:0-dCL. Fig. 4 shows the region of the C = O stretching bands in the original spectra and after band narrowing. As can be seen already from the spectra in Fig. 4A, but more clearly from Fig. 4B, the carbonyl band of 16:0-dCL consists of two components at all temperatures. At T_m (58°C) both bands shift to higher frequencies by approx. 6 cm^{-1} (from 1736 to 1742 cm^{-1} and from 1718 to 1725 cm^{-1}); at the same time the latter band increases in intensity. The high frequency band can be assigned to free carbonyl groups and the low frequency band to carbonyl groups hydrogen bonded to D_2O [17,22]. The intensity of the low frequency band increases at T_m, indicating an increase in the number of hydrogen

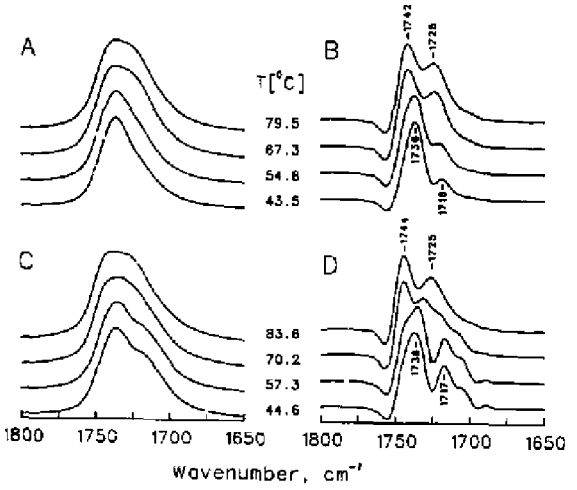


Fig. 4. FT-IR spectra of the ammonium salts of 16:0-dCL (panel A) and 18:0-CL (panel C) as dispersions in D_2O in the region of the carbonyl stretching bands at the indicated temperatures. Panels B and D show the spectra of the left-hand side after band narrowing by Fourier self deconvolution (band narrowing factor 2.2).

bonded C = O species above T_m. The spectra of 18:0-CL (Fig. 4C) show a pronounced increase in intensity on the low-frequency side of the absorption maximum compared to the spectra of 16:0-dCL (Fig. 4A). Band narrowing (Fig. 4D) reveals the presence of at least three C = O bands in the spectrum of 18:0-CL at room temperature, of which two match the frequencies of the two C = O stretching bands in 16:0-dCL, with the third occurring at a much lower frequency, around 1703 cm^{-1}. The latter band must result from strong (most likely intramolecular) hydrogen-bonded C = O groups. Fig. 5 shows the temperature dependence of the individual component bands. With 18:0-CL (filled circles), there is a splitting of the non-hydrogen bonded C = O band at 1736 cm^{-1}, while the hydrogen-bonded C = O bands at 1703 and 1717 cm^{-1} shift to higher frequencies. The low frequency C = O band (below 1710 cm^{-1}) and the band at 1733 cm^{-1} disappear above T_m (70 °C). Above their respective T_m the frequencies and relative intensities of the two remaining C = O bands at 1725 and 1743 cm^{-1} become identical in 18:0-CL and 16:0-dCL (compare Fig. 4A with Fig. 4C, or Fig. 4B with Fig. 4D). The loss of the C = O component below 1710 cm^{-1} and the high mobility of the acyl chains in the liquid-crystalline phase of 18:0-CL indicate a disruption of the intramolecular hydrogen bonding between the ester carbonyl groups and the glycerol C-OH and/or P-OH groups that seem to exist in the gel phase.

CH_2 scissoring region. The question arises as to how intramolecular hydrogen bonding in the gel phase of 18:0-CL affects the chain packing. This question was addressed by use of the CH_2 scissoring vibration of the acyl chain methylene groups. In the spectra of 16:0-dCL, in which intramolecular hydrogen bonding does not exist, there is a single CH_2 scissoring band with a frequency of 1467 cm^{-1} at all temperatures (see Fig. 6). Such a band is characteristic of hexagonally packed acyl chains, which are able to rotate around their long axis, as *n*-paraffins in the solid rotator phase [23]. Above T_m (58 °C) the band remains at 1467 cm^{-1} but broadens considerably, indicating increased disorder and a higher mobility of the acyl chains. This behavior is typical of many phospholipids when changing from the gel to a liquid-crystalline state.

On the other hand, the spectra of 18:0-CL in the gel phase show two CH_2 scissoring bands, at 1471 and 1468 cm^{-1} (Fig. 6B). The additional band at 1471 cm^{-1} is characteristic of a rigid, crystal-like lattice in which rotation around the long axis of the molecules cannot occur [24]. For a correlation field splitting (as in an orthorhombic lattice) two bands at ≈ 1472 and ≈ 1464 cm^{-1} would be expected [24]; thus, the two CH_2 scissoring bands at 1471 and 1468 cm^{-1} are not compatible with an orthorhombic subcell and we must conclude that the gel phase of 18:0-CL contains two

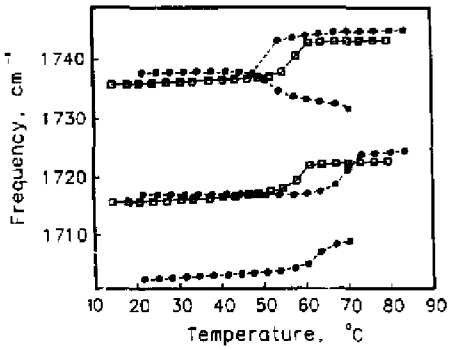


Fig. 5. Frequency vs. temperature plots of the components of the carbonyl stretching band in aqueous (D_2O) dispersions of 16:0-dCL (open squares) and 18:0-CL (filled circles). Frequencies were obtained from third order derivative spectra (see Methods).

populations of acyl chains. We postulate that the immobilization of the chains in the gel phase is caused by intramolecular hydrogen bonding of the glycerol OH to the ester carbonyl groups. The acyl chains bearing no hydrogen bonded C = O groups can rotate freely around their long axis, and may be attributed to the absorption band at 1468 cm^{-1}, whereas the band at 1471 cm^{-1} may be attributed to acyl chains with intramolecular hydrogen-bonded C = O groups.

The CH_2 scissoring band arising from the α-CH_2 group of the acyl chains next to the ester group is spectroscopically separated from the other CH_2 scissoring bands, and usually occurs at 1418 cm^{-1} [25]. An inspection of Figs. 6A and 6B shows that in the gel phase there is a weak band at 1417 cm^{-1} in both 16:0-dCL and in 18:0-CL. However, in the gel phase of 18:0-CL there is an additional band at 1425 cm^{-1} which is narrower than the 1417 cm^{-1} band and thus may be assigned to immobilized α-CH_2 groups. Since the latter group is located next to the C = O double

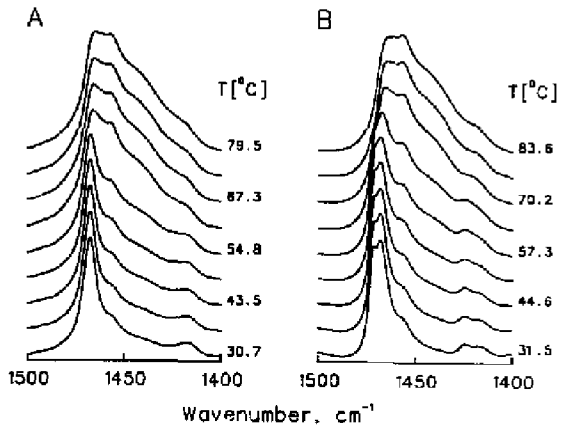


Fig. 6. FT-IR spectra of the ammonium salts of 16:0-dCL (A) and 18:0-CL (B) as dispersions in D_2O in the region of the CH_2 scissoring bands.

bond, immobilization of the ester group by intramolecular hydrogen bonding should affect the CH_2 scissoring vibration, and indeed a shift of 8 cm^{-1} to higher frequencies of this absorption band is observed.

Phosphate region. Fig. 7 illustrates the 1300–1000 cm^{-1} region of the infrared spectra of aqueous dispersions of 16:0-dCL (Fig. 7A) and 18:0-CL (Fig. 7B), below (solid lines) and above the phase transition temperature (broken lines). The antisymmetric PO_2^- stretching vibration ($\nu_{as}PO_2^-$) of non-hydrated phosphodiesters usually gives a strong band in the frequency range between 1240 and 1260 cm^{-1} [26], which shifts to lower frequency values (1230–1220 cm^{-1}) when the PO_2^- group is hydrated and/or involved in hydrogen bonding. In the gel phase of 16:0-dCL and 18:0-CL the $\nu_{as}PO_2^-$ band overlaps with the CH_2 wagging band progression, a series of equally spaced narrow bands (see solid lines in Fig. 7), which hampers the precise determination of the maximum of the much broader $\nu_{as}PO_2^-$ band. Nonetheless, in 16:0-dCL as well as in 18:0-CL the $\nu_{as}PO_2^-$ band is found below 1230 cm^{-1}, indicating that in both lipids the phosphate group is hydrated. Above T_m this band is located at 1215 cm^{-1} (see broken lines in Fig. 7). In aqueous dispersions of natural beef heart CL at 20°C the $\nu_{as}PO_2^-$ band also occurs at $\approx$ 1215 cm^{-1} regardless of whether the sodium or the ammonium salts are used (data not shown).

The symmetric PO_2^- stretching vibration, $\nu_sPO_2^-$, which is generally found in the frequency range 1115–1085 cm^{-1} [27], occurs at $\approx$ 1090 cm^{-1} in all aqueous cardiolipin dispersions, irrespective of the physical state of the system. Thus, in aqueous CL dispersions the PO_2^- stretching vibrations of the phosphodiester group

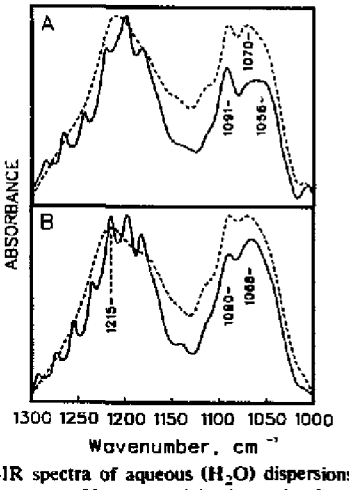


Fig. 7. FT-IR spectra of aqueous (H_2O) dispersions of 16:0-dCL (panel A) and 18:0-CL (panel B) in the region between 1300 and 1000 cm^{-1}. Solid lines denote spectra recorded below the phase transition, and broken lines spectra recorded above the phase transition of the respective lipid.

are not well suited for detecting specific internal hydrogen bonding involving the free glycerol OH group in 18:0-CL (and which is lacking in 16:0-dCL) because the water molecules can also act as proton donors to the hydrated phosphate groups and therefore mask any specific internal hydrogen bonds in these cardiolipins. Nonetheless, there are small differences between the spectra of 16:0-dCL and 18:0-CL in the region 1050–1070 cm^{-1}, which contains infrared absorption bands assigned to C-O-P-O-C vibrations [28,29]. In the gel phase of 16:0-dCL there is a band with a maximum at 1056 cm^{-1} which shifts to 1070 cm^{-1} above T_m (see Fig. 7A). In 18:0-CL (Fig. 7B), as well as in aqueous dispersions of beef heart CL, the band maximum occurs always near 1070 cm^{-1} irrespective of the temperature.

Cardiolipin films on ZnSe

In order to circumvent the possibility of hydrogen bonding of water molecules to the phosphate groups of cardiolipin we also examined the samples as dry films deposited on ZnSe plates. Interestingly, we found significant differences between the spectra when the counterion was NH_4^+ or Na^+. The sodium salt of beef heart CL deposited as a dry film on ZnSe (as well as when dissolved in CCl_4), exhibits the $\nu_{as}PO_2^-$ band at 1247 cm^{-1} and the $\nu_sPO_2^-$ band at 1103 cm^{-1}. In the ammonium salt the frequencies are shifted to 1218 and 1093 cm^{-1}, respectively. The shifts to lower wavenumbers clearly indicate hydrogen bonding between the phosphate groups and the ammonium counterions. Therefore, in order to detect possible differences between 16:0-dCL and 18:0-CL with respect to hydrogen bonding due to the additional glycerol OH group in 18:0-CL we also investigated the Na^+ salts deposited as thin dry films on ZnSe.

Fig. 8 shows ATR-IR spectra of the sodium salts of 16:0-dCL (Fig. 8A) and 18:0-CL (Fig. 8B) recorded with parallel polarization (A_p, solid line) and perpendicular polarization (A_s, broken line), along with the corresponding difference spectrum (A_p–A_s). Negative bands in the difference spectrum are the result of a dichroic ratio $R = A_p/A_s < 1$, indicating that the direction of the oscillating dipole moment (transition moment) of the respective vibration is in the plane of the crystal surface. When A_p–A_s is positive and/or comparable to A_p ($R \gg 1$), then the transition moment of the vibration is perpendicular to the crystal surface. R values of approx. 1 (A_p–$A_s = 0$) for all absorption bands would result in a flat line in the difference spectrum, indicating completely random orientation of the lipid molecules on the crystal surface. We found this to be the case with beef heart CL (data not shown), which demonstrates that this compound cannot form bilayers. The negative bands observed for the antisymmetric and symmetric CH_2 stretching bands at 2918 and 2850 cm^{-1} and for the CH_2 scissoring band at 1470 cm^{-1} (which originate from the methylene groups in the all-*trans* acyl chains with a transition moment in the CH_2 plane), constitute direct evidence that 16:0-dCL and 18:0-CL are arranged in flat bilayer arrays on the surface of the ZnSe crystal. Furthermore, the ester carbonyl groups in 16:0-dCL are also oriented predominantly in the bilayer plane, as judged from the negative absorption band in the difference spectrum at 1740 cm^{-1} (Fig. 8A). In contrast, the C = O stretching band in 18:0-CL (Fig. 8B) exhibits an R value slightly greater than 1, indicating that at least some of the carbonyl groups must be oriented more towards the bilayer normal. This is to be expected for internal hydrogen bonding between the glycerol OH group and the ester C = O groups in 18:0-CL.

The difference spectra in Fig. 8 show a series of sharp, equally spaced CH_2 wagging bands in the region

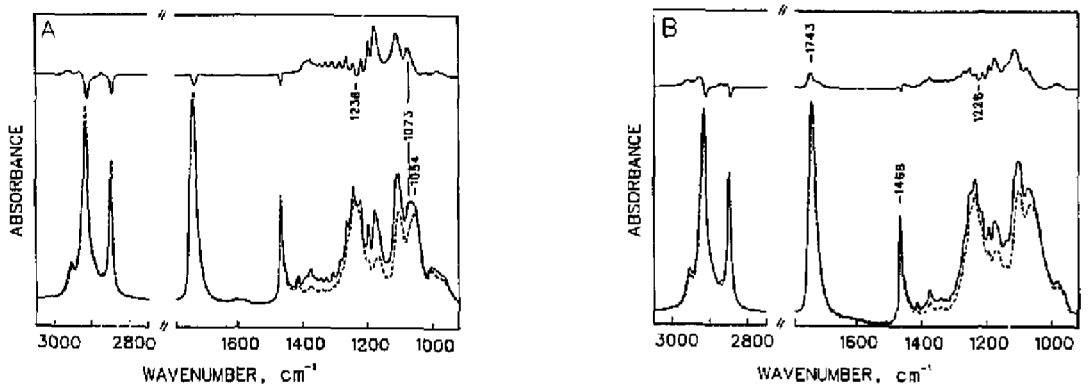


Fig. 8. ATR-IR spectra of dry films of the sodium salts of 16:0-dCL (panel A) and 18:0-CL (panel B) recorded with parallel (A_p; solid lines) and perpendicular (A_s; broken lines) polarized light. The top spectrum in each panel displays the difference spectrum A_p–A_s. In the frequency range between 3000 and 2750 cm^{-1} the panel heights are equivalent to 1 absorbance unit, below 1800 cm^{-1} the equivalents are 0.33 absorbance units.

1195–1330 cm^{-1}. From the number (n) of these bands, i.e., 7 in 16:0-dCL and 8 in 18:0-CL, one can derive the number (N) of all-*trans* acyl chain carbon atoms $N = 2(n + 1)$, namely 16 for 16:0-dCL and primarily 18 for 18:0-CL. Because the transition moment of this vibration lies along the chain axis of the lipid [10], the dichroic ratios of $R > 5$ in 16:0-dCL and $R > 2.5$ in 18:0-CL indicate predominantly perpendicular orientation of the all-*trans* acyl chains relative to the crystal surface. This also confirms the bilayer structure of the two lipids.

The presence of the CH_2 wagging bands in the gel phase of these spectra makes it difficult to establish the exact position of the broad antisymetric PO_2^- stretching vibration, especially in the A_p spectra. However, since the direction of the transition moment of this vibration (i.e., the connecting line between the two non-esterified phosphate oxygens) is oriented towards the bilayer plane, this shoud result in a negative band in the difference spectrum A_p–A_s. Therefore, a good way to estimate the peak maximum of the broad $\nu_{as}PO_2^-$ band is to look for a minimum in the difference spectra of Figs. 8A and 8B. This minimum is located at 1228 cm^{-1} in 18:0-CL, whereas it is at 1236 cm^{-1} in 16:0-dCL. The frequency shift of 8 cm^{-1} to lower wavenumbers can be rationalized if one assumes that in 18:0-CL there is internal hydrogen bonding to the phosphate group, absent in 16:0-dCL. This would support the concept of a proton conduction pathway in 18:0-CL, but not in 16:0-dCL. The simultaneous shift of the $\nu_sPO_2^-$ band by 3 cm^{-1} (from 1107 cm^{-1} in 16:0-dCL to 1104 cm^{-1} in 18:0-CL) endorses this assumption. The dichroic ratio R of the $\nu_sPO_2^-$ band is approx. 1.3 in both lipids. From this, the orientation of the transition moment of the symmetric phosphate stretching vibration (along the bisector of the PO_2^- group) is calculated to deviate by 40° from the bilayer normal. Since the vibrations of the phosphate group of DPPC bilayers deposited on ZnSe show the same dichroic ratios, namely $R = 1.2$–1.3 for the $\nu_sPO_2^-$ band, and $R < 1$ for the $\nu_{as}PO_2^-$ band [10], we conclude that the PO_2^- groups in 16:0-dCL and 18:0-CL adopt similar orientations relative to the bilayer plane as those in phosphatidylcholine.

In both 16:0-dCL and 18:0-CL there are two infrared bands, at 1054 and 1073 cm^{-1}, assignable to P-O-(C)- stretching vibrations of the phosphodiester group. The high frequency band at 1073 cm^{-1} shows parallel polarization with $R \gg 1$ (especially in 16:0-dCL) and may be assigned to the fragment with the carbon atom originating from the diacyldiglycerol moiety, whereas the low frequency band at $\approx$ 1054 cm^{-1} shows no preferred polarization with $R \approx 1$ (indicating random orientation or a 54° angle between the transition moment of this P-O-(C)- vibration and the bilayer normal), which may be assigned to the fragment with the carbon atom originating from the glycerol moiety in 18:0-CL or the 1,3-propanediol moiety in 16:0-dCL.

Concluding remarks

Aqueous dispersions of 16:0-CL and of the hydrogenated beef heart CL (18:0-CL) show a thermotropic phase behavior consistent with a bilayer structure in the gel phase at room temperature, and a liquid-crystalline bilayer phase above their respective phase transition temperatures. Differences between the two lipids in the gel phase can be explained by intramolecular hydrogen bonding of the free glycerol C-OH group to the carbonyl oxygens of the ester groups and to the phosphate oxygens of the head group in 18:0-CL. This reduces the mobility of the lipid molecules in the bilayers of 18:0-CL compared to that in 16:0-dCL. Intramolecular hydrogen bonding in 18:0-CL is further supported by the increase of its phase transition temperature from 61°C to approx. 70°C, observed on changing the aqueous medium from H_2O to D_2O. In 16:0-dCL this change has only a minor effect on the phase transition temperature. Aqueous dispersions of natural beef heart CL at room temperature resemble 16:0-dCL and 18:0-CL dispersions above their T_m. From the ATR polarization experiments of dry cardiolipin films we conclude that, in contrast to 18:0-CL and 16:0-dCL, beef heart CL alone cannot form bilayers and has to be embedded in a bilayer matrix to function as a possible proton carrier in nature. Specific hydrogen bonded C-OH $\cdots$ PO_2^- species cannot be identified from the phosphate vibrations in the infrared spectra of aqueous 16:0-dCL and 18:0-CL dispersions, because water molecules also compete for hydrogen bonding to the PO_2^- group. Consequently, the frequency values of the phosphate vibrations are similar in both lipids. However, differences in the phosphate vibrations of 16:0-dCL and 18:0-CL as dry sodium salts can be explained by assuming the existence of hydrogen bonding between the free glycerol C-OH group in 18:0-CL and the PO_2^- groups (see Scheme II). With the ammonium salts the NH_4^+ ions can also act as proton donors to the phosphate groups and therefore no dfferences were detected between dry bilayer films or CCl_4 solutions of the ammonium salts of 16:0-dCL and 18:0-CL. In summary, our infrared data lead us to the conclusion that as in the diphytanyl ether analogues of phosphatidylglycerophosphate, the central C-OH group of 18:0-CL provides a structural basis for proton conduction, except that the pathway of proton conduction via hydrogen bonded species seems to involve not only the phosphate groups but also the ester carbonyl groups. Natural beef heart CL, on the other hand, has to be embedded in an ordered lipid bilayer matrix prior to being able to function in the same way as does 18:0-CL. It will be recalled that in

the inner mitochondrial membrane, phosphatidylcholine and phosphatidylethanolamine provide such an ordered lipid bilayer matrix in which the cardiolipin is embedded [1].

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