Conformational Study of Phospholipids in Crystalline State and Hydrated Bilayers by ^{13}C and ^{31}P CP-MAS NMR

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Abstract: Crystalline forms of the racemic and enantiomerically pure saturated zwitterionic phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyl-*N*-methylethanolamine (PEM_1), and phosphatidyl-*N*,*N*-dimethylethanolamine (PEM_2) have been investigated using ^{13}C and ^{31}P cross-polarization magic angle spinning (CP-MAS) NMR. The observed spectra indicate two types of packing behavior depending on the optical purity of phospholipids and the structure of a head group. The crystalline lattices of the enantiomerically pure PC, PE, PEM_1, and PEM_2 contained two conformations, whereas the corresponding racemic phospholipids contained only a single conformational species. On the basis of the known crystal structures of phospholipids, these results can be explained in terms of different ways the various zwitterionic phospholipids achieve packing into lattices with a minimal residual dipole moment. In enantiomerically pure PC and PE, the presence of two conformations, combining one configurational isomer of the diacyl glycerol moiety with two conformational mirror images of the head group, is necessary to eliminate the residual in-plane dipole moment of head groups and still achieve tight packing of the chiral diacylglycerol residue. In racemates, the in-plane dipole moment is eliminated by packing a single conformation into a true centrosymmetric lattice. The hydration of the crystalline phospholipid samples produced significant spectral alterations such as changes in the ^{13}C chemical shifts of the diacylglycerol C2 carbon and carbonyl groups, and ^{31}P NMR signals, suggesting conformational changes. The hydrated bilayers of both racemic and enantiomerically pure dipalmitoyl PC (DPPC) gave rise to identical ^{13}C NMR spectra regardless the phase state, except for the most rigid L_c subgel phase. The invariance of chemical shifts in these gel phases indicated that no major conformational changes occurred during phase transitions of hydrated bilayers of PC. The NMR data presented in this work provide evidence that conformations of phospholipids in the crystalline state are strongly affected by packing forces and the tendency of the head groups to pack in true- or quasi-centrosymmetric lattices. On the other hand, conformations in hydrated bilayers are largely governed by the intrinsic conformational properties of monomeric molecules. The data presented address the problem of the relevance of the X-ray structures of crystalline phospholipids for evaluation of the conformations in the less ordered hydrated bilayers.

Introduction

Conformation, dynamics, and packing of individual phospholipid molecules are important in determining physical properties of phospholipid bilayers,[1−3] as well as various biological functions of phospholipids in biomembranes.[4−6] Much effort has been devoted to studying these problems using X-ray diffraction of single crystals,[7−9] X-ray diffraction of hydrated bilayers,[10,11] high-resolution NMR in solution,[12−16] high-resolution NMR of oriented hydrated bilayers,[17−21] and wide-line NMR of unoriented hydrated bilayers.[22−24] Recently, several studies have been reported employing a cross-polarization magic angle spinning (CP-MAS) ^{13}C NMR technique for elucidation of properties of polar[12,25−36] and nonpolar[37] lipids. The advan-

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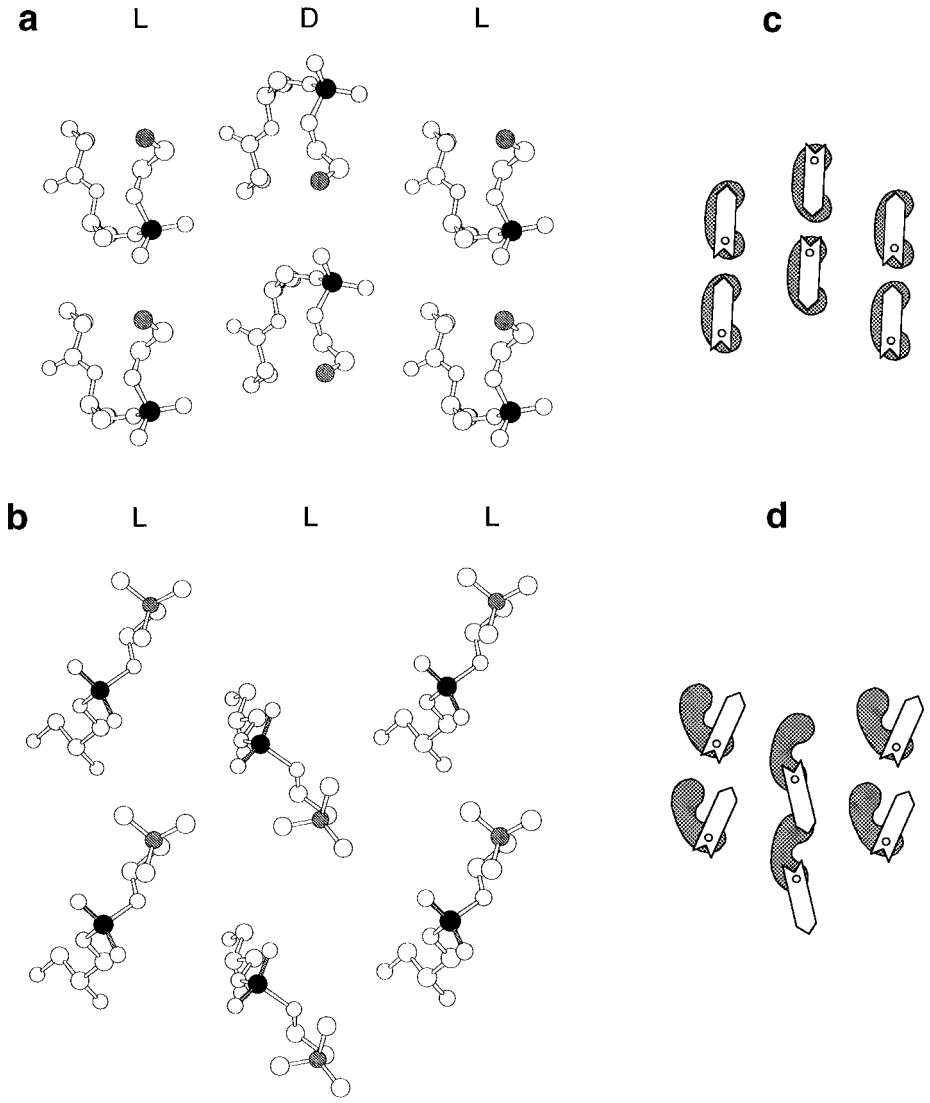

Figure 1. Representation of the head group packing in DL-DPPE (a) and L-α-DMPC (b) in the bilayer plane. The phosphorus atom is shown as black, the nitrogen atom as a shaded, and all other atoms as blank spheres. The packing pattern of these phospholipids is simplified as in c and d, respectively, where the blank arrows represent the head group dipoles and the curved dumbbell shapes represent the cross section through the chiral diacyl glycerol moiety. Note that to accomplish a proper DAG stacking and an antiparallel head group arrangement in L-α-DMPC, each second row of molecules features a rotated head group with regard to DAG.

tage of this technique is that it can provide structural information for molecular assemblies which due to their slow reorientation cannot be studied by solution NMR techniques, but which due to their packing disorder are not amenable to high-resolution X-ray crystallographic studies. This report describes application of ^{13}C and ^{31}P CP-MAS NMR to study conformational differences of racemic and enantiomerically pure zwitterionic phospholipids of various types of packing order ranging from solid polycrystalline samples to hydrated bilayers in the gel and liquid−crystalline state.

The structure of phospholipid assemblies depends to a large extent on the nature of the phospholipid head group, its relative size with respect to the cross-sectional area of hydrocarbon chains, and its propensity to form lateral interactions.[7,38,39] The feature that affects the structure of the bilayer to a significant extent is the packing of the polar head groups (Figure 1).[7] Two major types of head group packing pattern were found for crystalline zwitterionic phospholipids by using X-ray diffraction:[7] (i) head group dipoles of phosphatidylethanolamines (PE) and phosphatidylcholines (PC) are oriented parallel to the bilayer plane, causing little interaction between the head groups of adjacent bilayers;[7−9] (ii) head groups of partially N-methylated PE are oriented perpendicularly to the bilayer plane and are interdigitated with those of another bilayer.[7,40] The crystal structure of the racemic dilauroylphosphatidylethanolamine[7,9,38] (DL-DLPE) contains a single conformational isomer, with molecules of opposite configurations arranged in adjacent

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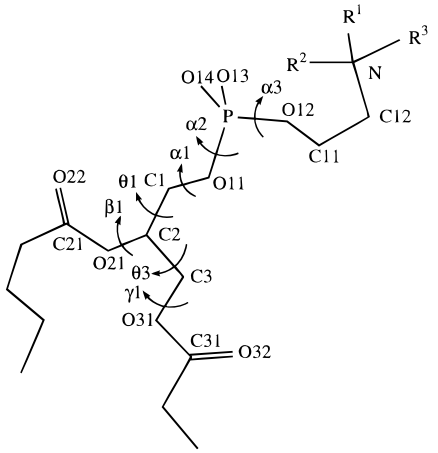

Figure 2. Structure of the polar head group of zwitterionic phospholipids with atom numbering and torsion angle nomenclature used in this work.[7,61] $R^1 = R^2 = R^3 =$ H, PE; $R^1 = R^2 =$ H, $R^3 =$ Me, PEM_1; $R^1 = R^2 =$ Me, $R^3 =$ H, PEM_2; $R^1 = R^2 = R^3 =$ Me, PC. The nomenclature of rotamers conforms that of Klyne and Prelog[60] and the designation of torsional angles is according to Sundaralingam.[61]

antiparallel ribbons within the same monolayer (Figure 1a,c) and with each ribbon containing exclusively a single enantiomer. The large resultant dipole moment of the PE head groups in one ribbon is neutralized by an equal antiparallel dipole moment of another adjacent ribbon. An analogous head group packing behavior is observed in phosphatidylcholines. In the only molecular structure of the enantiomerically pure lecithin reported to date,[8] dimirystoylphosphatidylcholine (L-α-DMPC), the phosphocholine (PC) head groups form a herringbone pattern, whereby the head groups are arranged into the antiparallel ribbons (Figure 1b,d). The adjacent antiparallel ribbons contain distinct conformers which differ largely by the rotation of the polar head group about C1−C2 bond[7,8,41] (see Figure 2 for atom numbering). The polar fragments of the two diastereomeric conformers, extending from the N-terminal methyl groups of the choline residue to the C2 carbon atom of glycerol, are quasi-mirror images of one another.

In sharp contrast to PE and PC, crystalline racemic dilauroylphosphatidyl-*N*-methylethanolamine (DL-DLPEM1) and dilauroylphosphatidyl-*N*,*N*-dimethylethanolamine (DL-DLPEM2) pack in bilayers with layer-perpendicular, interdigitating head group dipoles, whereby alternating monolayers are composed of opposite enantiomers.[7,40] The head groups are organized within the monolayers into parallel ribbons, and a large layer-perpendicular dipole moment of one monolayer is counterbalanced by the dipole moment of the alternating monolayer oriented in the opposite direction. Unlike the case of DL-DLPE or L-α-DMPC, in the lattices of DL-$DLPEM_1$ and DL-$DLPEM_2$ there is a strong interlayer coupling due to the penetration of head groups of one bilayer into the head group region of the adjacent bilayer.

Due to the high intrinsic conformational flexibility of phospholipid molecules, it is unclear how the conformations present in the crystalline multibilayer state relate to those featured in hydrated bilayers. Because phospholipids are difficult to crystallize, generally only one type of the X-ray structure is available for a given type of a head group[7] at a given hydration level, and with a given stereoisomeric form of diacylglycerol. For example, for phosphatidylethanolamines, the crystal structure of the racemic, but not the enantiomerically pure, form is known, whereas the opposite is true for phosphatidylcholines. Although the earlier NMR studies indicated

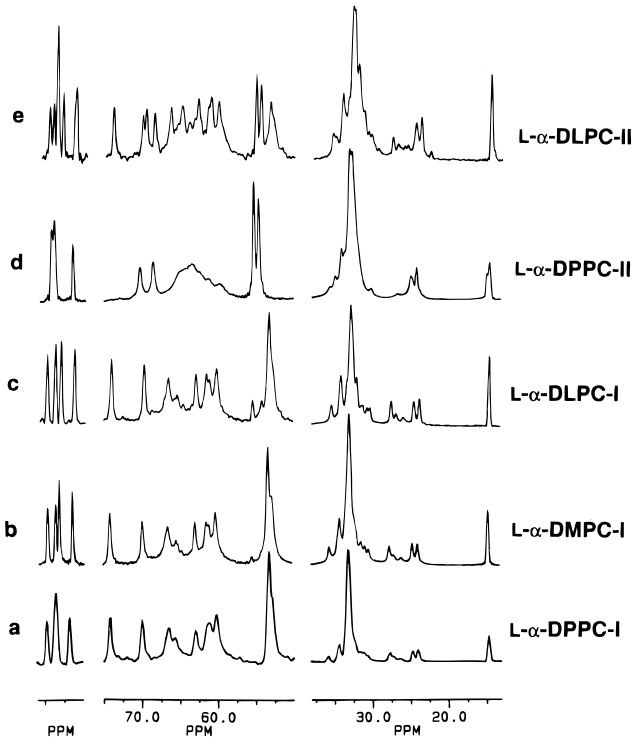

Figure 3. 75.44 MHz ^{13}C CP-MAS NMR spectra of crystalline L-α-DPPC-I (a), L-α-DMPC-I (b), L-α-DLPC-I (c), L-α-DPPC-II (d), and L-α-DLPC-II (e). All spectra were obtained and processed as described in the Experimental Section. The intensities of the spectra in the left panel were scaled down 10-fold as compared to the middle and the right panel.

that conformations of phosphatidylcholine,[1,13,41] sphingomyelin,[14] and cerebroside[12] do not change significantly upon aggregation from a monomeric into a micellar state, and that there is no detectable difference in NMR spectra of racemic and enantiomerically pure phosphatidylcholines in the micellar state,[42] it is still possible that intermolecular interactions exert a significant effect on molecular conformations in the more tightly packed lipid bilayer gel phases and in crystalline state. Consistent with this suggestion, differential scanning calorimetry (DSC) studies indicated differences in thermotropic properties of the racemic and enantiomerically pure phosphatidylcholines[43] and sphingolipids.[44−46] It is also not known how the hydration of a phospholipid affects the packing and conformations in crystalline and bilayer states. This study is dedicated to exploring the conformational behavior of racemic and enantiomerically pure zwitterionic phospholipids in crystalline state and hydrated bilayers.

Results

Crystalline Forms of Enantiomerically Pure Phosphatidylcholines. ^{13}C and ^{31}P CP-MAS NMR spectra of polycrystalline phosphatidylcholines are presented in Figures 3 and 4, respectively. Most of these spectra feature well-resolved resonances, and several ^{13}C signals can be tentatively assigned based on the previously reported spectra of phospholipids in solution and in liquid crystalline bilayers (Table 1, see also refs 18, 20, and 31). The most interesting result manifested in these spectra is that samples produced by different crystallization

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Table 1. ^{13}C and ^{31}P NMR Chemical Shifts of the Crystalline Phospholipid Samples

	$\delta_{^{13}C}$				
sample	C31,C21	C2	NMe	C12	$\delta_{^{31}P}$
L-α-DPPC-I	174.7, 173.5, 171.7	74.2, 70.0	53.4	na[c]	1.1, 0.5
L-α-DPPC-II	174.4, 174.0, 171.6	70.2, 68.5	55.2, 54.5	na	−1.5
DL-DPPC-I	174.7, 172.0	74.3	53.0	60.1	1.55
L-α-DPPE[a]	173.0, 171.9, 170.1, 168.5	70.2, 69.2		39.9	−0.7, −1.3
DL-DPPE[a]	174.3, 172.0	70.8		41.2	−0.5
L-α-DPPEM1[a]	173.2, 172.2	72.3, 69.2	35.3[b]	50.4	0.6
L-α-DPPEM2[a]	172.7, 171.6	71.9, 69.2	41.4, 39.5	56.6	−2.9
DL-DPPEM2[a]	172.9	72.4	42.0, 40.1	56.0	−0.9
L-α-DPPC-hydr	173.1	70.9	54.3	66.2	
DL-DPPC-hydr	173.0	70.9	54.3	66.2	

[a] Type I samples. [b] Tentative assignment. [c] na, not assigned.

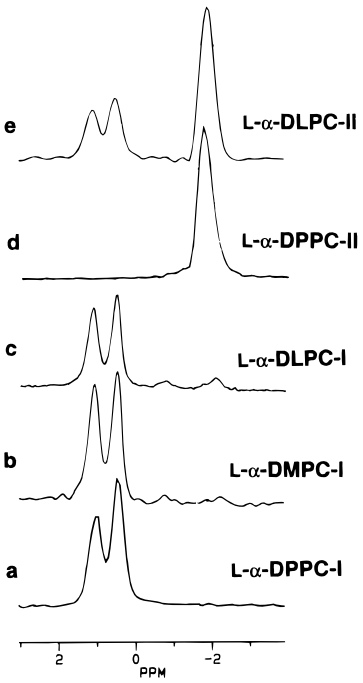

Figure 4. 121.49 MHz ^{31}P CP-MAS NMR spectra of crystalline L-α-DPPC-I (a), L-α-DMPC-I (b), L-α-DLPC-I (c), L-α-DPPC-II (d), and L-α-DLPC-II (e). All spectra were obtained and processed as described in the Experimental Section. Each ^{31}P NMR spectrum was obtained directly after acquiring the ^{13}C spectrum shown in Figure 2.

protocols gave NMR spectra with vastly different chemical shifts and different signal multiplicity.

The spectra of the samples of dipalmitoylphosphatidylcholine (L-α-DPPC), L-α-DMPC, and dilauroylphosphatidylcholine (L-α-DLPC) obtained by ether diffusion into a phospholipid solution in anhydrous ethanol (type I samples, Figure 3a−c) showed duplication of most of the discernible resonances: C═O (175.0, 173.9, 173.2, and 171.4 ppm), C2 (74.2, 69.7 ppm), C1 (62.8, 61.45 ppm), C11 (61.0, 60.1 ppm) (shifts for L-α-DMPC-I). The sample of L-α-DPPC obtained by the low-temperature crystallization method (type II sample, Figure 3d) also displayed two sets of resonances, but different from those of type I samples, as evidenced by the duplication of ^{13}C signals from the carbonyl (174.4, 174.0, 171.6 ppm), C2 (70.2, 68.5 ppm), and NMe (55.2, 54.5 ppm) groups (see also Table 1). Other carbon atoms of the polar head group gave rise to a single broad and unresolved signal at 58−68 ppm. On the other hand, the spectrum of L-α-DLPC-II (Figure 3e) can be closely described as a superposition of the above-mentioned two types of spectra (such as 3a and 3d), most likely due to a coexistence of two types of crystals, each containing two noncongruent molecules. The presence of a total of four conformers in this sample is best evidenced by a quadruplication of the C2 signal (two pairs of signals at 73.9 and 69.7 ppm, and at 70.1 and 68.6 ppm),

Table 2. Oxygen Content and Hydration Level of the Samples of Crystalline Phospholipids[a]

phospholipid	crystallization method	molecular formula	calcd	found	hydrate
L-α-DPPC	I	$C_{40}H_{84}NO_{10}P$	20.78	21.22	dihydrate
L-α-DPPC	II	$C_{40}H_{86}NO_{11}P$	22.33	22.01	trihydrate
DL-DPPC	I	$C_{40}H_{84}NO_{10}P$	20.78	20.21	dihydrate
L-α-DPPE	I	$C_{37}H_{78}NO_{10}P$	22.20	21.98	dihydrate
DL-DPPE	I	$C_{37}H_{76}NO_9P$	20.28	20.64	monohydrate
L-α-DPPEM$_2$	I	$C_{39}H_{82}NO_{11}P$	22.79	22.57	trihydrate
DL-DPPEM$_2$	I	$C_{39}H_{82}NO_{11}P$	22.79	23.03	trihydrate

[a] Method accuracy is ±0.3%. Deviation of the oxygen content from the calculated value reflects probably some heterogeneity of the sample and/or hydration of the sample during handling.

and multiplication of signals arising from the carbonyl carbon atoms, with five resonances observed of the approximate relative intensities 1:1:3:1:2. The determination of the oxygen content in the samples of L-α-DPPC indicated that the type I sample was a dihydrate and the type II sample was a trihydrate (see Experimental Section, Table 2).

The above conformer multiplicity is also apparent from the ^{31}P NMR spectra (Figure 4). The ^{31}P NMR spectra of L-α-DPPC-I, L-α-DMPC-I, and L-α-DLPC-I display two equal-intensity lines at 1.1 and 0.5 ppm (Figure 4a,b,c, respectively), and that of L-α-DPPC-II consists of only one signal at −1.7 ppm (Figure 4d), which could not be further resolved. On the other hand, the ^{31}P NMR spectrum of L-α-DLPC-II (Figure 4e) is a close superposition of the spectra 4c and 4d.

Crystalline Form of Racemic Phosphatidylcholines. Interestingly, only one type of the polycrystalline solid could be obtained from DL-DPPC and DL-DMPC, regardless of the crystallization method used (I or II), and both samples contained only one spectroscopically discernible molecule, as evidenced by the observation of only a single line from each ^{13}C site (Figure 5a,b), and a single ^{31}P NMR signal (Figure 5c,d). The ^{13}C spectra are highly analogous to those reported earlier[29] for anhydrous DL-DPPC, however, the oxygen content determination indicated that our sample of DL-DPPC was a dihydrate (Experimental Section, Table 2). The ^{13}C signals of the C1, C2, C11, *N*-methyl group, one of the carbonyl groups, and hydrocarbon atoms closely matched the corresponding signals of one of the components in the spectra of the enantiomerically pure type I samples (Table 1).

Crystalline Forms of Phosphatidylethanolamine and Its Partially Methylated Derivatives. In agreement with the above observations, each of the samples of the enantiomerically pure dipalmitoylphosphatidylethanolamine (L-α-DPPE), dipalmitoylphosphatidyl-*N*-methylethanolamine (L-α-DPPEM), and dipalmitoylphosphatidyl-*N*,*N*-dimethylethanolamine (L-α-DPPEM$_2$) contained two conformational isomers. This is apparent from the doubling of resonances of the carbonyl groups, glycerol C2, and ethanolamine carbon atoms in the DPPE

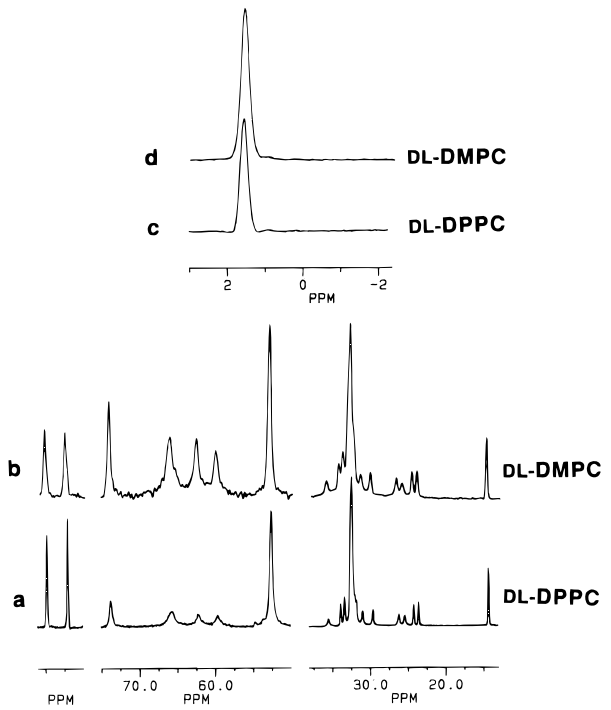

Figure 5. 75.44 MHz ^{13}C (a, b) and 121.49 MHz ^{31}P (c, d) CP-MAS NMR spectra of DL-DPPC (a, c) and DL-DMPC (b, d). Spectral conditions and processing were the same as those in Figures 2 and 3. All samples were of the type I.

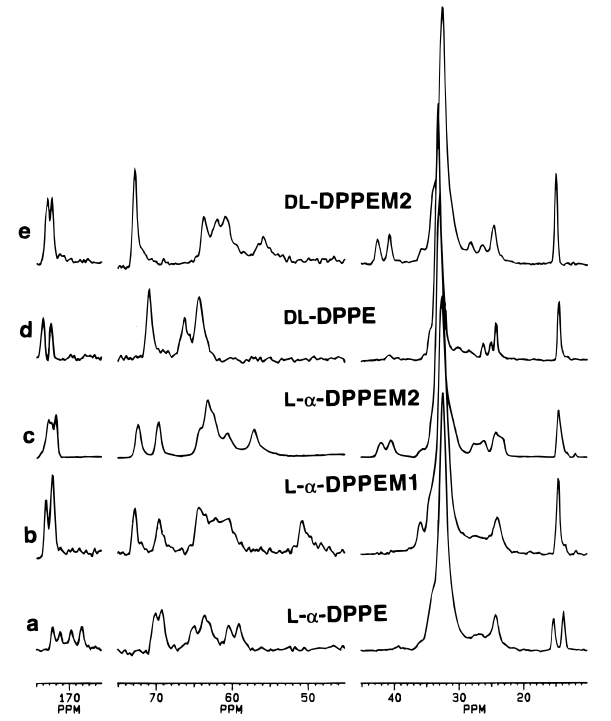

Figure 6. 100.61 MHz ^{13}C CP-MAS spectra of L-α-DPPE (a), L-α-$DPPEM_1$ (b), L-α-$DPPEM_2$ (c), DL-DPPE (d), and DL-$DPPEM_2$ (e). All spectral conditions and processing parameters were as described in the Experimental Section. The intensities of the spectra in the left panel were scaled down 10-fold as compared to the middle and the right panel.

sample (Figure 6a), and of the C2 glycerol resonances of $DPPEM_1$ (Figure 6b) and $DPPEM_2$ (Figure 6c). In contrast to phosphatidylcholines, however, these conformational differences gave rise only to splitting of the ^{31}P NMR signal of L-α-DPPE (Figure 7a), but not of its partially methylated derivatives (Figure 7c,d). On the other hand, both ^{13}C and ^{31}P spectra indicated the presence of only a single conformational species in the

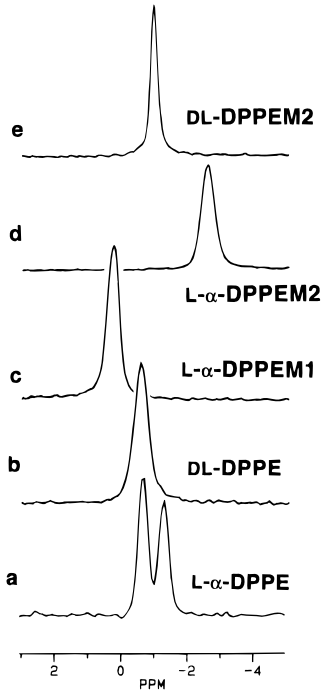

Figure 7. 161.98 MHz ^{31}P CP-MAS NMR spectra of L-α-DPPE (a), DL-DPPE (b), L-α-$DPPEM_1$ (c), L-α-$DPPEM_2$ (d), and DL-$DPPEM_2$ (e). The samples were identical to the corresponding ones shown in Figure 6.

racemic samples of DPPE (Figures 6d and 7b, respectively), and $DPPEM_2$ (Figures 6e and 7e, respectively). The chemical shift of the ^{31}P NMR signal of DL-DPPE (−0.5 ppm) closely matched the chemical shift of one of the signals in L-α-DPPE (−0.7, −1.3 ppm). The chemical shifts of other phoshatidylethanolamines, L-α-$DPPEM_1$ (0.6 ppm), DL-$DPPEM_2$ (−0.9 ppm), and L-α-$DPPEM_2$ (−2.9 ppm) displayed much greater variability, the origin of which is unclear at the moment.

Hydrated Phosphatidylcholines. ^{13}C CP-MAS NMR spectra of the hydrated bilayers of L-α-DPPC and DL-DPPC (DPPC−water, 1:1, w/w) in their gel phases, the $P_{\beta'}$ phase at 310 K, the $L_{\beta'}$ gel phase at 297 K, and the L_c subgel phase at 278 K are shown in Figure 8. The spectra of L-α- and DL-DPPC in the $L_{\beta'}$ and $P_{\beta'}$ are identical, whereas the low-temperature phases of L-α-DPPC and DL-DPPC do display significant spectral differences such as splitting of the signal of the carbonyl groups in the sample of L-α-DPPC into a doublet and some less well defined changes of signals of the polar head group. To obtain samples represented by the spectra at 278 K, both samples of DPPC were stored at 4 °C for 3 weeks, and caution was used not to warm the samples during their transfer into the MAS probe. The observed spectral differences reflect the fact that under such conditions the bilayers of L-α-DPPC, but not DL-DPPC, are converted into the rigid subgel (L_c) phase.[43]

Discussion

Conformations of Phosphatidylcholines and Phosphatidylethanolamines in the Crystalline Form. (A) Glycerol Backbone. The two procedures employed in this study for crystallization of enantiomerically pure PC produced polycrystalline samples, one with two (type I) and another one with three (type II) water molecules per phospholipid. It has to be stressed that the type I samples could be reproducibly obtained from the different chain length phosphatidylcholines using only the anhydrous solvents for crystallizations. On the basis of the same hydration level and the presence of two noncongruent molecules, it is believed that the type I samples closely correspond to the sample of a single crystal studied by Pearson and Pascher.[8] It

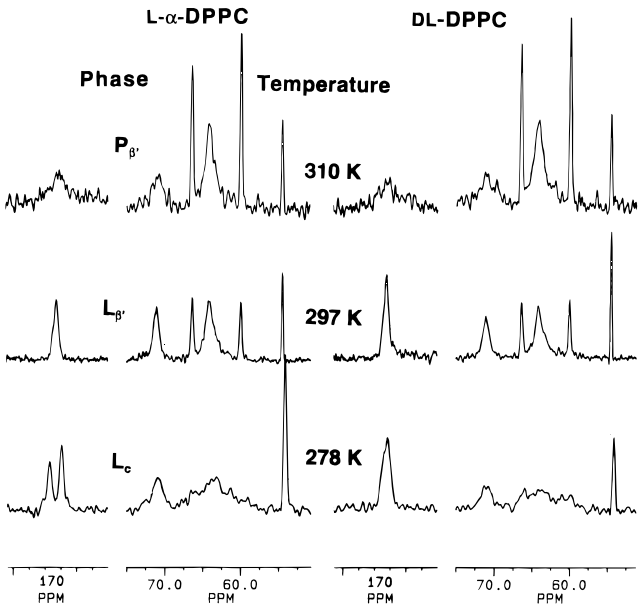

Figure 8. 75.44 MHz ^{13}C CP-MAS NMR spectra of the hydrated bilayers of L-α-DPPC (left panel) and DL-DPPC (right panel) at the indicated temperatures. All conditions were the same as those of Figure 3.

should be emphasized, however, that the polycrystalline samples used in this work were not of a crystallographic quality and that no X-ray diffraction of these crystalline solids was performed. It is further assumed that the two sets of ^{13}C and ^{31}P signals observed in the type I crystals correspond to the molecules A and B (Figure 9) found in the single crystal. These conformers have the same $\theta_3 = ap$ conformations (see Figure 2 for definition of torsional angles), but differ in other torsional angles around C2 such as θ_1 (58° and 168°, respectively), β_1 (82° and 120°), and γ_1 (−177° and 102°). The conformers A and B are expected to give rise to two separate sets of ^{13}C and ^{31}P signals in NMR spectra due to conformationally induced changes in chemical shifts.[47,48] Below, we examine the effect of stereochemical differences of molecules A and B[8] on the expected ^{13}C chemical shift of the C2 signal in order to assign the observed resonances to those specific conformers. (i) A survey of chemical shifts of hydroxylated carbon atoms in cyclic systems in solution spectra suggests that a change in the θ_1 angle from $+sc$ (molecule A) to ap (molecule B) should not significantly alter the chemical shift of the C2 signal. For example, the glycerol backbone of the molecules A and B can be quite well mimicked by the C3−C1 fragment of α- and β-mannose (Figure 10), respectively, with the δ_{C2} 71.7 and 72.3 ppm, respectively. Other analogous examples include C5 of glucose and galactose (δ_{C5} 76.8 and 76 ppm, respectively), and C1 of α-mannose and β-mannose (δ_{C1} 95.0 and 94.6 ppm, respectively).[49] (ii) The rotation of the head group from $\theta_1 = +sc$ to $\theta_1 = ap$ (from A to B) would bring the O11 and O22 oxygen atoms into a close proximity (from 3.8 to 2.2 Å); therefore, a change of θ_1 causes a simultaneous change of $\beta_1 = +sc$ to $\beta_1 = +ac$, the latter which is representative of most phospholipid structures.[7] This conformational change brings H2 and O22 into an eclipse (Figure 9), which should affect the chemical shift of C2. No experimental data base is available, however, for closely related molecules to serve as a basis for prediction of the direction and the magnitude of such change. The change in β_1 also changes the steric relationships C21/C3 = ap and C21/C1 = sc into ac, however, the net effect of such changes on the chemical shift of C21 should be rather small due to the mutually compensating γ-effects of C3 and C1. (iii) The decrease in $\gamma_1 = ap$ angle in the molecule A to $\gamma_1 = ac$ in the rotamer B is expected to shift both C2 and C31 signals upfield by several ppm due to the γ-gauche effect. This is in agreement with our other observations that typically a low chemical shift of C2 is accompanied by a low chemical shift of carbonyl signals (and vice versa, e.g. compare DL-DPPC and DL-DPPE). On the basis of such a putative γ-gauche effect of the C31 carbon, it would appear that the upfield C2 signal (δ 70.0 ppm) belongs to the molecule B (with the $\gamma_1 = ac$) and that the downfield signal (δ 74.2 ppm) belongs to the molecule A (with the $\gamma_1 = ap$).

The type II sample of L-α-DPPC, which was found to be a trihydrate, displayed two noncongruent molecules, neither of which was identical to molecule A or B of the type I phosphatidylcholine samples. The C2 signals of the two conformers (70.2 and 68.4 ppm) are shifted upfield, and the two signals of NMe group are shifted downfield, as compared to the type I sample, and therefore they are more close to those of the fully hydrated DPPC (see Table 2). The ^{13}C spectrum of the L-α-DLPC obtained by low temperature crystallization was a combination of type I and type II spectra. A possible explanation of this result is that partial hydration of type I crystals took place during the crystal growth or isolation procedure. This suggests that the type II crystals can be derived from type I by limited hydration, and also emphasizes the effect of hydration on the head group conformation in bilayers.

(B) Phosphocholine Group. In the absence of rotation about the C12−N bond the methyl groups of the choline residue in PC should be inequivalent because they are in sc ($-sc$) or ap relation with respect to C12. Unexpectedly, the type I sample showed only a single slightly broadened NMe signal for all methyl groups in both global rotamers (Figure 3a−c), and the type II sample showed a single signal for all methyl groups in each global conformational species (Figure 3d). Observation of such a pattern suggests a limited rotational freedom of the trimethylammonium group about the C12−N bond, exchanging the positions of the methyl groups and resulting in a rotationally averaged signal. Conversely, observation of the two distinct methyl groups in $DPPEM_2$ (signals at 42.0 and 40.1 ppm in L-α-$DPPEM_2$, and 41.8 and 40.3 ppm in DL-$DPPEM_2$) suggests that no such rotation about the C12−N bond takes place. This result is consistent with the presence of the intermolecular hydrogen bond between the phosphoryl oxygen and the ammonium atom in the partially methylated PEs.[7,40] The degree of proton transfer, and the related magnitude of positive charge on the nitrogen atom, should vary depending on the pK_b of the amino group, and hence also on the number of methyl groups. The increasing positive charge on the nitrogen atom with an increasing number of methyl groups in the series of partially methylated PEs is indeed reflected in the downfield shift of the C12 and NMe signals. Thus, the chemical shift of the *N*-methyl group changes from 35.3 ppm (tentative assignment) in $DPPEM_1$ to ca. 40−42 ppm in $DPPEM_2$, and to 55 ppm in DPPC (full formal positive charge). This trend is paralleled by the shift of the position of the C12 signal from 40.2 ppm in DPPE to 50.4 ppm in $DPPEM_1$, to ca. 56 ppm in $DPPEM_2$ and to 66.2 ppm in DPPC (note that positions of the *N*-Me and C12 change by a similar increment with a given structure modification).

(C) Comparison of Racemic and Enantiomerically Pure Crystalline Phosphatidylcholines. The disparity in the X-ray diffraction patterns[50] and melting curves[29] of the presumably

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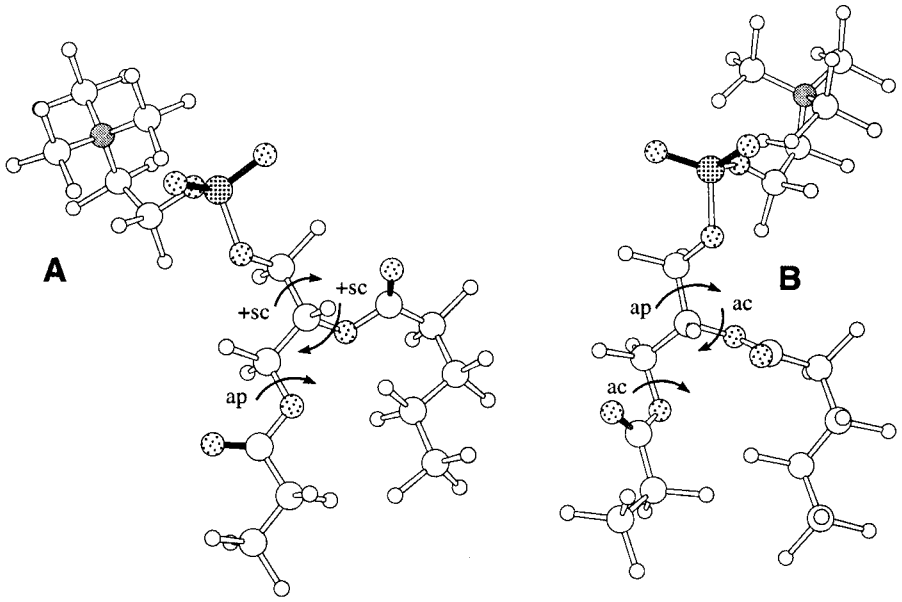

Figure 9. Representation of polar fragments of conformers A and B observed in the crystal structure of L-α-DMPC.[9]

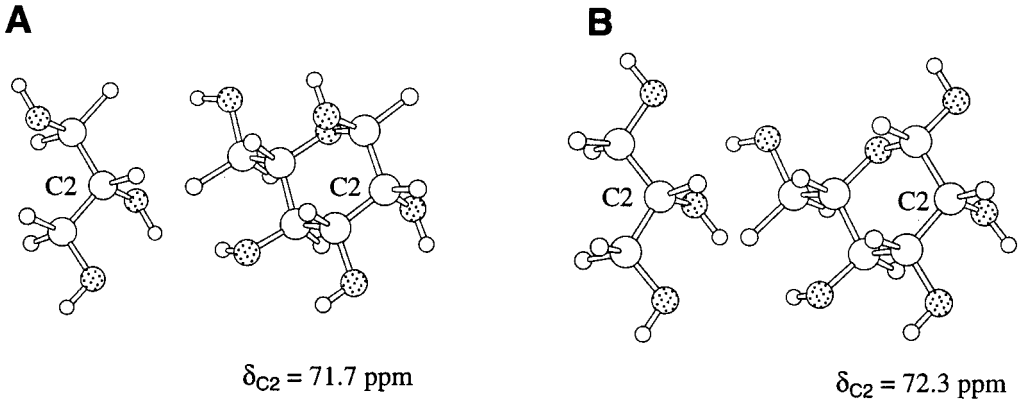

Figure 10. Comparison of the glycerol backbone conformation of molecules A and B of L-α-DMPC with the C1−C3 fragments of α- and β-mannose.

anhydrous crystalline L-α-DPPC and DL-DPPC indicated different packing and conformational properties of the two phospholipids, although the molecular structure for the racemic phosphatidylcholine has not been solved to date. The differences between enantiomerically pure and racemic samples are clearly seen in the ^{13}C and ^{31}P NMR spectra. The positions of the C2 signal (74.3 ppm) and of the ^{31}P signal (1.55 ppm) in DL-DPPC, matching the corresponding signals in L-α-DMPC-I (74.0 and 1.1 ppm, respectively), suggest that the single conformational species found in the racemic samples is similar to one of the molecules in enantiomerically pure samples and is perhaps analogous to the molecule A of L-α-DMPC.[8] The observation of a single conformer in racemic phosphatidylcholines is interesting in view of the notion[7] that a large size of the PC head group makes it difficult to be accommodated within a single bilayer plane. To achieve close packing in L-α-DMPC, one of the head groups is elevated by 2.5 Å with respect to another.[7] Most likely, a larger head group cross-sectional area needed to pack head groups within a single plane in the racemic DPPC is generated by a larger tilt angle.[50]

(D) DPPE and Partially Methylated DPPE. Due to low solubility of DPPE, $DPPEM_1$, and $DPPEM_2$ in ethanol, crystallization of these samples was performed by ether diffusion into the mixture of ethanol−chloroform−water (type I samples). The obtained specimen of the DL-DPPE sample was a monohydrate, that of L-α-DPPE was a dihydrate, and both DL- and L-α-$DPPEM_2$ samples were trihydrates. Similar to the behavior of DL-phosphatidylcholines, and consistent with the X-ray study,[9] DL-DPPE displayed a single conformational isomer in the crystalline lattice; however, since the crystallization was performed under different conditions than those used in the crystallographic work, it is not known if this isomer is the same as that of the published X-ray data.[9] The ^{13}C spectrum of the enantiomerically pure L-α-DPPE showed two conformational isomers, which also were differentiated by ^{31}P NMR. One of the two conformational isomers in this sample showed an unusually high field shift of one of the carbonyl groups at 168.5 ppm, and both displayed rather high field shifts of the C2 signals (70.1 and 69.1 ppm). The behavior of the partially methylated phosphatidylethanolamines, $DPPEM_1$ and $DPPEM_2$, is clearly different from those of either DPPC or DPPE. The enantiomerically pure $DPPEM_1$ and $DPPEM_2$ contain two conformational species differing in the position of the C2, and the racemic $DPPEM_2$ displays a single conformational isomer, however, *no major differences in packing of the carbonyl groups are detected between racemic and enantiomeric samples*. This observation suggests that for these phospholipids conformational changes are limited to the polar head group.

Effect of Hydration On the Head Group Conformation. Earlier studies indicated that hydration of phosphatidylcholine produces large spectral changes,[29,33,51,52] most likely due to an increase in conformational exchange rates, which could result from the hydration-induced uncoupling of head group interactions of apposing monolayers. The effect of hydration on head group conformations is very clear from the ^{13}C spectral data

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presented in this work. First, most type I samples of PCs (dihydrates) show a small signal from NMe_3 group at 55 ppm (in addition to the main signal at 53.2 ppm, Figure 3b,c), which closely coincides with one of the two signals of the NMe_3 group in DPPC-II (Figure 3d) and with the analogous signal in the fully hydrated bilayers (all spectra in Figure 8). A small signal is also present at −1.9 ppm in ^{31}P NMR spectra of DMPC-I and DLPC-I samples (Figure 4b,c), coinciding with the signal of DPPC-II (Figure 4d). This indicates that these type I samples are contaminated with the type II (trihydrate) conformational species, which could be due to additional hydration either during sample handling or data accumulation. The DLPC-II sample (Figure 3e) is thus most likely a 1:1 mixture of the dihydrate and the trihydrate. A similar (but less clear-cut) observation was reported by Meulendijks and co-workers,[29] who showed the downfield shift of NMe_3 group in DL-DPPC upon hydration. The upfield shift of the ^{31}P NMR signal upon increase of hydration (Figure 4a,d) is likely due to the hydrogen bonding to the phosphoryl oxygen.

In contrast to the crystalline samples, the ^{13}C NMR spectra of the fully hydrated racemic and enantiomeric DPPC (50% w/w water content) were identical within the limit of detection of ^{13}C NMR spectroscopy in the $L_{\beta'}$ and $P_{\beta'}$ gel phases and were very different from the corresponding crystalline samples. The biggest spectral change upon the full hydration of the crystalline DPPC is the upfield shift of the C2 resonance to ca. 71 ppm, the downfield shift of the NMe_3 signal, and the collapse of the carbonyl signals into a single broad line. The chemical shift changes of the C2 upon hydration observed in this work are consistent with the dipolar coupling data,[17,20,21] suggesting that the conformer B is a dominant species under the hydrated bilayer conditions.[17] In view of the large range of conformationally induced ^{13}C chemical shift changes possible,[47,48] the invariability of the ^{13}C chemical shifts observed in several phases of the hydrated bilayers of DPPC (see also ref. 31) indicates that *conformational equilibria of the glycerol backbone and the phosphocholine head group are not altered significantly during these phase transitions*. Rather, the observed changes in signal width are caused by the slower conformational dynamics in the lower temperature gel phases,[2,53−55] resulting in an incomplete motional averaging of several conformers. The only observable difference in the behavior of DL- and L-α-DPPC occurs during the transition from the $L_{\beta'}$ gel to the L_c subgel phase of L-α-DPPC. In the latter phase at 278 K, two separate resonances of the carbonyl groups are observed for L-α-DPPC vs one broad resonance for DL-DPPC at the same temperature. This difference can be explained by the fact that DL-DPPC does not rearrange into the subgel phase,[43,56−58] therefore the two specimens are in fact in different phases.

Centrosymmetry of Phospholipid Head Group Packing. (A) Crystalline Solids. In most of the reported X-ray crystal structures of zwitterionic phospholipids the head groups pack in true- or quasi-centrosymmetric arrangements,[7] where the axial dipole moment of the layer-perpendicular head group one monolayer is canceled out by the antiparallel dipole orientation of the head group in the adjacent monolayer, and where the in-plane (lateral) dipole moment of the layer-parallel head groups (such as of the phosphocholine or phosphoethanolamine) is zeroed by ordering the head groups into antiparallel ribbons of equal, but opposite, dipole moments (Figure 1). Such an arrangement is suitable for racemic molecules, where the antiparallel ribbons alternate in the configuration at the glycerol C2 center, which is best illustrated by the molecular structure of DL-DLPE.[9] In this case the favorable centrosymmetric packing can be achieved using mirror images of a single molecular conformation (Figure 1a,c). As illustrated by the example of DMPC,[8] this centrosymmetric arrangement, however, cannot be adopted by enantiomerically pure phospholipids. In this case the antiparallel arrangement of the head group dipoles generates two noncongruent molecules A and B, differing by the head group rotation versus the diacylglycerol residue. Our results augment the existing crystal X-ray data by showing that the same type of packing obtained for DMPC exists also for other phosphatidylcholines. On the basis of the results obtained here for phospholipids with different chain length, enantiomeric purity, and extent of methylation, it is possible to draw a general conclusion that the tendency to adopt *centrosymmetrical or quasi-centrosymmetrical packing in the crystalline state significantly affects molecular conformations of zwitterionic phospholipid molecules and, in the case of enantiomerically pure phospholipids, results in the presence of two conformations differing in the angle of the head group rotation with regard to the glycerol backbone*. At least one of these conformations therefore deviate significantly from the minimum energy conformations attainable in the solution, or in the liquid−crystalline state. From the comparison of the hydrated and polycrystalline samples it is apparent that *the effect of head group packing on phospholipid conformation is observed in the physical states featuring static molecules with little or no rotation about the long molecular axis*.

(B) Hydrated Phospholipid Bilayers. The quasi-centrosymmetric nature of the lateral head group packing should still remain in sphingolipid bilayers, where the axial rotation of whole molecules is restricted by intramolecular hydrogen bonds between the polar ceramide functions, especially in the gel phases. Consequently, sphingolipid phase transitions depend very strongly on the enantiomeric purity.[45,46] The low-temperature stable phase of the hydrated D-*erythro*-stearoylsphingomyelin, which shows a complete immobilization of the phosphocholine group,[28] displays the presence of two conformers differing in the ^{13}C chemical shifts of the carbonyl, C3, and C2 signals,[28] analogously to the polycrystalline phosphatidylcholines described here. On the basis of the results described here, we believe that the two molecules observed in the low-temperature, stable phase of sphingomyelin represent two conformers differing in their head group rotation, induced by quasi-centrosymmetric packing of head group dipoles. In contrast to sphingolipids, the glycerol-based phospholipids lack chemical functions capable of restricting their axial rotation in the hydrated bilayer structure. The lack of the difference between time-averaged conformations of racemic and enantiomeric phosphatidylcholines in the liquid−crystalline and the metastable gel phases can be explaind as a consequence of a disappearance of any in-plane orientational coherence, due to the fast axial rotation of whole phospholipid molecules or their head groups. This lack of coherence nullifies the resultant in-plane dipole moment of phospholipid head groups and removes a necessity for quasi-centrosymmetric packing. The conformation of the rapidly axially rotating phospholipid molecule (such as in liquid−crystalline phases) is therefore mainly decided by inherent conformational properties of the monomer, rather than by molecular packing forces. For the reasons described above we believe that the conformation of most phospholipid molecules

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in the hydrated liquid−crystalline bilayers is more closely represented by the conformation in the nonaggregated solution state[1,12,15] than by the conformation in crystalline state. The tendency of the bilayer to adopt quasi-centrosymmetric packing of head groups should increase, however, as the rate of axial and head group rotation decreases. In fact, the two-dimensional nature of L_c phase of L-α-DPPC[59] (laterally ordered), could be due to head group packing in a quasi-centrosymmetric lattice.

Conclusion

The ^{13}C and ^{31}P CP-MAS NMR spectra of crystalline phosphatidylcholines and phosphatidylethanolamines show a remarkable uniformity in that all racemic samples display the presence of a single conformer, and the enantiomerically pure ones, two distinct conformers. This behavior results from the tendency of zwitterionic head groups to pack in quasi-centrosymmetrical pattern, which necessitates the presence of two conformers in the enantiomerically pure glycero- and sphingophospholipids. These conformers differ primarily in the azimuthal orientation of the head group with respect to the diacylglycerol (ceramide) backbone in order to eliminate the resultant lateral dipole moment of the bilayer. The effect of dipole orientation is small in the liquid crystalline phases and metastable gel phases where, most likely, there is little angular in-plane coherence between head group dipoles. The results also indicate that valuable information on phospholipid conformations can be extracted from CP-MAS NMR spectra using samples of low crystallographic quality, linking the spectral information with the available high-resolution X-ray crystal structure data, and comparison of the spectroscopic data for crystalline samples with those of hydrated bilayers.

Experimental Section

Phospholipid Samples. All phospholipid samples were of the highest purity available from commercial sources and were not further purified. Solutions of L-α-DLPC, L-α-DMPC, L-α-DPPC, DL-DMPC, and DL-DPPC (100−200 mg in 2−3 mL of anhydrous ethanol) in open-end test tubes were placed in a closed dessicator containing anhydrous ether (500 mL) and were maintained in such ether-saturated atmosphere at room temperature for several days. The diffusion of ether into ethanol solutions resulted in phospholipid precipitation. The crystalline deposits were filtered or centrifuged off, and the solids were dried under vacuum to give type I samples. Type II samples of L-α-DLPC and L-α-DPPC were obtained by dissolving the lipid (100−200 mg) in ethanol (5 mL) and hexane (100 mL), storing the solution at 258 K for several days to form crystalline precipitate, filtration of the suspension under vacuum, and drying crystals also under vacuum. Both types of samples were stored at room temperature in the evacuated screw cap vials in the dessicator. Due to a low solubility of DPPE, $DPPEM_1$, and $DPPEM_2$ in ethanol, the crystallization of these phospholipids was performed analogously as in method I, but using the mixture of chloroform−ethanol−water (1:3:0.4) for solubilization of L-α-DPPE and DL-DPPE, and the mixture of chloroform−ethanol−water (1:1:0.05) for solubilization of L-α-$DPPEM_1$, L-α-$DPPEM_2$, DL-$DLPEM_1$, and DL-$DPPEM_2$. An analogous crystallization method was used earlier to prepare crystals of DL-$DPPEM_2$.[40] All solid samples appeared polycrystalline in nature and not suitable for high-resolution X-ray diffraction work. The major types of phospholipid samples were analyzed for oxygen content to determine the hydration levels (Midwest Microlabs). The results of analyses are listed in Table 2. The samples of the crystalline L-α-DPPC and DL-DPPC obtained by method I (Figures 3a and 4a) were dihydrates, and the L-α-DPPC-II (Figures 3d and 4d) was a trihydrate. The sample of L-α-DPPC obtained by ether diffusion into the ethanol−water (95:5) solution at room temperature, as described by Pearson and Pascher,[8] had the oxygen content of a trihydrate, but displayed a more complex spectral characteristics, suggesting it was not homogeneous in composition. The sample of DL-DPPE was a monohydrate (Figures 6d and 7b), L-α-DPPE was a dihydrate (Figures 6a and 7a), and both samples of $DPPEM_2$ were trihydrates (Figures 6c and 7d, and 6e and 7e). The fully hydrated samples of DPPC for CP-MAS experiments were prepared by adding an equal amount of water to the dry phospholipid and centrifuging the resulting semisolid sample several times through a narrow passage. Such semisolid samples were placed in the epoxy-sealed glass ampoules which fitted tightly in the MAS rotor.

NMR Methods. All CP-MAS NMR spectra were obtained using a single-contact cross-polarization pulse sequence. ^{13}C NMR spectra of phosphatidylcholines were obtained using at the 75.44 MHz frequency (Bruker MSL) with 20 kHz sweep width, 4.5 μs ^1H 90° pulse, a 2 ms contact time, a 52 ms acquisition time, and a 4 s recycle delay time. ^{31}P NMR spectra of phosphatidylcholines were obtained at the 121.49 MHz frequency with 100 kHz sweep width, and using other parameters as above. The ^{13}C spectra of all phosphatidylethanolamines were obtained at the 100.6 MHz frequency (Bruker AM-400) with 24 kHz sweep width, a 6 μs 90° ^1H pulse, a 2 ms contact time, a 22 ms acquisition time, and a 3 s recycle delay time. The ^{31}P NMR spectra of these samples were obtained at the 191.98 MHz frequency with 50 kHz sweep width, a 1 ms contact time, and with other parameters as above. Samples were rotated at a 3.5−6.0 kHz rate, using a 7 mm MAS rotor for all spectra of phosphatidylcholines, and a 4 mm rotor for all other spectra. For FID workup, 1−2K data points were transformed in a 4K memory after gaussian apodization using GB = 0.3−0.5 and LB = −10 Hz parameters. The crystalline samples were packed into the MAS rotor within a short time (1−2 min) and stored in a capped rotor until all analyzes were performed. NMR spectra of the same sample obtained within several days indicated that no detectable changes in its identity took place. ^{31}P and ^{13}C NMR spectra were always obtained with the same specimen, and within a short time needed only to retune the probe to the desired frequency. Chemical shifts in ^{13}C CP-MAS spectra were indirectly referenced to tetramethylsilane via the glycine carbonyl carbon (δ 176.06 ppm), and chemical shifts in ^{31}P NMR spectra were indirectly referenced to the 85% phosphoric acid standard. Spectra of the glycine and phosphoric acid standards were taken prior to each measurement session, and the shim parameters obtained for the standard glycine sample were kept constant throughout the measurement session to avoid shim-induced field change.

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