Effect of Phospholipids and a Transmembrane Peptide on the Stability of the Cubic Phase of Monoolein: Implication for Protein Crystalization from a Cubic Phase

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ABSTRACT The cubic phase of monoolein has successfully been used for crystallization of a number of membrane proteins. However, the mechanism of protein crystallization in the cubic phase is still unknown. It was hypothesized, that crystallization occurs at locally formed patches of bilayers. To get insight into the stability of the cubic phase, we investigated the effect of different phospholipids and a model transmembrane peptide on the lipid organization in mixed monoolein systems. Deuterium-labeled 1-oleoyl-*rac*-[$^2H_5$]-glycerol was used as a selective probe for ^2H NMR. The phase behavior of the phospholipids was followed by ^{31}P NMR. Upon incorporation of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, or phosphatidic acid, the cubic phase of monoolein transformed into the L_α or H_{II} phase depending on the phase preference of the phospholipid and its concentration. The ability of phospholipids to destabilize the cubic phase was found to be dependent on the phospholipid packing properties. Electrostatic repulsion facilitated the cubic-to-L_α transition. Incorporation of the transmembrane peptide KALP31 induced formation of the L_α phase with tightly packed lipid molecules. In all cases when phase separation occurs, monoolein and phospholipid participate in both phases. The implications of these findings for protein crystallization are discussed.

INTRODUCTION

Monoglycerides are amphiphatic neutral lipid molecules in which a hydrophobic fatty acid is attached at the *rac*-1 position of a hydrophilic glycerol backbone via an ester bond. Despite their relatively simple chemical structure, monoglycerides can form various phases found in membrane phospholipid/water systems. The ability of monoglycerides to form bilayer as well as nonbilayer structures offers many interesting opportunities for studies of membrane lipid organization (Boots et al., 1999, 2001; Chupin et al., 2001, 2002).

Recently a novel approach has been devised for the crystallization of membrane proteins by using monoolein cubic phases (Landau and Rosenbusch, 1996; Pebay-Peyroula et al., 1997; Rummel et al., 1998; Chiu et al., 2000). These phases are ordered, three-dimensional water-lipid systems, in which lipids are organized in a highly curved bicontinuous bilayer network (Hyde and Andersson, 1984; Lindblom and Rilfors, 1989). Such matrices support growth of crystals by lateral diffusion of protein molecules in the bilayer network. The mechanism of protein crystallization in the cubic phase is still not known. Recently, it was hypothesized, that protein crystallization occurs in locally formed liquid-crystalline lamellar L_α phases (Caffrey, 2000; Nollert et al., 2001). One indication for that is a lamellar-type packing arrangement of protein molecules within crystals. In addition, the cubic-to-L_α phase transition can be induced by different additives, which are always present during crystallization. For example, membrane protein samples contain phospholipids and detergents (Nollert et al., 2001). Potentially, phospholipids, detergents, and proteins can change the lipid organization in the cubic phase of monoolein and cause a transition to a liquid crystalline bilayer, thereby modulating the crystallization process. The effect of detergents on the cubic phase of monoolein was reported previously (Ai and Caffrey, 2000). However, little is known about how phospholipids and proteins interact with the cubic phase of monoolein. To analyze a possible effect of phospholipids and proteins we investigated the lipid organization of monoolein in the presence of different phospholipids and in the presence of a model transmembrane peptide.

To allow systematic analysis of the effect of different phospholipids we used phospholipids with the same acyl chain as monoolein, but with the headgroups differing in size and electrical charge: 1,2-dioleoylphosphatidylcholine (DOPC), 1,2-dioleoylphosphatidylethanolamine (DOPE), 1,2-dioleoylphosphatidylglycerol (DOPG), and 1,2-dioleoylphosphatidylphosphatidic acid (DOPA). DOPC and DOPE are zwitterionic and electrically neutral, whereas DOPG and DOPA are negatively charged at neutral pH. DOPC and DOPG are typical bilayer-forming phospholipids with relatively large headgroups, whereas DOPE prefers to organize in nonbilayer structures like the inverted hexagonal H_{II} phase (de Kruijff, 1997a). DOPA displays intermediate behavior and can form bilayer and inverted structures depending on a range of factors, including pH and the presence of cations (Verkleij et al., 1982; Lindblom et al.,

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1991). To mimic the situation when membrane spanning proteins are present we used a model peptide, KALP31, with a hydrophobic core of alternating leucine and alanine, flanked by lysine residues. This peptide forms a transmembrane α-helix in lipid bilayers (de Planque et al., 1999).

The lipid organization of these mixed systems was studied by means of solid state ^{2}H and ^{31}P NMR spectroscopy. Deuterium-labeled monoolein 1-oleoyl-*rac*-[$^2H_5$]-glycerol ([$^2H_5$]-MO) (Fig. 1) with a fully deuterated headgroup was used as a selective probe for ^{2}H NMR. Such labels can provide not only information on the phase behavior, but also on the lipid/water interface and glycerol conformation in monoglyceride systems. The behavior of phospholipids was followed by ^{31}P NMR. In this approach, the behavior of individual molecular components can be monitored in the same sample, which is important because monoglyceride/water systems are known to form long-living metastable phases.

MATERIALS AND METHODS

Materials

Oleic acid was obtained from Sigma Chemical Company (St. Louis, MO). Deuterated glycerol-d5 and deuterium-depleted water were obtained from Cambridge Isotope Laboratories (Cambridge, MA). 1,2–Dioleoyl-*sn*-glycerophosphocholine, 1,2–dioleoyl-*sn*-glycerophosphoethanolamine, 1,2–dioleoyl-*sn*-glycerophospho-*rac*-glycerol and 1,2–dioleoyl-*sn*-glycerophosphate were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Oleoyl-*rac*-[$^2H_5$]-glycerol was synthesized via acylation of 1,2-isopropylidene-[$^2H_5$]-glycerol, and removal of the protective group. Monoglyceride was purified on a silica column and crystallized from acetone. The KALP31 peptide (Ac-GKK(LA)$_{12}$LKKA-amide) was synthesized on an automatic ABI 433A peptide synthesizer using the ABI FastMoc 0.25 mmol protocols (de Planque et al., 1999).

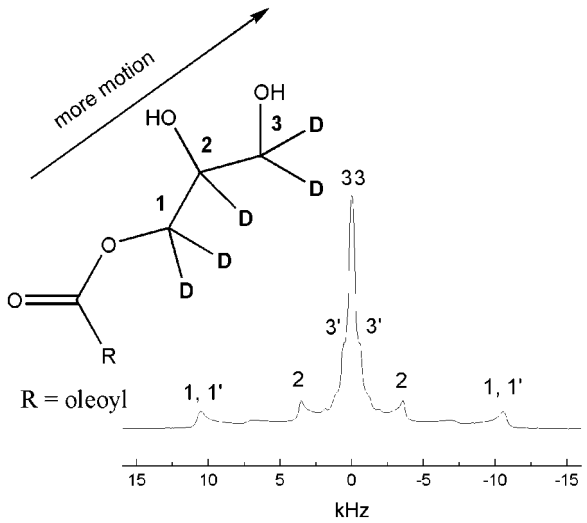

FIGURE 1 Deuterium-labeled 1-monooleoyl-*rac*-[$^2H_5$]-glycerol ([$^2H_5$]-MO) and signal assignment in the ^{2}H NMR spectrum of [$^2H_5$]-MO in the liquid-crystalline phase at 5°C (85 wt % lipid). The undercooled liquid-crystalline phase was obtained by subsequent heating up to 50°C and cooling down to 5°C of the sample.

Sample preparation

Samples were prepared by mixing known amounts of [$^2H_5$]-MO and phospholipid stock solutions in $CHCl_3$/MeOH (3:1). KALP31 was dissolved in TFE and added to a lipid solution. The solvents were evaporated and residual solvent was removed under high vacuum for at least 12 h. The lipids were subsequently hydrated by adding buffer at room temperature (20 mM Tris, pH 7, prepared with deuterium-depleted water). The samples consisted of 70–100 μmol lipid. A lipid-to-buffer ratio of 3/2 (wt/wt) was used. To study pure [$^2H_5$]-MO in the L_α phase, a water content of 15 wt % was used. Above 35 wt % water, MO can form only the cubic and crystalline phase (Qiu and Caffrey, 2000). Before heating the sample of [$^2H_5$]-MO was incubated at −20°C during 24 h to obtain the thermodynamically stable crystalline phase (Qiu and Caffrey, 2000). In the NMR probe, the sample was incubated for 10 min at each temperature and then measured for 20 min.

NMR measurements

NMR spectra were recorded on Bruker MSL 300 and Avance 500 WB spectrometers. ^{2}H NMR spectra were obtained using a quadrupolar echo technique (Davis et al., 1976). The recycling delay was 200 ms. ^{31}P NMR spectra were recorded using a high resolution 10 mm broad-band probe with broad-band gated proton decoupling. The recycling delay was 1.5 s and the $\pi/4$ pulse width 7 μs. An exponential multiplication with a line-broadening factor of 100 Hz was used before performing the Fourier transformation. All ^{2}H NMR spectra were symmetrized. Chemical shifts in ^{31}P NMR spectra were measured relatively to the isotropic signal.

Theory and application of ^{2}H NMR (Davis, 1983; Seelig and MacDonald, 1987; Smith, 1989) and ^{31}P NMR (Seelig, 1978; Smith and Ekiel, 1984) spectroscopy in lipid systems are described in the literature.

RESULTS

Phase characterization of deuterium headgroup labeled monoglycerides by ^{2}H NMR

We previously demonstrated that ^{2}H NMR spectroscopy on deuterated acyl chain monoglycerides is a convenient technique to monitor phase transitions in monoglyceride-water systems (Chupin et al., 2001), because all different phases give rise to characteristic features in the ^{2}H NMR spectra. In this study, the headgroup-deuterated [$^2H_5$]-MO was chosen to investigate not only the phase behavior but also the properties of the interface and glycerol conformation in mixed monoglyceride/phospholipid systems. However, the line shape of ^{2}H NMR spectra of headgroup-labeled monoglycerides in different phases is not known. Therefore we first characterized different phases of [$^2H_5$]-MO by ^{2}H NMR.

Signal assignment

In deuterated glycerol of chiral [$^2H_5$]-MO, all five deuterons are chemically nonequivalent (Fig. 1) and can give five different resonances in a ^{2}H NMR spectrum. The two

enantiomers present in the racemic mixture cannot be distinguished in the NMR spectra. The signal assignment in the hydrated liquid-crystalline L_α phase of [$^2H_5$]-MO is shown in Fig. 1. The mobility of the deuterium labels increases in the direction from the *rac*-1 position to the *rac*-3 in the glycerol moiety due to intramolecular rotations around C-C bonds and glycerol wobbling as it was shown for headgroup-deuterated phosphatidylglycerol (Wohlgemuth et al., 1980). As a result, three groups of resonances are present in the spectrum (Fig. 1). The signal with the largest quadrupolar splitting of 22 kHz corresponds to two nonequivalent deuterons at the *rac*-1 position, which are not resolved in this spectrum. The signal at the *rac*-2 position exhibits a reduced quadrupolar splitting of 8 kHz, indicating motional averaging compared with the labels at the *rac*-1 position, which is mainly due to rotation around the C(1)-C(2) bond. Deuterium labels at the *rac*-3 position give two resolved resonances with further decreased quadrupolar splittings of 1.3 kHz and less than 0.4 kHz. The quadrupolar splittings of the headgroup-deuterated lipids are also dependent on the conformation of the headgroup and can differ within the same phase (Scherer and Seelig, 1989; Marassi and Macdonald, 1991) as will be shown below for mixed [$^2H_5$]-MO/phospholipid systems.

Phase characterization

According to the phase diagram of MO (Qiu and Caffrey, 2000), different phases can be obtained by varying the temperature and/or water content. As an example, the spectra of [$^2H_5$]-MO at 15 wt % water concentration are shown in Fig. 2 recorded upon subsequent heating (Fig. 2, *A–C*) and cooling (Fig. 2 *D*). In the crystalline phase of [$^2H_5$]-MO, the spectrum consists of a broad powder pattern with a quadrupolar splitting of ~120 kHz (Fig. 2 *A*), which is characteristic for an immobilized headgroup with nonresolved signals from the different labeled sites. In the L_α phase at 40°C, the spectrum of [$^2H_5$]-MO shows three group of resolved signals with reduced quadrupolar splittings of 14 kHz, 4, and 1.2/0.2 kHz from the labeled sites *rac*-1, *rac*-2, and *rac*-3 (Fig. 2 *B*), indicating motional averaging in this phase as compared to the crystalline phase. In the cubic phase, the ^2H NMR spectrum of [$^2H_5$]-MO shows a narrow signal originating from isotropically moving lipid molecules (Fig. 2 *C*). Once heated above the crystalline-to-liquid crystalline transition temperature, [$^2H_5$]-MO at 5°C forms the undercooled L_α phase (Fig. 2 *D*), which is in line with the x-ray data (Qiu and Caffrey, 2000). The increased quadrupolar splitings in the L_α phase at 5°C (see also Fig. 1) as compared to 40°C reflects the decrease in motion of the [$^2H_5$]-MO at the lower temperature.

The data presented above demonstrate that ^2H NMR on deuterium-headgroup-labeled monoglycerides is a sensitive technique to monitor lipid organization in monoglyceride systems.

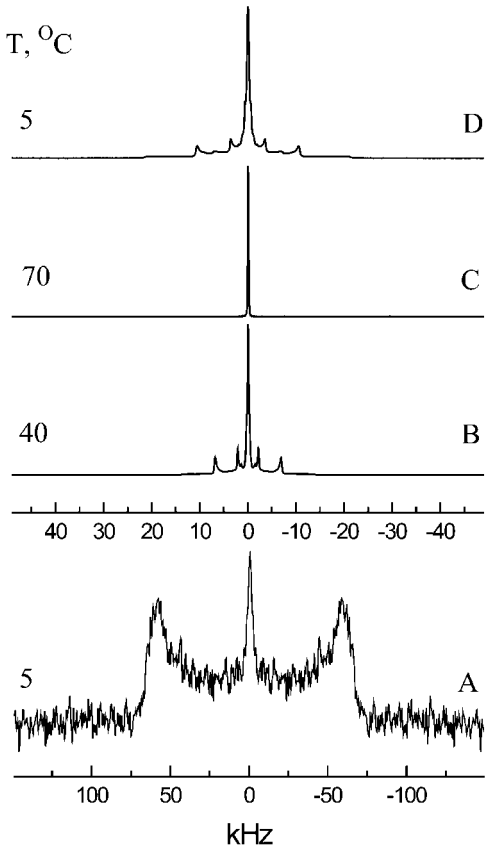

FIGURE 2 ^2H NMR spectra representing the different phases of [$^2H_5$]-MO (85 wt % lipid) crystalline phase. (*A*) Liquid-crystalline phase. (*B*) Cubic phase of [$^2H_5$]-MS. (*C*) Undercooled liquid crystalline phase of [$^2H_5$]-MO. (*D*) The thermodynamically stable crystalline phase was obtained by incubation of the sample at −20°C during 24 h.

Effect of phospholipids on phase behavior of monoolein

To elucidate a possible effect of phospholipids on the cubic phase of MO we investigated the lipid organization in mixtures with [$^2H_5$]-MO/phospholipid ratio's of 1:0, 9:1, 7:3, 1:1, 3:7, 1:9, and 0:1 (wt/wt) by ^2H and ^{31}P NMR. The weight concentration was used rather than molar ratio because this is commonly accepted for phase diagrams in monoglyceride systems. All experiments were performed at a lipid/water ratio of 3/2 (wt/wt) and at a temperature of 20°C, which are the same conditions that were used for protein crystallization from the cubic phase (Nollert et al., 2001). Under these conditions, pure [$^2H_5$]-MO forms a cubic phase.

Phosphatidylcholine

Phosphatidylcholine is a bilayer preferring lipid (de Kruijff, 1997a). The ^{31}P NMR spectrum of DOPC (Fig. 3 *A*) exhibits a line shape with a high field peak and a low field shoulder,

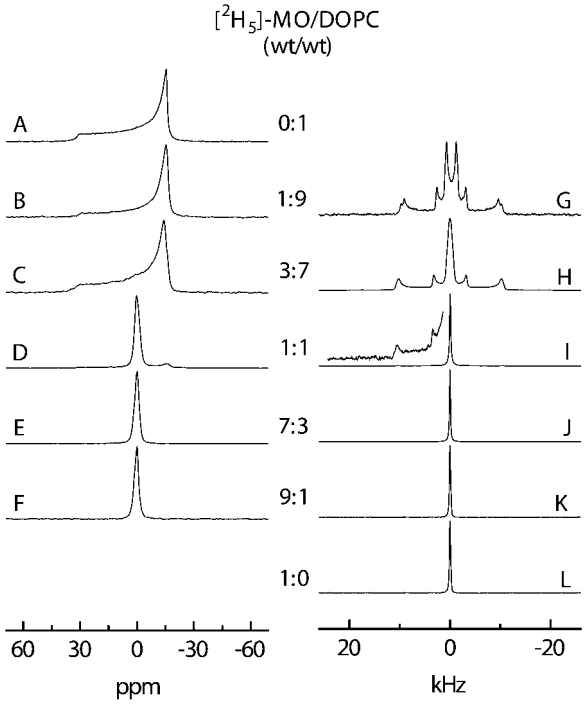

FIGURE 3 ^{31}P NMR spectra (*left*) and ^{2}H NMR (*right*) of [$^{2}H_5$]-MO/DOPC at different [$^{2}H_5$]-MO/phospholipid ratios, as indicated in the figure.

which is characteristic of the L_α phase. The isotropic signal of the pure [$^{2}H_5$]-MO cubic phase is shown in Fig. 3 *L*. At concentrations of DOPC below 50 wt %, ^{31}P, and ^{2}H NMR spectra show isotropic signals (Fig. 3, *E*, *F*, *J*, and *K*), indicating that both phospholipid and monoglyceride molecules are organized in the isotropic cubic phase. At a concentration of DOPC of 50 wt %, both ^{31}P and ^{2}H NMR spectra show a superposition of isotropic and anisotropic signals (Fig. 3, *D* and *I*). The line shapes of the anisotropic signals indicate formation of the L_α phase. The ^{31}P NMR spectrum (Fig. 3 *D*) shows a peak at the high field like pure DOPC (compare with Fig. 3 *A*). In the ^{2}H NMR spectrum (Fig. 3 *I*), the signal of the *rac*-1 labels exhibits a quadrupolar splitting of 21 kHz, which corresponds to that of the L_α phase of pure [$^{2}H_5$]-MO. Thus, at this concentration phase separation occurs between an L_α and a cubic phase, with both lipids participating in both phases. At concentrations of DOPC higher than 50 wt %, both ^{31}P and ^{2}H NMR spectra (Fig. 3, *B*, *C*, *G*, and *H*) show a line shape, which is characteristic of the L_α phase. The phase behavior of the [$^{2}H_5$]-MO/DOPC mixture at 28°C (not shown) is in line with the phase diagram reported previously for MO/DOPC/water mixtures, obtained by using ^{31}P NMR on DOPC and ^{2}H NMR on D_2O (Lindblom and Rilfors, 1989). However, in our approach we can follow the behavior of both DOPC and [$^{2}H_5$]-MO. No lipid separation of DOPC and [$^{2}H_5$]-MO was detected indicating that both phospholipid and monoglyceride are homogeneously mixed in the cubic and L_α phase.

Phosphatidylethanolamine

In contrast to phosphatidylcholine, phosphatidylethanolamine has a strong propensity to form inverted structures with a negative curvature (de Kruijff, 1977a). At 20°C, DOPE forms a hexagonal H_{II} phase, as indicated by the ^{31}P NMR spectrum with a low field peak and a high field shoulder and a twofold-reduced chemical shift anisotropy (Fig. 4 *A*). At concentrations of DOPE up to 50 wt % the [$^{2}H_5$]-MO:DOPE mixture still forms the H_{II} phase as evidenced by the ^{31}P NMR spectra (Fig. 4, *B–D*). In the corresponding ^{2}H NMR spectra (Fig. 4, *F–H*), the quadrupolar splitting of the *rac*-1 labels of [$^{2}H_5$]-MO is reduced by a factor of ~2 as compared with that in the L_α phase of [$^{2}H_5$]-MO/DOPC mixtures. This is typical for an organization of [$^{2}H_5$]-MO in the H_{II} phase, in which additional fast rotation of lipid molecules around the cylinders of the H_{II} phase causes a twofold decrease in the quadrupolar splitting (Davis, 1983; Sternin et al., 1988). At DOPE content of 30% (Fig. 4, *E* and *I*) and below (not shown), [$^{2}H_5$]-MO/DOPE mixtures show isotropic signals in ^{31}P and ^{2}H NMR spectra, indicating that both lipids are co-organized in the isotropic phase.

Phosphatidic acid

Phosphatidic acid is a minor but very important membrane lipid as key intermediate in lipid biosynthesis and because it is involved in different cellular functions (Schmidt et al., 1999; Weigert et al., 1999; Barr and Shorter, 2000). Phosphatidic acid shows a rich polymorphism (Verkleij et al., 1982; Lindblom et al., 1991). At neutral pH and in the absence of divalent cations, DOPA forms a liquid crystalline

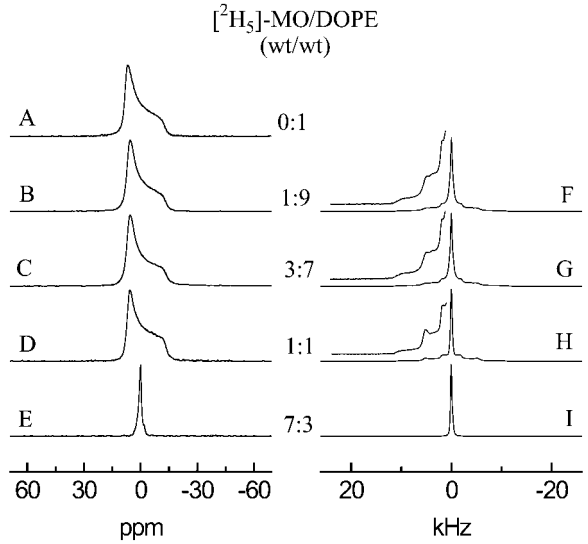

FIGURE 4 ^{31}P NMR spectra (*left*) and ^{2}H NMR (*right*) of [$^{2}H_5$]-MO/DOPE at different [$^{2}H_5$]-MO/phospholipid ratios, as indicated in the figure.

bilayer at 20°C, as indicated by the ^{31}P NMR spectrum (Fig. 5 *A*). At a DOPA content of 90 wt %, the ^{2}H and ^{31}P NMR spectra show a line shape of the liquid-crystalline bilayer (Fig. 5, *B* and *F*). In the presence of 30 wt % [$^{2}H_5$]-MO phase separation occurs between an L_α and an isotropic phase, in which both molecules participate (Fig. 5, *C* and *G*). Interestingly, upon further decreasing the DOPA content, the mixture with a [$^{2}H_5$]-MO/DOPA ratio of 1:1 (wt/wt) transforms into a mixture of the H_{II} and isotropic phase as indicated by a twofold decrease of the quadrupolar splitting and chemical shift anisotropy of the anisotropic signal in ^{2}H NMR and ^{31}P NMR spectra (Fig. 5, *D* and *H*). A further decrease of the DOPA content to 30 wt % and lower (not shown) again leads to isotropic signals in both ^{31}P NMR and ^{2}H NMR spectra of [$^{2}H_5$]-MO/DOPA (Fig. 5, *E* and *I*). Subsequent transformation from the L_α to the H_{II} and then to the cubic phase as observed upon increasing the [$^{2}H_5$]-MO content is very unusual, because normally the isotropic phase is intermediate between the L_α and H_{II} phase.

Phosphatidylglycerol

Phosphatidylglycerol is a common anionic bacterial lipid, which organizes by itself in a lamellar phase (Fig. 6 *A*). Up to 50 wt % of [$^{2}H_5$]-MO can be homogeneously incorporated into the L_α phase of DOPG (Fig. 6, *B–D*, *G–I*). At 30 wt % of DOPG, phase separation occurs between the L_α and an isotropic phase but the lamellar phase prevails (Fig. 6, *E* and *J*). At a [$^{2}H_5$]-MO/DOPG ratio of 9:1 (wt/wt), ^{31}P and ^{2}H NMR spectra show isotropic signals (Fig. 6, *F* and *K*). Comparison with Fig. 3 reveals that DOPG is much more effective in stabilization of the L_α phase of [$^{2}H_5$]-MO/PL as compared to DOPC.

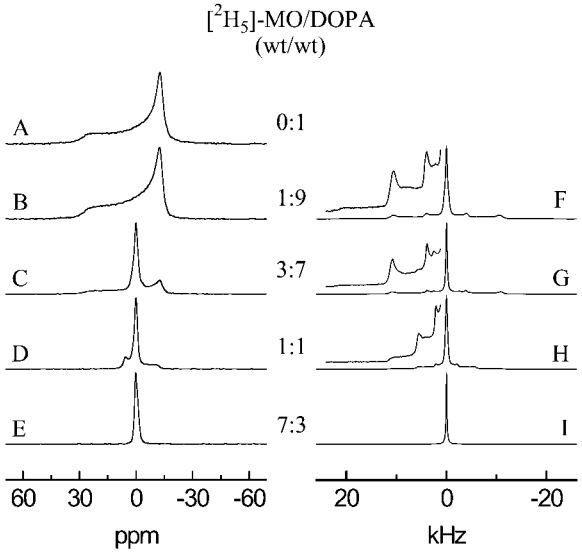

FIGURE 5 ^{31}P NMR spectra (*left*) and ^{2}H NMR (*right*) of [$^{2}H_5$]-MO/DOPA at different [$^{2}H_5$]-MO/phospholipid ratios, as indicated in the figure.

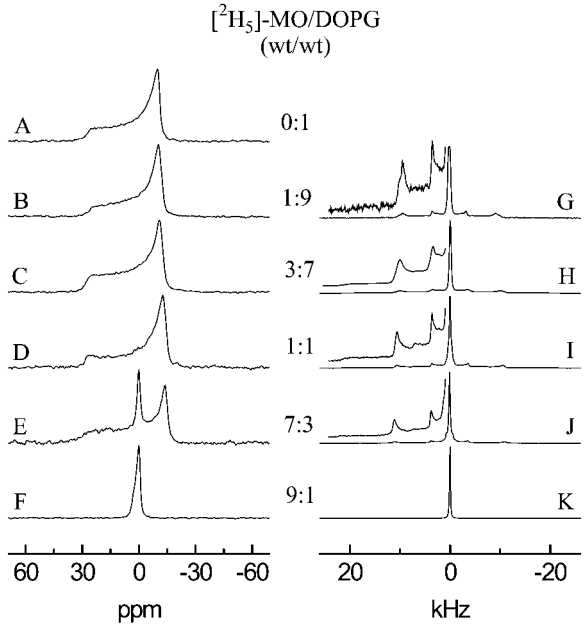

FIGURE 6 ^{31}P NMR spectra (*left*) and ^{2}H NMR (*right*) of [$^{2}H_5$]-MO/DOPG at different [$^{2}H_5$]-MO/phospholipid ratios, as indicated in the figure.

Another interesting difference between the [$^{2}H_5$]-MO/DOPC and [$^{2}H_5$]-MO/DOPG systems is seen in the line shape of the ^{2}H NMR spectra of [$^{2}H_5$]-MO in the L_α phase (PL/[$^{2}H_5$]-MO, 9:1 wt/wt) (Fig. 3 *G* and Fig. 6 *G*). The expanded spectra are shown in Fig. 7. The quadrupolar splittings of ~19 kHz of *rac*-1 labels of [$^{2}H_5$]-MO are identical in both [$^{2}H_5$]-MO/DOPC and [$^{2}H_5$]-MO/DOPG bilayers. However, the quadrupolar splitting of the *rac*-2 label in DOPC bilayer is 5 kHz as compared to 6 kHz in DOPG. For labels at the *rac*-3 position the effect is opposite. The quadrupolar splitting in the DOPC bilayer (2 kHz) is much larger compared to the value of 0.6 kHz in the DOPG

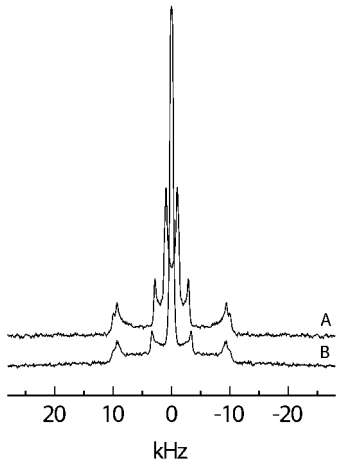

FIGURE 7 ^{2}H NMR spectra of [$^{2}H_5$]-MO/DOPC (*A*) and [$^{2}H_5$]-MO/DOPG (*B*) in the L_α phase. [$^{2}H_5$]-MO/phospholipid ratio 1:9 (wt/wt).

bilayer. In the [$^2H_5$]-MO/DOPA bilayer, the quadrupolar splitting of the *rac*-2 label (7.9 kHz) is even more increased, whereas quadrupolar splittings of the *rac*-3 labels (0 and 0.6 kHz) are decreased compared with these in the DOPC and DOPG bilayers. Such changes strongly suggest (Seelig and Macdonald, 1987; Scherer and Seelig, 1989) that the orientation of the glycerol headgroup of [$^2H_5$]-MO with respect to the bilayer surface is different in DOPC and DOPG bilayers.

Effect of [$^2H_5$]-MO on the lipid packing in the L_α phase of [$^2H_5$]-MO/PL

The quadrupolar splittings arising from the deuterium labels at the *rac*-1 position of [$^2H_5$]-MO are well resolved and can be used as a measure for the molecular order of the [$^2H_5$]-MO headgroup. As shown in Fig. 8, these quadrupolar splittings are dependent on the presence of [$^2H_5$]-MO in the L_α phase. Upon increasing the [$^2H_5$]-MO content in the [$^2H_5$]-MO/DOPC and [$^2H_5$]-MO/DOPG bilayers, the quadrupolar splittings also increase, indicating that the packing density of the lipid molecules increases. The results suggest that when a critical packing density level corresponding to the quadrupolar splitting of ~21.5 kHz is reached, the system transforms from the L_α phase into the cubic phase. The quadrupolar splittings of the *rac*-1 labels of [$^2H_5$]-MO are almost identical in the [$^2H_5$]-MO/DOPC and [$^2H_5$]-MO/DOPG bilayers with the same [$^2H_5$]-MO/PL ratio (Fig. 8). This is in line with the similar packing properties of DOPC and DOPG as can be concluded from their similar gel-to-L_α transition temperatures (March, 1990). The quadrupolar splittings of the *rac*-1 labels of [$^2H_5$]-MO in the [$^2H_5$]-MO/DOPA bilayer are larger as compared to the bilayers of [$^2H_5$]-MO/DOPC and [$^2H_5$]-MO/DOPG (Fig. 8), which is most likely due to the small size of the polar headgroup of DOPA and more tight packing of the lipid molecules in the liquid crystalline bilayers of [$^2H_5$]-MO/DOPA .

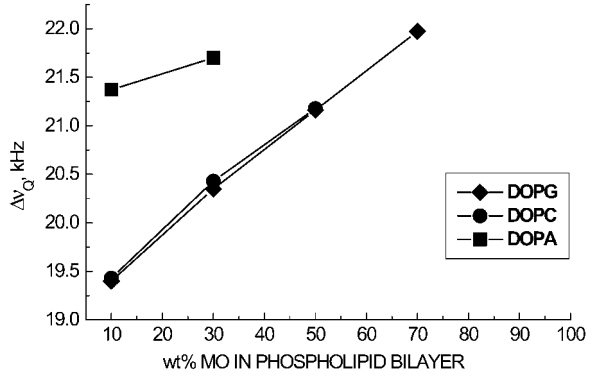

FIGURE 8 Quadrupolar splitting Dn_Q of *rac*-1 labels of [$^2H_5$]-MO in the liquid crystalline lamellar L_α phase of DOPG, DOPC, and DOPA as a function of the [$^2H_5$]-MO content.

Effect of a transmembrane α-helical peptide KALP31 on the cubic phase

To get insight into effects of proteins on the cubic phase, we investigated the lipid organization of a [$^2H_5$]-MO/DOPC, 7:3 (w/w) mixture in the presence of a model peptide KALP31. KALP31 is a hydrophobic peptide, which forms a transmembrane α-helix in lipid bilayers that is interfacially stabilized by the N- and C-terminal lysines (de Planque et al., 1999). The mixture of [$^2H_5$]-MO/DOPC, 7:3 (wt/wt), was chosen because it forms a cubic phase by itself but contains the bilayer preferring DOPC.

^{31}P and ^2H NMR spectra of a [$^2H_5$]-MO/DOPC, 7:3 (w/w) mixture in the presence of increasing amounts of KALP31 are shown in Fig. 9. In each panel, all spectra were normalized to the same height. The ^2H NMR spectra on the right panel are shown in the expanded vertical scale. In the absence of KALP31, anisotropic signals were not detected (Fig. 9, *top left* and *right*). In the presence of even very low concentration of KALP31, an anisotropic signal was observed in both ^{31}P and ^2H NMR spectra with a line shape, which is characteristic of the L_α phase (Fig. 9, *bottom three, left* and *right*). The intensity of the anisotropic signal increases upon increasing the KALP31 content (Fig. 9, *bottom three, left* and *right*) demonstrating that KALP31 stabilizes the L_α phase. The quadrupolar splitting of 22 kHz of the *rac*-1 labels in the anisotropic ^2H NMR signals indicates that lipid molecules are tightly packed in the L_α phase (compare with Fig. 8).

DISCUSSION

Upon incorporation of increasing amounts of different phospholipids, the cubic phase of monoolein transforms into the L_α or H_{II} phase depending on the type of phospholipid and its concentration. The ability of phospholipids to rearrange the lipid organization in the cubic phase of mono-

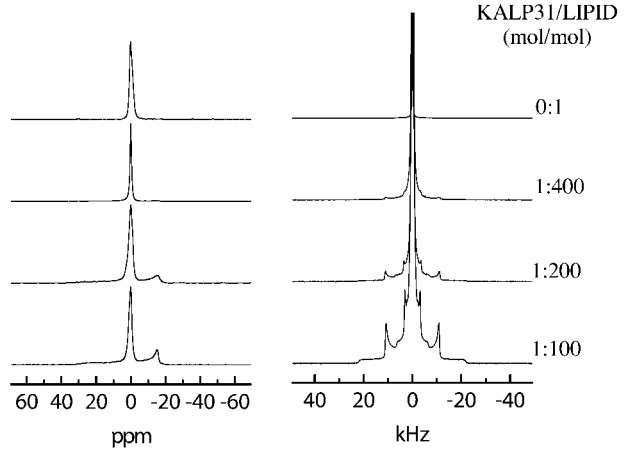

FIGURE 9 ^{31}P NMR and ^2H NMR spectra of [$^2H_5$]-MO/DOPC, 7:3 (wt/wt) mixture at different KALP31/lipid ratios, as indicated in the figure.

olein is correlated with the phospholipid headgroup size and charge, which both contribute to the lipid packing in the mixed [$^2H_5$]-MO/PL systems.

Effect of phospholipids on phase behavior of MO

Lipid organization can be understood by relating the packing properties of lipid molecules to their shape via a packing parameter p (Israelachvili et al., 1977):

$$p = V/Al, \quad (1)$$

where V is the acyl chains volume, A is the area of the lipid headgroup at the hydrocarbon-water interface, and l is the length of the acyl chains

If $p \cong 1$, lipid molecules form a bilayer. If $p > 1$, they tend to form structures with a negative curvature like the H_{II} phase and if $p < 1$, they tend to form structures with a positive curvature, such as micelles. Cubic phases can be considered as intermediate between the bilayer and H_{II} phase.

Zwitterionic DOPC and DOPE are electrically neutral but differ in the size of the polar headgroup. DOPE has a small headgroup, which increases p (Eq. 1). As a result, incorporation of the inverted phase forming DOPE in [$^2H_5$]-MO destabilizes the cubic phase-inducing formation of the H_{II} phase. In contrast to DOPE, DOPC has a large headgroup, which decreases p (Eq. 1). DOPC transforms the cubic phase of [$^2H_5$]-MO into the L_α phase.

Like DOPC, negatively-charged DOPG is a bilayer-forming phospholipid with a relatively large headgroup (de Kruijff, 1997a). Moreover, for the same fatty acid composition the acyl chain packing in the liquid crystalline bilayers of phosphatidylglycerols is similar to that of phosphatidylcholines, as indicated by the remarkably close values of the gel-to-liquid crystalline transition temperatures of these phospholipids (March, 1990). Therefore, the effect of electrostatic interactions on the stability of the cubic phase of monoolein can be derived by comparison of the [$^2H_5$]-MO/DOPC and [$^2H_5$]-MO/DOPG systems. Both DOPC and DOPG destabilize the cubic phase of [$^2H_5$]-MO. However, despite the similar packing properties of DOPC and DOPG, the cubic-to-L_α phase transition occurs at a significantly lower content of DOPG as compared to that of DOPC (Fig. 6). This higher efficiency of DOPG to destabilize the cubic phase of [$^2H_5$]-MO indicates that electrostatic interactions play an important role. A likely possibility is that electrostatic repulsion between the negatively-charged headgroup of DOPG induces an increase in the diameter of the water channels of the cubic phase as observed for MO/PA (Shu et al., 2001), leading to subsequent disruption of the cubic phase, which transforms in the liquid crystalline bilayer.

The behavior of [$^2H_5$]-MO/DOPA is more complex. Upon increasing the DOPA content in [$^2H_5$]-MO/DOPA mixture, the cubic phase subsequently transforms into the H_{II} phase and then into the L_α phase. Such polymorphic behavior is not usual for lipid systems, in which the cubic phases are structures that are intermediate between a bilayer and inverted phase organization (de Kruijff, 1997b). The behavior of the [$^2H_5$]-MO/DOPA system can be explained in terms of electrostatic interactions. When the content of DOPA is high, strong electrostatic repulsive forces cause an increase of the area of the lipid headgroup at the lipid-water interface A (Eq. 1), which leads to stabilization of the L_α phase. Upon decreasing the DOPA content in the neutral [$^2H_5$]-MO matrix, the electrical charge density is decreased at the lipid/water interface, decreasing the electrostatic repulsion. To some extent, this is equivalent to a screening of the charges of DOPA, which is known to induce the L_α-to-H_{II} transition in pure DOPA (Verkleij et al., 1982; Lindblom et al., 1991). At a [$^2H_5$]-MO/DOPA ratio of 1:1 (wt/wt), the electrostatic repulsion is not strong enough to overcome the propensity of DOPA to form a nonbilayer structure and the system transforms into the mixture of the H_{II} and isotropic phase. Upon further decreasing the DOPA content, the system transforms into the cubic phase because the lipid organization now is determined by the packing properties of [$^2H_5$]-MO.

Lipid packing and cubic phase formation in [$^2H_5$]-MO/PL systems

The combination of ^2H NMR on [$^2H_5$]-MO and ^{31}P NMR on phospholipids allows to monitor the phase organization of monoolein and phospholipids in the mixed [$^2H_5$]-MO/PL systems. In transitions from the cubic-to-L_α, cubic-to-H_{II}, or L_α-to-H_{II} mediated by the lipid composition of [$^2H_5$]-MO/PL mixtures, both monoolein and phospholipid are involved. The ^2H NMR data also allow to get insight in the lipid packing of the different systems. In the L_α phase of [$^2H_5$]-MO/DOPC and [$^2H_5$]-MO/DOPG, increasing amounts of [$^2H_5$]-MO lead to increase lipid packing density, as indicated by the increase of the quadrupolar splitting of the *rac*-1 labels (Fig. 8). When a critical packing density is reached the system transforms to the cubic phase. Similarly in [$^2H_5$]-MO/DOPA, the L_α phase transforms into the H_{II} phase at the same lipid packing density as the cubic phase is formed in [$^2H_5$]-MO/DOPG and [$^2H_5$]-MO/DOPC, as indicated by the close values of the quadrupolar splittings of the *rac*-1 labels (Fig. 8). The molecular order increase in the L_α phase upon incorporation of nonbilayer-forming lipids with a small headgroup has a general character and was also observed for a number of other lipid systems (Lafleur et al., 1990a,b; Killian et al., 1992; Chupin et al., 1994; Orädd et al., 2000).

Conformation of the monoolein headgroup in the L_α phase

In the L_α phase of the MO/water system, the *rac*-3 hydroxyl group forms an intramolecular hydrogen bond to the carbonyl group, which by addition of DOPC is broken and

hydrogen bonding between the hydroxyl groups of MO and phosphate group of DOPC begins to form (Nilsson et al., 1991). Such interaction with phospholipids can change the conformation of the monolein headgroup. In this study, we observed that the quadrupolar splittings of the *rac*-2 label of [$^2H_5$]-MO are decreasing, whereas the quadrupolar splittings of the *rac*-1 labels are increasing in bilayers of DOPC, DOPG, and DOPA, respectively. This indicates a different conformation of the headgroup of [$^2H_5$]-MO in the DOPC, DOPG, and DOPA bilayers. Previously such effect was observed for headgroup-deuterated phosphocholine, in which the headgroup can change its orientation with respect to the bilayer surface due to electrostatic interactions (Seelig and Macdonald, 1987; Scherer and Seelig, 1989). Based on those data, our observation can be interpreted as follows. The tilt of the glycerol headgroup of [$^2H_5$]-MO with respect to the plane of the lipid-water interface is increasing in the DOPC $<$ DOPG $<$ DOPA bilayers, which correlates with the geometrical headgroup size of these phospholipids. Most likely, the bulky choline headgroups of DOPC decrease the tilt of the headgroups of [$^2H_5$]-MO due to spatial hindrance, which is lower in the DOPG and especially low in the DOPA bilayer.

Implication for protein crystallization

It was proposed, that crystallization of proteins in the cubic phase of monoolein occurs in the locally formed L_α phase (Caffrey, 2000; Nollert et al., 2001). Our results show that the presence of the bilayer-forming phospholipids DOPC and DOPG facilitates formation of the liquid crystalline bilayer in the cubic phase of monoolein. Moreover, the effect of DOPG is larger compared to that of DOPC, indicating that electrostatic repulsion is important for the bilayer formation. The presence of proteins can also destabilize the cubic phase of monoolein, as concluded from the effect of the transmembrane KALP31 peptide, which induces formation of a bilayer coexisting with a cubic phase. Within this bilayer, the presence of the nonbilayer-forming monoolein creates a tight packing of the lipid molecules, as indicated by the quadrupolar splitting of the *rac*-1 labels of [$^2H_5$]-MO. One could speculate that as a result of this, protein molecules are forced to aggregate, which also can facilitate crystallization (de Kruijff, 1997b).

In the case of membrane proteins, which were successfully crystallized from the cubic phase, the crystallization conditions can facilitate formation of the L_α phase. For example, crystallization of bacteriorhodopsin in the cubic phase of monoolein always occurs in the presence of anionic lipids of purple membrane, phosphatidylglycerophosphate, and phosphatidylglycerosulfate (Nollert et al., 2001), which can facilitate the cubic-to-L_α phase transition. In this study, we demonstrated that the stability of the cubic phase of monoolein can be regulated by the presence of phospholipids. It is possible that crystallization of different membrane proteins can be facilitated by incorporation in the cubic phase of lipids, which can facilitate formation of the liquid crystalline bilayer, thereby creating crystallization sites.

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