Membrane Protein Crystallization In Meso: Lipid Type-Tailoring of the Cubic Phase

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ABSTRACT Hydrated monoolein forms the cubic-Pn3m mesophase that has been used for in meso crystallization of membrane proteins. The crystals have subsequently provided high-resolution structures by crystallographic means. It is possible that the hosting cubic phase created by monoolein alone, which itself is not a common membrane component, will limit the range of membrane proteins crystallizable by the in meso method. With a view to expanding the range of applicability of the method, we investigated by x-ray diffraction the degree to which the reference cubic-Pn3m phase formed by hydrated monoolein could be modified by other lipid types. These included phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine, cardiolipin, lyso-PC, a polyethylene glycol-lipid, 2-monoolein, oleamide, and cholesterol. The results show that all nine lipids were accommodated in the cubic phase to some extent without altering phase identity. The positional isomer, 2-monoolein, was tolerated to the highest level. The least well tolerated were the anionic lipids, followed by lyso-PC. The others were accommodated to the extent of 20–25 mol %. Beyond a certain concentration limit, the lipid additives either triggered one or a series of phase transitions or saturated the phase and separated out as crystals, as seen with oleamide and cholesterol. The series of phases observed and their order of appearance were consistent with expectations in terms of interfacial curvature changes. The changes in phase type and microstructure have been rationalized on the basis of lipid molecular shape, interfacial curvature, and chain packing energy. The data should prove useful in the rational design of cubic phase crystallization matrices with different lipid profiles that match the needs of a greater range of membrane proteins.

INTRODUCTION

The lipidic cubic mesophase (cubic-Pn3m, Fig. 1) has proven to be a useful host for the growth of well ordered crystals of membrane proteins (Chiu et al., 2000; Nollert et al., 2001). The crystals have subsequently been used in diffraction measurements for structure determination, at or close to atomic resolution. The mesophase-based (in meso) method makes use of the monoacylglycerol monoolein as the primary lipid ingredient. According to the temperature-composition phase diagram for the monoolein/water system, the relevant cubic phase forms spontaneously at 20°C above a limiting hydration level of ~35% (w/w) water (Fig. 2).

A model for how crystals of membrane proteins grow in meso has been presented (Caffrey, 2000). It includes a conduit between the bulk cubic phase and the crystal that consists of planes of lipid bilayers as in the lamellar liquid crystalline (L_α) phase in which the protein diffuses. The model also proposes that the protein becomes reconstituted uniformly into the lipid bilayer of the cubic phase during its spontaneous formation. Some evidence in support of this aspect of the model has been reported (Nollert et al., 2001).

There are several different cubic phases (Lindblom and Rilfors, 1989), two of which are formed in the monoolein/water system (Fig. 2). These are shown schematically in Fig. 1. Although not investigated in any great detail, preliminary measurements with bacteriorhodopsin (bR), suggest that the bulk medium giving rise to in-meso-grown crystals is of the cubic-Pn3m phase type (Nollert et al., 2001).

The in meso method was developed with bR as the test membrane protein and with hydrated monoolein as the hosting lipid for use at 20°C (Landau and Rosenbusch, 1996). However, monoolein is not a common membrane lipid. Thus, the relatively sparse bilayer environment it creates in the cubic phase might be recognized as foreign from the protein's perspective. With a view to making it (the cubic phase bilayer) more natural and the in meso method more generally applicable, we set out to determine whether, and to what extent, lipids of the type normally found in biomembranes could be accommodated in the cubic phase formed by hydrated monoolein. The lipids examined included phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), cardiolipin, and cholesterol. Also included in the study were lyso-PC; a polyethylene glycol (PEG)-lipid, dimyristoylphosphoethanolamine (DMPE)-mPEG550, of stealth lipid fame (Lasic, 1997); the sleep-inducing long-chain amide, oleamide (Boger et al., 1998); and 2-monoolein, an isomer that forms spontaneously by acyl chain migration in hydrated monoolein. As much as possible, the study was done with lipids having chains of the oleoyl type (Fig. 3). It is one of the more common acyl chains in biological membranes.

All of the phase behavior measurements were performed with monoolein as the reference lipid to which the other

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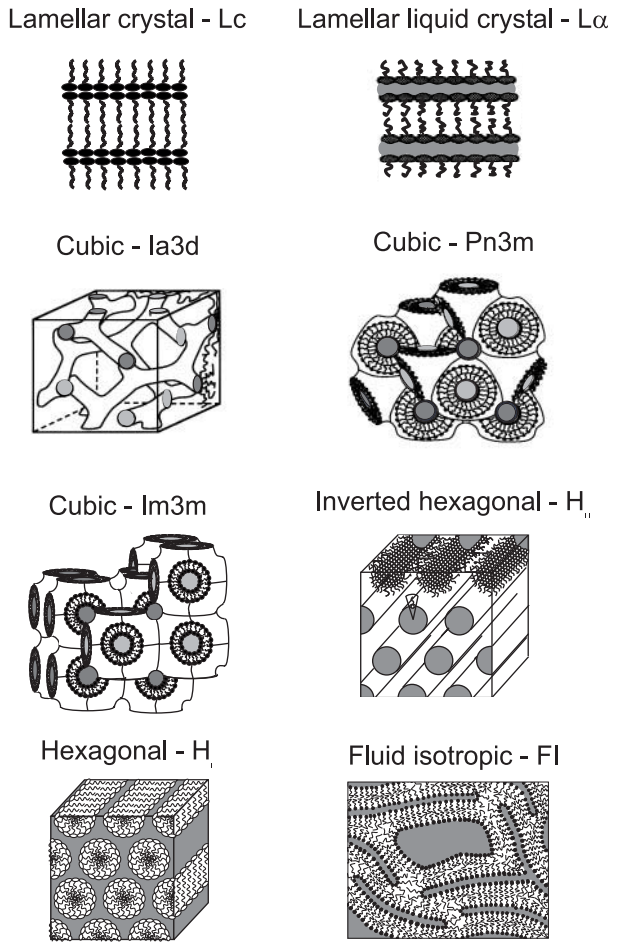

FIGURE 1 Lipid phases. Cartoon representation of the various solid (lamellar crystal phase), mesophase (lamellar liquid crystal phase, cubic-Pn3m phase (space group number 224, diamond type D), cubic-Ia3d phase (space group number 230, gyroid type I), cubic-Im3m phase (space group number 229, primitive type P), normal hexagonal, and inverted hexagonal phase), and liquid (fluid isotropic phase (Larsson, 1994)) states adopted by lipids dispersed in water. Individual lipids are shown as lollipop figures with the pop and stick parts representing the polar headgroup and the apolar acyl chain, respectively. The shaded regions represent water. The normal and inverted designations refer to the curvature of the lipid/water interface that is convex and concave, respectively, when viewed from the aqueous medium.

lipids were added. In most cases, the entire range of concentration from 0 to 100 mol % lipid additive was examined. Data were collected at a sample hydration level of 60% (w/w) water and at 20°C to mimic the conditions under which typical in meso crystallization trials are performed (Cherezov et al., 2001). Additional measurements were made at 4°C (in the cooling direction), as is commonly used in crystallization trials (McPherson, 1999). Under this condition, the system is wont to express its well documented tendency to undercool, as will be discussed (Misquitta and Caffrey, 2001; Qiu and Caffrey, 2000).

Low- and wide-angle x-ray diffraction were used for phase identification and microstructure characterization

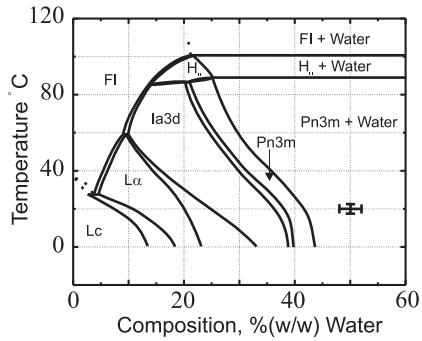

FIGURE 2 Temperature-composition phase diagram for the monoolein/water system (adapted from Briggs et al., 1996). The phase diagram was constructed in the heating and cooling directions beginning at 25°C. Boundary positions have an estimated error of ±2.0% (w/w) water in composition and ±2.5°C in temperature, as described (Misquitta and Caffrey, 2001) and as indicated.

(Fig. 4). Thus, we were able to monitor not just the effect that a given lipid additive had on phase type but also the manner in which it caused each phase to swell or to shrink, depending on the stresses and strains induced. Many of the additive effects observed, which included phase type and microstructure changes as well as phase separation, have been rationalized on the basis of molecular shape, interfacial curvature, and chain-packing energies.

MATERIALS AND METHODS

Materials

Monoolein (1-oleoyl-rac-glycerol, lot M239-M3-L and lot M239−029-L, 356.54 g/mol) was purchased from Nu Chek Prep (Elysian, MN) and from Sigma (lot 108H5168; St. Louis, MO) with a reported purity in excess of 99% and was used as supplied. Thin layer chromatography of fresh monoolein was used to verify purity. For this purpose, 1-, 5-, 50-, and 200-μg samples of monoolein dissolved in chloroform were run on Adsorbosil Plus plates (Alltech, Deerfield, IL) using three different solvent systems: chloroform/acetone (96/4, v/v), chloroform/acetone/methanol/acetic acid (73.5/25/1/0.5, v/v) and hexane/toluene/acetic acid (70/30/1, v/v). The plates were pre-run twice in chloroform/methanol (10/1, v/v). Spots were visualized by spraying with 4.2 M sulfuric acid followed by charring on a hot plate (250°C). Estimated purity of the lipid was in excess of 99.5%. Cholesterol (lot CH-800-N22-K, 386.66 g/mol) was from Nu Chek Prep, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine sodium salt (DOPS; lot 181PS-192, 810.03 g/mol), 1,2-dioleoyl-*sn*-glyero-3-phosphoethanolamine (DOPE; lot 181PE-236, 744.04 g/mol), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; lot 181PC-170, 786.15 g/mol), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethyleneglycol)-550] sodium salt (DMPE-mPEG550; lot 140PEG550PE-12, 1221.52 g/mol), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PC; lot 181LPC-39, 521.67 g/mol), and 1,1′,2,2′-tetraoleoyl cardiolipin sodium salt (cardiolipin; lot 181CA-17, 1501.18 g/mol) were from Avanti Polar Lipids (Alabaster, AL), and 2-monooleoylglycerol (lot 51k1624, 356.5 g/mol) and cis-9-octadecenamide (oleamide; lot 100K5210, 281.5 g/mol) were from Sigma. Water (resistivity > 18 MΩ cm) was purified by using a Milli-Q Water System (Millipore Corp., Bedford, MA) consisting of a carbon filter cartridge, two ion-exchange filter cartridges, and an organic removal cartridge.

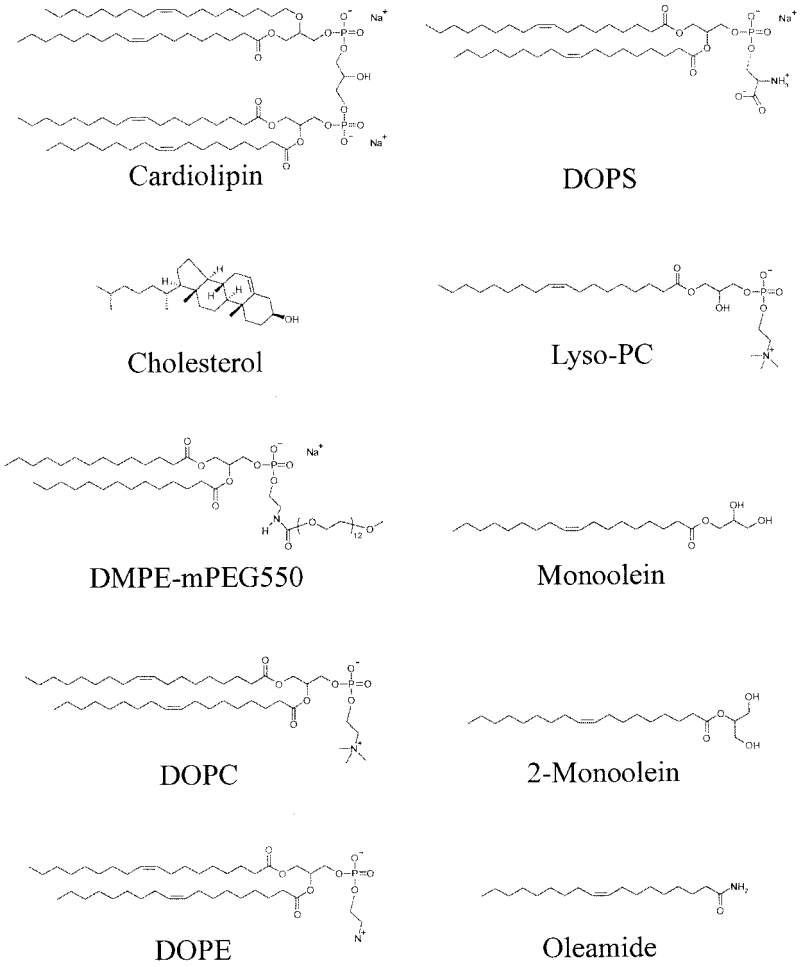

FIGURE 3 Molecular structures of the lipids used in this study.

Methods

Sample preparation

Stock solutions of monoolein and lipid additive in chloroform (typically 10–100 mg/ml) were co-dissolved in appropriate ratios with a total lipid mass between 15 and 20 mg. The samples were dried with a stream of inert (nitrogen or argon) gas and were subjected to subsequent vacuum drying at 30 mtorr and 20°C for at least 48 h. The only exceptions were samples with >60 mol % 2-monoolein. These were prepared by weighing out ~4 mg of the 2-isomer into a microsyringe and adding to it the required amount of reference monoolein (1-monoolein). Hydrated lipid samples were prepared at room temperature (20–24°C) using a home-built syringe mixer (Cheng et al., 1998; Qiu and Caffrey, 1998) at a concentration of 60% (w/w) water (and 40% (w/w) total lipid) with ~10–20 mg of lipid in each sample. The resultant aqueous dispersions were transferred to 1-mm quartz capillary tubes (Hampton Research, Laguna Niguel, CA) and flame-sealed using a propane/oxygen torch (Smith Equipment, Watertown, SD). A bead of 5-min epoxy (Devcon, Danvers, MA) was applied to protect and ensure the integrity of the flame seal. Capillaries were then centrifuged for ~5 min at ~2000 × *g* (clinical centrifuge, IEC, Needham, MA) to pellet the sample as necessary. Samples were stored at room temperature for at least 24 h and then incubated at 20°C for at least 4 h before being used for diffraction experiments. Subsequently, sample temperature was lowered to 4°C, and measurements were performed following an incubation of at least 4 h.

X-ray diffraction

X-ray diffraction measurements were performed using a rotating anode x-ray generator (Rigaku RU-300 operating at 45 kV and 250 mA) producing Ni-filtered Cu K_α radiation (wavelength $\lambda = 1.5418$ Å) as described (Cherezov et al., 2002a). Sample-to-detector distance (typically 340 mm) was measured using a silver behenate standard (Blanton et al., 1995). Samples were continuously translated at a rate of 3 mm/min back and forth along a 3-mm section of the sample to average the contributions to total scattering from different parts of the sample and to minimize possible radiation damage effects (Cherezov et al., 2002b). The temperature inside the sample holder (Zhu and Caffrey, 1993) was regulated by two thermoelectric Peltier effect elements controlled by a computer feedback system. Measurements were performed at 20.0 ± 0.05°C. A typical exposure time was 30 min. Diffraction pattern registration on high-resolution image plates and subsequent analysis have been described (Cherezov et al., 2002a).

RESULTS

The results presented below concern the effects that a variety of lipid additives have on the phase identity and phase microstructure of hydrated monoolein. Phase characteristics were quantified by low- and wide-angle x-ray diffraction. Measurements were made at 20°C, the tem-

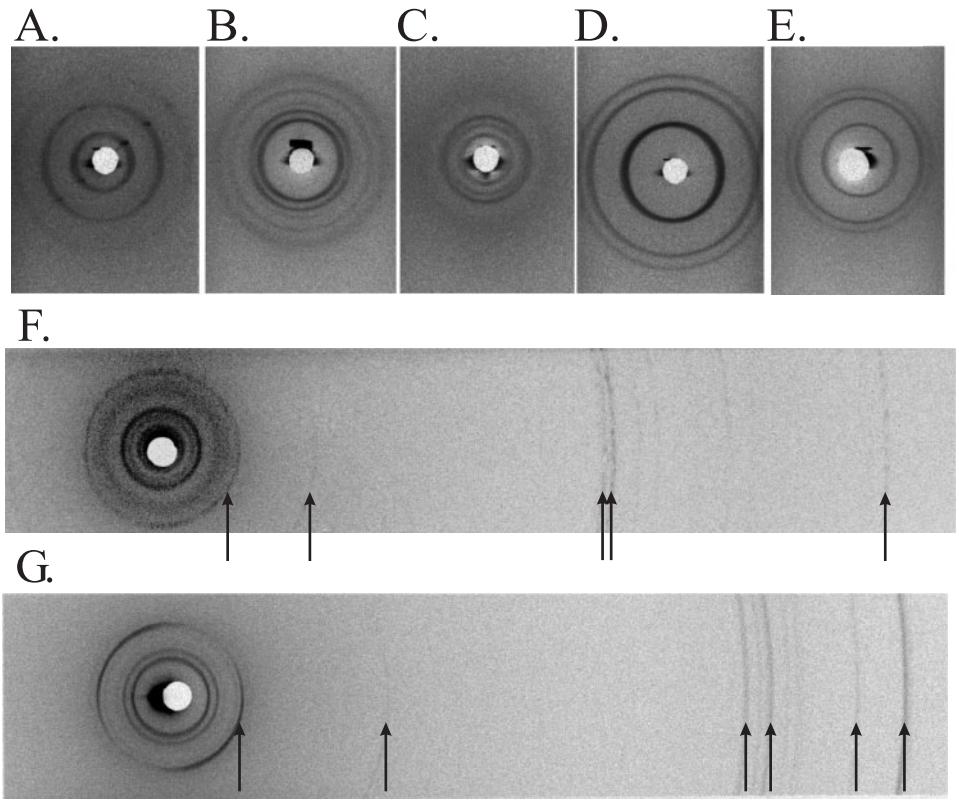

FIGURE 4 X-ray diffraction patterns of the various phases identified in the monoolein/water system with lipid additives. Phase identity and sample composition are as follows: (*A*) L_α phase, DMPE-mPEG550 (4.8 mol % in monoolein, 20°C); (*B*) Cubic-Pn3m phase, DOPC (0.17 mol % in monoolein, 4°C); (*C*) Cubic-Im3m phase, DMPE-mPEG550 (0.8 mol % in monoolein, 20°C); (*D*) H_{II} phase, DOPE (23.1 mol % in monoolein, 20°C); (*E*) H_I phase, DMPE-mPEG550 (83.1 mol % in monoolein, 20°C); (*F*) Cubic-Im3m plus crystals of cholesterol monohydrate (33.3 mol % in monoolein, 20°C); (*G*) Cubic-Pn3m plus oleamide crystals (50 mol % in monoolein, 20°C). Crystalline reflections in *F* and *G* are marked with arrows.

perature used in the development of the in meso method. It is often desirable to perform crystallization trials at temperatures considerably below room temperature, and accordingly, data were collected also at 4°C for most of the systems studied.

The reference state for monoolein at 60% (w/w) water and 20°C is the cubic-Pn3m phase in equilibrium with excess water (Fig. 2). Here, the lattice parameter of the cubic phase is ~106 Å. At 4°C, where undercooling is observed, the same phase state prevails and the relevant lattice parameter is ~108 Å. The monoolein used in this study originated from two commercial sources (see Methods). They exhibited slightly different lattice parameters when measured under standard conditions.

Lipid additive effects

Phosphatidylcholine

The particular phosphatidylcholine (PC) used in this study was DOPC (Fig. 3). Under fully hydrated conditions, DOPC as the sole lipid forms the lamellar liquid crystalline (L_α) phase at both 4°C and 20°C. In contrast, pure monoolein exists in the cubic-Pn3m phase as noted. Additions of DOPC to hydrated monoolein to the extent of ~25 mol % had no effect on phase type in that the cubic-Pn3m phase was retained (Fig. 5 *A*). At higher levels, however, the system transformed to the L_α phase, which remained stable in the composition range from ~26 to 100 mol % DOPC.

The lattice parameter of the cubic phase was very sensitive to DOPC and rose rapidly to a limiting value of 148.4 Å at ~24 mol % DOPC (20°C, Fig. 5 *A*). A similar trend was observed in related work (Templer et al., 1992). In the L_α phase, however, the lamellar repeat was quite insensitive to membrane composition.

The lattice parameters of the two phases encountered in this system were not particularly sensitive to temperature. The general trend was for lattice constants in both the cubic and lamellar phases to drop slightly with temperature in the presence of DOPC.

Phosphatidylethanolamine

As with PC, the particular chain variant of PE chosen for use in this study was of the dioleoyl type (DOPE). DOPE is a lipid with a propensity to form nonlamellar phases (Koy-

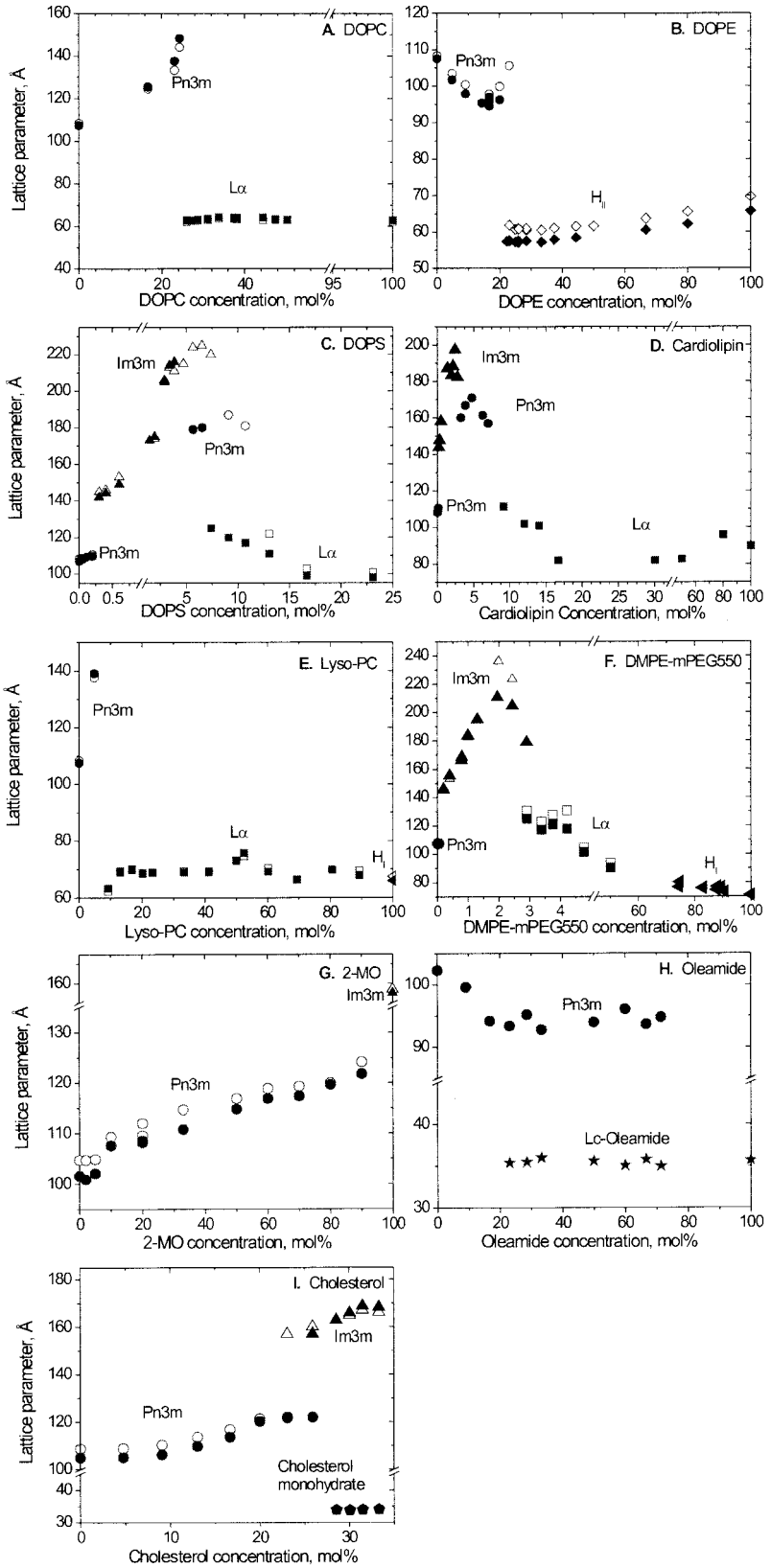

FIGURE 5 Dependence of the lattice parameters of the phases formed by mixtures of DOPC (*A*), DOPE (*B*), DOPS (*C*), cardiolipin (*D*), lyso-PC (*E*), DMPE-mPEG550 (*F*), 2-MO (*G*), oleamide (*H*), and cholesterol (*I*) and monoolein at 60% (w/w) water determined by small-angle x-ray diffraction. Measurements were made at 20°C (*solid symbols*) and in the cooling direction at 4°C (*open symbols*) where undercooling is expressed (Qiu and Caffrey, 2000). The identity of each of the phases is as follows: ■, L_α (d_{001}); ●, cubic-Pn3m (d_{100}); ▲, cubic-Im3m (d_{100}); ◀, H_I (d_{10}), ◆, H_{II} (d_{10}); ⬟, cholesterol monohydrate; ★, Lc-oleamide. The lattice parameter values reported are accurate to ±0.2 Å for the Lc, ±0.5 Å for the L_α and H_{II} phases, ±2 Å for the cubic-Pn3m and cubic-Im3m phases, and ±5 Å for the highly swollen cubic-Pn3m (with lattice parameter $>$ 140 Å) and cubic-Im3m phases (with lattice parameter $>$ 180 Å). Lipid concentration is expressed as mol % and is calculated as [100 (moles of lipid)/(moles of lipid + moles of monoolein)].

nova and Caffrey, 1994). In isolation, it exists in the H_{II} phase under conditions of full hydration at 4°C and 20°C (Fig. 5 *B*). The cubic-Pn3m phase of hydrated monoolein was found to tolerate DOPE to the extent of ~20 mol %.

Above this cutoff, the phase state of pure DOPE, namely the H_{II} phase, prevailed.

The lattice size of the cubic phase contracted significantly upon the addition of DOPE (Fig. 5 *B*). The value reached at maximum DOPE carrying capacity for the cubic phase was ~96 Å, down by ~10 Å compared with the reference state for pure monoolein. In the H_{II} phase, the lattice parameter was relatively insensitive to DOPE additions in the 22–38 mol % range and then rose slowly with DOPE concentration in the range from 44 to 80 mol % at 20°C. This behavior has been reported previously (Templer et al., 1992). The H_{II} phase of fully hydrated DOPE in isolation had a lattice parameter (d_{10}) of 65.7 Å. The effect of temperature on lattice parameter was consistent for both phases in that the lower temperature of 4°C favored a slightly larger size.

The cubic-to-H_{II} phase transition was sharp and well defined at 20°C (Fig. 5 *B*). However, at 4°C the two phases coexisted at a composition of ~23 mol %. The lattice parameter of the cubic phase in the coexistence region was unexpectedly large as was that of the H_{II} phase. It is possible that this reflects the fact that the samples were incubated for just 4 h after cooling to 4°C from the 20°C measurement and that the sample had not fully equilibrated. This issue was not examined further in this study. The existence of similarly highly swollen cubic phases in fully hydrated mixtures of monoolein/DOPC/DOPE has been reported (Templer et al., 1992).

Phosphatidylserine

As with PC and PE, DOPS was the chain variant used (Fig. 3). However, unlike PC and PE, DOPS is a negatively charged lipid. It had a profound effect on the phase state of hydrated monoolein (Fig. 5 *C*). When included at a level of ~0.25 mol %, it triggered a transformation from the cubic-Pn3m to the cubic-Im3m phase (Fig. 1). The cubic-Im3m phase persisted up to ~5 mol % DOPS at which point it reverted to the cubic-Pn3m phase. Further additions of DOPS led to the formation of the lamellar phase at the expense of the cubic-Pn3m phase. The former represents the stable phase for hydrated DOPS (Caffrey, 1987a). Qualitatively, the same phases in the same sequence were observed with increasing levels of DOPS at 4°C and at 20°C.

The lattice parameter of the cubic-Im3m phase responded in a dramatic way to DOPS in that it rose by ~70 Å as its concentration in the mixed lipid system went from 0.3 to 5 mol % at 20°C (Fig. 5 *C*). In the cubic-Pn3m phase that formed above 5 mol % DOPS, the lattice parameter was again ~70 Å larger than that seen in the original cubic-Pn3m phase below 0.25 mol % DOPS. The lamellar repeat of the L_α phase in the DOPS concentration range where the cubic phases were no longer stable dropped with increasing additive concentration to a limiting value (d_{001}) of 98 Å at 20°C. The corresponding value for pure DOPS was 100 Å. The general effect of lowering temperature from 20°C to 4°C was to raise the lattice parameters of all phases fractionally and to shift transition boundaries to slightly higher PS concentrations.

Cardiolipin

Cardiolipin, like DOPS, is an anionic lipid (Fig. 3). Despite the considerable differences in molecular constitution of DOPS and cardiolipin, they exhibited remarkably similar phase behavior in combination with hydrated monoolein at 20°C (Fig. 5 *D*). Thus, we see the same loss and return of the cubic-Pn3m phase in the 0–7 mol % cardiolipin range with the cubic-Im3m phase emerging at intermediate concentrations from 0.2 to 2.8 mol % cardiolipin. Above 9 mol % cardiolipin, the stable phase was of the L_α type.

The microstructure of the phases formed by hydrated monoolein in the presence of increasing amounts of cardiolipin followed the same profile seen with DOPS. The unit cell size of the initial cubic-Pn3m phase grew with added cardiolipin and continued to grow in the cubic-Im3m phase. The unit cell size of the high-cardiolipin-concentration cubic-Pn3m phase was 50–60 Å larger than that of the original seen below 0.2 mol %. With increasing levels of cardiolipin, the lamellar phase emerged and remained as the sole liquid-crystalline phase in pure cardiolipin. The lattice parameter of the lamellar phase dropped to a low value of 82 Å at 17 mol % and then rose slightly to a final value of 93 Å in the pure cardiolipin.

The phase behavior of the monoolein/cardiolipin system at 4°C was not examined.

Lyso-phosphatidylcholine

In this study, we used 1-oleoyl-PC (Fig. 3). Pure lyso-PC, with its relatively large polar headgroup and solitary acyl tail, forms the normal hexagonal or H_I phase (Fig. 1) at relatively high water concentrations (~31–69% (w/w) water (Arvidson et al., 1985)). At even higher dilution, it forms a normal micellar solution (Arvidson et al., 1985). In this study, we found that lyso-PC did not alter the phase behavior of hydrated monoolein at concentrations below 5 mol % where the cubic-Pn3m phase remained stable (Fig. 5 *E*). However, further additions of the lyso lipid induced a conversion to the L_α phase that persisted up to 90 mol % lyso-PC. Our only other datum in this series was for pure lyso-PC, which as noted, existed in the H_I phase.

The unit cell size of the cubic-Pn3m phase rose rapidly with lyso-PC addition (Fig. 5 *E*). At 4.8 mol % lyso-PC, the lattice parameter had increased by more than 30 Å compared with pure monoolein. In contrast, the lattice parameter of the L_α phase was insensitive to lyso-PC concentration in the range from 5 to 90 mol % lyso lipid.

The phase behavior and phase microstructure of the hydrated monoolein/water system were not particularly sensitive to temperature in the range from 4°C to 20°C (Fig. 5 *E*).

DMPE-mPEG550

DMPE-mPEG550 is a synthetic lipid with two 14-carbon acyl chains and a relatively large polyethylene-glycol-containing headgroup (Fig. 3). In isolation, the hydrated lipid forms the normal hexagonal, H_I, phase at 4°C and 20°C (Fig. 5 *F*). When combined with monoolein at 60% (w/w) water, the cubic-Pn3m phase was destabilized and was replaced by the cubic-Im3m phase in the low concentration range from 0.2 to ~3 mol % PEG-lipid at 20°C. At intermediate concentrations, the L_α phase emerged and was replaced by the H_I phase characteristic of the pure synthetic lipid at higher levels of the PEG-lipid (Fig. 5 *F*).

Once formed, the unit cell of the cubic-Im3m phase grew with added DMPE-mPEG550 as evidenced by the rise in lattice parameter (Fig. 5 *F*). In contrast, the lamellar repeat of the L_α phase fell with increasing PEG-lipid concentration. A limiting value of 71 Å was recorded for the lattice constant of the H_I phase in pure DMPE-mPEG550.

The same general phase behavior and phase microstructure changes were observed as a function of DMPE-mPEG550 concentration in hydrated monoolein at 4°C and at 20°C (Fig. 5 *F*).

2-Monoolein

Commercial suppliers provide 1- and 2-monoolein with an isomeric purity that ranges from 97% to 99% and 90% to 92%, respectively, based on ^{1}H- and ^{13}C-NMR analysis (data not shown). These are the materials used in the current study. To minimize acyl chain migration and isomeric equilibration, all materials were stored at the lowest available temperatures commensurate with their form and intended use. Thus, for example, the as-purchased lipids were stored in the dark at −70°C until used for sample preparation. Furthermore, samples were used in diffraction work as soon as possible after they were prepared to the correct composition.

2-Monoolein is an achiral isomer of 1-monoolein (referred to simply as monoolein up to this point in the paper). In isolation (note that this does not signify 100% pure 2-monoolein, as emphasized in the previous paragraph), 2-monoolein formed the cubic-Im3m phase when hydrated to the extent of 60% (w/w) water at 20°C (Fig. 5 *G*). In contrast, 1-monoolein accessed the cubic-Pn3m phase under identical conditions. Interestingly, the latter cubic phase modification remained stable as the relative amounts of 2-monoolein in the mix increased up to a concentration of at least 90 mol %.

As the concentration of 2-monoolein in the mixed lipid system grew, the lattice parameter of the cubic-Pn3m phase rose steadily. Specifically, the unit cell axis length increased by at least 20 Å compared with that observed for 1-monoolein alone and reached a value of 121.2 Å at 90 mol % 2-monoolein and 20°C.

With the exception that the cubic phase lattice parameters were generally higher at the lower temperature, the phase behavior of the 1-monoolein/2-monoolein system was the same at 4°C and 20°C (Fig. 5 *G*).

Oleamide

Oleamide is an amide of oleic acid (Fig. 3). When dispersed in water at 20°C, it remains in the solid lamellar crystalline phase (Fig. 5 *H*). The cubic-Pn3m phase of hydrated monoolein accommodated small amounts of the amide in that it persisted as the sole liquid-crystalline phase up to 16.7 mol % oleamide. Beyond this, the oleamide phase separated as the solid Lc phase coexisting with the cubic-Pn3m phase. A typical diffraction pattern showing phase coexistence is shown in Fig. 4 *G*.

The cubic-Pn3m phase lattice constant dropped by ~10 Å for the first few additions of oleamide (Fig. 5 *H*). Beyond the solubility limit of the hydrated monoolein system for oleamide of 20 mol %, the cubic phase lattice parameter remained fixed at 94 Å. The lamellar repeat of the coexisting Lc phase at 35.4 Å was also invariant in the range of oleamide concentration studied. The corresponding value recorded for pure oleamide dispersed in water was 35.7 Å.

The temperature sensitivity of monoolein phase behavior to oleamide additions was not examined in this study.

Cholesterol

When dispersed in bulk water, cholesterol is stable in the solid Lc state as a crystal monohydrate at temperatures up to 86°C (Loomis et al., 1979). In this study, the cubic-Pn3m phase of hydrated monoolein was found to accommodate relatively large amounts of the steroid (Fig. 5 *I*). Thus, it persisted up to 23 mol % cholesterol at 20°C. Beyond this, the cubic-Im3m phase emerged as the dominant liquid-crystalline phase, which, with further cholesterol additions, existed in equilibrium with crystals of the monohydrate. The solubility of cholesterol in the cubic phase of hydrated monoolein at 20°C is therefore ~28 mol %.

The presence of cholesterol crystals that have separated from the cubic phase was evidenced by a unique set of sharp diffraction powder rings in addition to the cubic phase reflections from the mixed lipid system (Fig. 4 *F*). The diffraction patterns of anhydrous cholesterol and cholesterol monohydrate are quite different and were used for purposes of identification (Brzustowicz et al., 2002; Craven, 1976; Loomis et al., 1979; Shieh et al., 1977). Loomis et al. (1979) showed that anhydrous cholesterol crystals convert to the monohydrate form within 24 h of being dispersed in water.

Because the samples used in this study were equilibrated after preparation for at least 24 h before measurement, the monohydrate form was expected.

Cholesterol caused the unit cell axes length of the cubic-Pn3m phase to grow by 15 Å upon incorporating 23 mol % sterol (Fig. 5 *I*). Beyond this, the unit cell of the cubic-Im3m phase continued to grow in size and appeared to have stabilized at a lattice parameter of 168 Å at 30 mol % cholesterol.

The effect of reducing temperature from 20°C to 4°C was to cause the lattice parameter of the cubic-Pn3m phase to rise slightly, particularly under conditions of low steroid loading. The other effect of cooling was to allow for the emergence of the cubic-Im3m phase at 23 mol % as opposed to 26 mol % cholesterol.

DISCUSSION

The in meso method for crystallizing membrane proteins was developed based on a cubic phase created by the hydrated lipid monoolein (Fig. 3). Despite its molecular simplicity, monoolein exhibits a rich mesomorphism as a function of temperature and hydration (Fig. 2). The in meso method was first described in 1996 (Landau and Rosenbusch, 1996). In the intervening 6-year period, it has been used successfully for high-resolution structure determination of bR (Luecke et al., 1999a; Pebay-Peyroula et al., 1997), halorhodopsin (Kolbe et al., 2000), sensory rhodopsin II (Luecke et al., 2001; Royant et al., 2001), and related mutants or photointermediates (Edman et al., 1999, 2002; Facciotti et al., 2001; Luecke et al., 1999b, 2000; Pebay-Peyroula et al., 2000; Rouhani et al., 2001; Royant et al., 2000; Sass et al., 2000). These are uniformly small, stable, and compact bacterial plasma membrane proteins consisting almost exclusively of transmembrane helices. The obvious question is whether the method is limited to such protein types.

It is entirely possible that the rather spare chemical character provided by monoolein, which creates the lipidic fabric and from which crystals grow, somehow limits the range of membrane proteins yielding to the method. Accordingly, the purpose of the current study was to explore the possibility of procuring a series of cubic phases with a more diverse lipid profile.

In the main, the focus of this study was on the commonly encountered biomembrane lipid types. In a separate investigation, the possibility of fine-tuning the cubic phase based solely on individual or mixtures of monoacylglycerols is being explored (Misquitta and Caffrey, 2001). A total of 10 lipid species were included in the current study. Monoolein served as the reference compound. DOPC, DOPE, DOPS, cardiolipin, and cholesterol served as representative membrane lipid types. The others are special cases, and each contributed a unique insight into phase microstructure and stability. Phase identity and microstructure were monitored continuously by low- and wide-angle x-ray diffraction as each of the nine lipids was added individually to hydrated monoolein.

Lipid additive effects

For purposes of this discussion, we will focus on data collected at 20°C. They are not much different at 4°C. The model for in meso crystallization described in the Introduction indicates that membrane proteins are first reconstituted into the lipid bilayer of the cubic phase and are ferried to the growing crystal face by way of an intermediate lamellar-type conduit. The implication is that anything that perturbs the cubic phase is likely to impact on the progress of crystallization. And it is this that we will focus on in the ensuing discussion. However, because so little is known about the crystallization mechanism in meso, the consequences of a given change in phase identity and/or microstructure for crystal growth and quality cannot be evaluated at this time. In what follows, the effects of the different lipid types on phase behavior were evaluated as much as possible in the context of molecular shape, interfacial curvature, and chain packing energy. Throughout the discussion, the assumption is made that monoolein is fully miscible with each lipid additive in all of the liquid-crystalline phases formed.

Neutral, lamellar-phase-forming lipids

The PCs are common components in many biomembranes. DOPC is a classic, lamellar-phase-forming zwitterionic lipid. In excess water at 20°C, it forms the L_α phase and is tolerated to the extent of ~25 mol % in the cubic-Pn3m phase of hydrated monoolein.

It is interesting to note that the lattice parameter of the cubic phase rises dramatically as PC replaces monoolein in the mixed lipid system. This is interpreted as reflecting the dynamically averaged shape of DOPC that is uniform in cross section along the long axis of the molecule. Close packing of such cylindrically shaped molecules will naturally lead to the planar sheets characteristic of the lipid bilayers that constitute the lamellar phase. Thus, DOPC in the cubic phase has the effect of lowering the degree of curvature at the lipid bilayer/water interface. A flatter interface will cause the cubic lattice to expand if excess water is available to satisfy the enhanced hydration needs of the phase. The hydration boundary for the monoolein/water system at 20°C occurs at ~45% (w/w) water (Fig. 2). Because the samples used in this study were prepared at 60% (w/w) water, excess water was available for imbibition and for limited swelling of the cubic phase. At 25 mol % DOPC, the lattice parameter increased by ~40 Å. Further additions of PC triggered a transition to the lamellar phase. A similar phase sequence has been reported in this system (Gutman et al., 1984; Nilsson et al., 1991; Templer et al., 1992).

Neutral, H_{II}-phase-forming lipid

PE is a typical H_{II}-phase-forming lipid and is a common membrane component. In excess water at 20°C, the PE used in this study, DOPE, forms the H_{II} phase with a lattice parameter, d_{10}, of 66 Å. This reflects the large negative spontaneous curvature associated with DOPE (Tate and Gruner, 1989). To accommodate to the H_{II} phase, the dynamically averaged shape of an individual DOPE molecule is that of a truncated wedge.

The cubic-Pn3m phase, formed by hydrated monoolein, is of the inverted type. The H_{II} phase is of the inverted type also. As DOPE is added to the hydrated monoolein system, the lattice size of the cubic-Pn3m phase drops, to the extent of ~10 Å at 20 mol % DOPE (Fig. 5 *B*). It is not clear to what we can ascribe this reduction because lattice size depends in a complex way on spontaneous curvature and curvature inhomogeneities in the cubic phase. Beyond this, however, the H_{II} phase formed and was stable over the entire composition range from ~20 to 100 mol % PE. With increasing concentration from 20 to 100 mol % DOPE, there was a small but steady rise in the lattice parameter of the H_{II} phase from 57 to 66 Å. A similar result has been described previously (Templer et al., 1992). Assuming that the system remained in equilibrium with excess water over the entire PE concentration range studied, the change must reflect in part an inherently higher spontaneous curvature associated with the monoolein component.

Anionic, lamellar-phase-forming lipids

PS and cardiolipin are negatively charged glycerophospholipids and are common to the biomembrane. PS has two acyl chains whereas cardiolipin has four. Under full hydration conditions at 20°C, both lipids form isolated vesicles reflecting the mutual electrostatic repulsion experienced by adjacent lamellae.

Unlike PC and PE, the DOPS and tetra-oleoyl cardiolipin used in this study were tolerated to a very minor degree in the cubic-Pn3m phase of hydrated monoolein. In both cases, as little as 0.3 mol % anionic lipid was enough to trigger a transition to a cubic phase modification of the Im3m type. However, within the narrow range of concentration in which the cubic-Pn3m phase was stable, its lattice parameter rose with increasing anionic lipid content. Presumably, this reflects a charging up of the continuous bilayer surface and thus a swelling of the unit cell. Once in the cubic-Im3m phase, the swelling was quite dramatic, rising by between ~60 and 70 Å with the addition of 2–4 mol % anionic lipid. Again, the increase likely originated from a charging up of the lipid/water interface and a small spontaneous curvature as reflected in the ability of both lipids to form planar lamellae at higher levels of hydration. Similar effects have been reported in the literature for other anionic lipids including dioleoylphosphatidic acid (Li et al., 2001) and oleic acid (Aota-Nakano et al., 1999).

At concentrations of PS and cardiolipin in the range from 2 to 4 mol %, the cubic phase imbibed all available water. This was evidenced by the fact that a relatively constant lattice parameter was reached with increasing proportions of added lipid in that same concentration range (Fig. 5, *C* and *D*). This coincided with the absence of bulk water in such samples, as noted by visual inspection. Further addition of anionic lipid induced a return to the cubic-Pn3m phase, presumably now under conditions of limited hydration. In both PS and cardiolipin systems, the lattice parameter of the new cubic-Pn3m phase was larger by 60–70 Å than that of the original phase reflecting the elevated hydration level.

At even higher concentrations of PS and cardiolipin (8–9 mol %), the L_α phase formed. The lamellar repeat distances measured had values in the vicinity of 90–100 Å (Fig. 5, *C* and *D*). The limiting d_{001} values observed at high anionic lipid content presumably reflect lamellar phases that have imbibed as much water as they can at this hydration level.

Normal hexagonal (H_I)-phase-forming lipids

Lyso-PC (Fig. 3), although not a common membrane lipid per se, is an important monoacylated intermediate in glycerophospholipid metabolism. It is a powerful detergent as the name lyso implies. In contrast, DMPE-mPEG550 is a synthetic anionic lipid where the PE headgroup has been modified with a hydrophilic polymer, PEG (Fig. 3). It is referred to as a PEG-lipid and, as a group, these modified lipids have found application in targeted drug delivery.

Lyso-PC and the PEG-lipid are discussed together here because they have in common a relatively large, hydrophilic headgroup and a small hydrocarbon region (Fig. 3). This gives them a dynamically averaged wedge shape that favors packing of the hydrated lipid as long, hexagonally arranged rods of lipid with a hydrocarbon core. The bulk phase is of the H_I type (Fig. 1).

Thus, whereas monoolein forms inverted structures, those created by lyso-PC and the PEG-lipid are of the normal type. When combined, the expectation is that an intermediate with a planar interface should be encountered. This is exactly what was seen for both lipids. In the case of lyso-PC, the L_α phase was observed between the inverted cubic phase at high monoolein concentrations (~90 mol % monoolein) and the H_I phase in pure lyso-lipid (Fig. 5 *E*). For the PEG-lipid, the H_I phase came in at even lower added lipid concentrations (3 mol % PEG-lipid) but was destabilized in the presence of ~75 mol % PEG-lipid, which led to H_I phase formation (Fig. 5 *F*).

At low levels of lyso-PC and before the L_α phase was formed, the cubic-Pn3m phase showed some limited ability to accommodate this additive (Fig. 5 *E*). And as expected, the lattice parameter of the cubic phase rose dramatically with a relatively small addition of the lyso-lipid, as was seen with DOPC (compare Fig. 5, *A* and *E*). In fact, the phase behaviors of both lyso-PC and DOPC in the presence of

hydrated monoolein are quite similar, as might be expected. The difference arises from the fact that the former, with its greater tendency to create a curved interface, goes beyond the L_α phase and eventually induces H_I phase formation.

In contrast to lyso-PC, DMPE-mPEG550 is quite potent in its ability to destabilize the cubic-Pn3m phase. Thus, at 0.2 mol % PEG-lipid, the system had transformed into the cubic-Im3m phase whose lattice parameter rose by ~70 Å in going from 0.2 to 2 mol % added lipid (Fig. 5 *F*).

Given the symmetry in the system, one might have expected to see one or several normal cubic phases as intermediates between the L_α and H_I phases in the lyso-PC and PEG-lipid systems. However, none was observed (Fig. 5, *E* and *F*). A careful investigation of the intermediate zone was not conducted because this was not the focus of the current work.

2-Monoolein

One of the reasons for including this lipid in the study has to do with the fact that monoolein has a tendency to isomerize in aqueous dispersions and to produce an equilibrium mix of the 1- and 2-isomers (Fureby et al., 1996; Ljusberg-Wahren et al., 1983). Thus, regardless of the identity of the isomer used at the beginning of a crystallization trial, the system will evolve in time and produce an equilibrium mix represented by 88% 1-monoolein and 12% 2-monoolein at 20°C (Ljusberg-Wahren et al., 1983). To what extent does this dynamic isomerization and attendant change in molecular structure of the hosting lipid affect phase behavior? We set out to answer this by preparing mixtures of the two isomers and making diffraction measurements as soon as possible after sample preparation to minimize the degree of isomerization. It should also be noted that the starting 1- and 2- isomers of monoolein were not 100% pure to begin with, as detailed under Results.

Under conditions of these measurements, the 1-monoolein forms the cubic-Pn3m whereas the 2-isomer forms the cubic-Im3m phase (Fig. 5 *G*). Remarkably, the cubic-Pn3m phase of 1-monoolein accommodated as much as 90 mol % of the 2-isomer. In so doing, the lattice parameter of the cubic-Pn3m phase rose by ~20 Å to a final value of 122 Å at 90 mol %. The 2-isomer has the acyl chain appended to the central carbon of glycerol, which produces an achiral molecule. The two terminal hydroxy methylene groups are arrayed symmetrically on either side and are expected to give the polar headgroup some lateral bulk (Fig. 3). This is in contrast to the 1-isomer where the glycerol carbons and those of the acyl chain could conceivably be colinear as drawn in Fig. 3. The expectation then is that the 2-isomer will tend to flatten out the lipid/water interface. This is consistent with the swelling of the cubic-Pn3m phase with increasing proportions of the 2-monoolein. The formation of the cubic-Im3m phase in pure 2-monoolein is perhaps also consistent with this line of reasoning given that the latter phase is associated with the more fully hydrated cubics (Caffrey, 1987b; Lindblom and Rilfors, 1989; Selstam et al., 1990).

As far as in meso crystallization is concerned, these results indicate that if we start out predominantly with the 1-isomer, as the standard protocol presumably calls for, then the 1-/2-isomerization will not trigger a conversion out of the cubic-Pn3m phase. However, the lattice parameter of the phase will change by a small amount (Fig. 5 *G*). The results also show that fine-tuning of the cubic-Pn3m phase lattice parameter over reasonably wide limits (115 ± 10 Å), while holding the chemistry of the lipid/water interface relatively constant, is possible by adjusting the 1-/2-isomer ratio.

One consequence to be mindful of as a result of chain isomerization is the attendant racemization in the subsequently formed 1-monoolein. Passing through the achiral 2-monoolein intermediate means that both the S and the R forms of the 1-monomer will be generated equally in the equilibration process. We have not evaluated the effect of enantiomeric purity on the phase properties of hydrated monoolein or on the in meso crystallization process. However, it is important to note that a crystallization trial initiated with enantiomerically pure monoolein will eventually end up as a mixture that includes the 2-isomer and racemic monoolein.

Phase-separating lipids: cholesterol and oleamide

Cholesterol is a very commonly encountered steroid in biomembranes, whereas oleamide is not. However, the latter is a potent signal transduction lipid with a claim to fame as a sleep inducer that interacts with gap junctions (Boger et al., 1998). The only reason for including these two, quite dissimilar molecules in the one section is that they share the ability to phase separate as a crystalline solid from the cubic phase of hydrated monoolein above a solubility limit of 20–28 mol % added lipid. Each will be discussed separately below.

We can attempt to rationalize the effect of oleamide in reducing the lattice parameter of the cubic-Pn3m phase in the 0–20-mol % concentration range as follows. The amide headgroup of oleamide is polar, with a strong tendency to hydrogen bond. Extensive hydration would be expected to increase the effective headgroup size and to produce a molecule with a shape similar to that of lyso-PC. However, the latter lyso-lipid causes the cubic-Pn3m phase to swell (Fig. 5 *E*), in contrast to the contraction seen with oleamide (Fig. 5 *H*). Thus, it may be that by virtue of being confined to the lipid bilayer of the cubic phase, the oleamide headgroup chooses instead to hydrogen bond to the adjacent hydroxyls of the hosting monoolein. The net effect may be to cause the interface to contract as it becomes more highly curved. In turn, this leads to a smaller unit cell. Beyond a certain concentration, added oleamide can no longer be accommodated in the bilayer, and it simply separates out as a crystal. The cutoff occurs at ~20 mol % oleamide where,

on average, each oleamide is surrounded by four molecules of monoolein.

A cursory inspection of a space-filling model of cholesterol (molecular structure shown in Fig. 3) suggests a dynamically averaged wedge shape similar to DOPE with a relatively small polar end. Combining such a molecule with hydrated monoolein in the cubic-Pn3m phase would be expected to cause the lattice parameter to fall, as observed with PE in Fig. 5 *B*. However, the opposite effect was seen with cholesterol (Fig. 5 I). This result calls for an alternative explanation, as follows. Cholesterol, with its rigid cyclic nucleus, restricts *trans*/*gauche* isomerization along (at least part of) the chain of the hosting lipid, monoolein. In turn, this leads to a combined dynamically averaged molecular shape that is less wedge shaped, allowing for a reduced curvature at the polar/apolar interface and an increase in the unit cell size.

As noted, the cubic-Im3m phase is associated with elevated levels of hydration. The emergence of this cubic phase modification at high concentrations of cholesterol where the cubic-Pn3m phase is already swollen is therefore not unexpected.

The crystals of sterol that form above the solubility limit of ~28 mol % (Fig. 5 *I*) were of the monohydrate form. Evidence in support of this came from the powder x-ray diffraction pattern of the corresponding monohydrate, which is recognizably different from the anhydrous sterol (Fig. 4 *F*) (Craven, 1976; Shieh et al., 1977). A similar value for cholesterol solubility in the cubic phase of hydrated monoolein has been reported (Larsson et al., 1978; Lindblom et al., 1979). Cholesterol adopts a layered arrangement in the crystal analogous to its orientation in the lamellae formed when combined with phospholipids. It would appear therefore that cholesterol has strong lamellar-phase-forming tendencies. Accordingly, it has neither the shape nor the flexibility needed to stabilize the highly curved interfaces that are integral to the cubic mesophases. Thus, beyond a certain concentration limit, cholesterol cannot support cubic phase formation and chooses instead to crystallize.

Effect of temperature

With the exception of cardiolipin and oleamide, the effect of temperature on the phase behavior of the hydrated lipid/monoolein mixtures was examined by making measurements at 4°C and 20°C. A perusal of the classic text on crystallizing proteins (McPherson, 1999) indicates that crystallization trials are commonly performed at these temperatures.

In all of the systems examined at 4°C in this study, undercooling (Qiu and Caffrey, 2000) was observed in that a conversion to the solid Lc phase did not occur, at least on the time scale of the experiment (Fig. 5). Furthermore, the effect of temperature on phase identity and microstructure was minimal. In some cases, phase sensitivity to lipid composition changed by a small amount upon cooling to 4°C (Fig. 5 *I*). In others, the lattice parameters either remained unchanged or rose slightly with a decrease in temperature. Such an effect is expected behavior for single liquid-crystalline phases (Briggs et al., 1996; Luzzati, 1968; Shipley, 1973). The situation is a little more complicated when the adjustment is made in excess water where the position of the hydration boundary can change with temperature (see Fig. 2, for example).

Lattice parameter ratio for the different cubics across all lipid systems and temperatures

The hydrated monoolein system exhibits two cubic phases (Fig. 2). The cubic-Ia3d (also known as the gyroid or G-type) with a space group designation Q^{230} occurs at low water contents. The phase in equilibrium with excess water is designated cubic-Pn3m (diamond, D-type, Q^{224}). A third cubic phase has been seen in the monoolein/water system but with little regularity (Caffrey, 1987b; Lindblom and Rilfors, 1989). It is associated with the most hydrated state of the lipid and is of the cubic-Im3m phase type (primitive, P-type, Q^{229}).

The bicontinuous nature of the latter phases has been described mathematically in terms of infinite periodic minimal surfaces (IPMSs) of types G, D, and P (Hyde et al., 1984). A curved surface with a mean curvature that is everywhere zero is a minimal surface. The IPMSs are intersection-free surfaces of this type that are periodic in three dimensions. They are mutually accessible by way of the Bonnet transformation that involves surface bending without a change in the Gaussian curvature. Accordingly, the relative lattice constants of the three phases are related as follows: a^{Q229}/a^{Q224} is 1.28 and a^{Q230}/a^{Q224} is 1.57 (Hyde et al., 1984).

The bicontinuous cubic phases can be viewed as arising by coating an IPMS with a continuous lipid bilayer where the chain methyl termini touch the minimal surface. Each lipid molecule in turn projects away from the surface with its long axis perpendicular to that surface. Interpenetrating but noncontacting aqueous channels are on either side of the contorted bilayer and fill the rest of the space (Fig. 1). The lattice constants for the transforming cubic phases observed in this study are assembled in Table 1. The agreement with the theoretical lattice constants ratios lends credence to the phase designations.

Implications for in meso crystallization

The in meso method has been described as requiring the protein to become associated with the lipid component of a bicontinuous phase. The latter takes the form of a solitary bilayer that represents an uninterrupted, three-dimensional reservoir in which the protein can move. The reservoir is

TABLE 1 Lattice parameter ratios for the different cubic phases formed by hydrated monoolein in combination with an assortment of membrane and other lipid types at 4°C and 20°C

Identity of added lipid	Concentration of added lipid (mol %)*	Temperature (°C)	Relevant cubic phases	Lattice constants (Å)†	Lattice constant ratio
DOPS	0.25	4	Pn3m/Im3m	111/142.5	1.28 ± 0.02
DOPS	0.25	20	Pn3m/Im3m	110.8/140.1	1.26 ± 0.02
DOPS	8	4	Im3m/Pn3m	225/184	1.22 ± 0.08
DOPS	5	20	Im3m/Pn3m	218/178	1.22 ± 0.08
Cardiolipin	0.2	20	Pn3m/Im3m	111/144	1.30 ± 0.03
Cardiolipin	3.0	20	Im3m/Pn3m	197/160	1.23 ± 0.08
PEG-lipid	0.1	4	Pn3m/Im3m	106.2/136.6	1.29 ± 0.02
	0.1	20	Pn3m/Im3m	107.7/143.5	1.33 ± 0.06
Cholesterol	23.1	4	Pn3m/Im3m	121.7/157	1.29 ± 0.02
	25.9	20	Pn3m/Im3m	122/157	1.29 ± 0.02

*The concentration of added lipid is the value used in calculating the lattice constants of the coexisting cubic phases.
†The lattice constants were obtained based on best-fit extrapolations of lattice constants recorded in regions of pure cubic phase stability into regions of cubic phase coexistence. The estimated concentration mid-point for phase coexistence was used for lattice constant ratio calculation. The original data upon which the estimates were made are shown in Fig. 5.

contiguous with a lamellar portal that conducts proteins reversibly between the bulk reservoir and the growing crystal face.

As noted, the bulk medium that gives rise to crystals, in the case of bR at least, would appear to be the cubic-Pn3m phase. We have no knowledge at this point as to the suitability or otherwise of the other bicontinuous cubic phases, or indeed other mesophases, to serve in this capacity. This represents work in progress. For purposes of the current discussion, however, we will focus on the cubic-Pn3m phase and assume that it is integral to the crystallization process.

The results presented in Fig. 5 tell us that of the nine lipids studied, all can be accommodated to some degree in the cubic-Pn3m phase. In the interests of space, the discussion that follows will be limited to the results obtained at 20°C. The three anionic lipids, DOPS, cardiolipin, and the PEG-lipid, were tolerated least of all by the cubic-Pn3m phase of hydrated monoolein. In all cases, the original cubic phase was lost as added lipid reached a level of 0.3 mol %. DOPS and cardiolipin exhibited behavior where the cubic-Pn3m phase reappeared in a narrow concentration range centered at ~5 mol % between the cubic-Im3m and the L_α phases. Lyso-PC was next in terms of amount tolerated in the cubic phase where the limit fell between 5 and 9 mol %. All of the other lipids were included in the cubic-Pn3m phase to the extent of 20 mol % or higher. DOPE and DOPC had limits of 20 and 25 mol %, respectively, whereas 2-monoolein did not destabilize the phase until its concentration rose above 90 mol %. Oleamide and cholesterol had solubilities of 20 and 28 mol %, respectively. Beyond these limits, crystals formed that coexisted with the cubic-Pn3m phase in the case of oleamide and the cubic-Im3m phase in the case of cholesterol. This might be considered as a simple model for in meso crystallization.

A summary of the effects just described is presented in Table 2. The phase sequence observed is consistent with the steady rise in interfacial curvature found in oil/water/detergent mixtures as follows: inverted micelles → inverted hexagonal → inverted cubic → lamellar → normal cubic → normal hexagonal → normal micelles. Although no evidence for a normal cubic phase was obtained for any of the additives examined in this study, the lyso-PC and PEG-lipid systems are candidates for the appearance of such an intermediate. The micellar solutions were not encountered either, because these are associated with extremes in concentration, conditions that were not examined in this study.

The lipid additives affected the lattice constant of the cubic-Pn3m phase to varying degrees and in different directions. DOPE and oleamide both caused the lattice to contract. The rest induced the unit cell to expand to various degrees. The largest increase was seen with DOPC. It is important to note that the lattice constant has two components, both of which can change with lipid composition. One of the components is the lipid bilayer thickness. The other is the water channel diameter. Both quantities can be measured, but with some effort (Briggs et al., 1996). This was not undertaken in the current study.

The information just described and contained in Fig. 5 can be used in designing crystallization trials where the lipid profile of the hosting lipid is to be adjusted. The data provide a quantitative measure of the limits to which different lipid types can be added to the monoolein cubic phase and of how its microstructure responds. The scope of the current study was restricted to a few disparate lipid types, but the conclusions drawn and behavior trends identified can be extended within limits to related lipid species. Thus, if the lipid in question is lamellar phase forming, then it is likely to follow the behavior exhibited by DOPC. An inverted hexagonal-phase-forming lipid will more than likely behave like DOPE. The behavior of lyso-PC and the PEG-lipid should be representative of normal hexagonal-phase-forming lipids, and so on.

It is important to note that the measurements were made with relatively simple systems consisting of water and a pair of lipids. In the actual crystallization trials, the system is considerably more complex and may include native membrane lipids, detergents, salts, and precipitants in addition to the protein(s) of interest. All of these components have the potential of affecting phase behavior (Cherezov et al., 2001). The results presented above must be evaluated with this cautionary note in mind.

CONCLUSIONS

The working model for membrane protein crystallization in meso has the protein moving from within a bicontinuous mesophase through a lamellar conduit to the crystal face. The original method calls for a lipidic mesophase composed solely of monoolein. With a view to making the method applicable to a wider range of membrane proteins, the sense was that more variety at the level of the lipid component would prove beneficial. Thus, the goal of the current study was to evaluate a number of lipid types for their compatibility with the cubic-Pn3m phase of hydrated monoolein, the phase that presumably hosts the protein before crystallization. This was approached by using x-ray diffraction to identify the phases formed and to characterize them structurally as different lipid types were combined with hydrated monoolein, initially in the cubic-Pn3m phase. The lipids used included DOPE, DOPC, DOPS, cardiolipin, lyso-PC, DMPE-mPEG550, 2-monoolein, oleamide, and cholesterol. Measurements were made at 4°C and 20°C. The work leads to the following conclusions.

All of the lipids examined were accommodated in the cubic-Pn3m phase of monoolein to some degree without altering phase identity. As might be expected, the positional isomer, 2-monoolein, was tolerated to the highest level. The least well tolerated were the anionic lipids, DOPS and cardiolipin, and the PEG-lipid, followed by lyso-PC. The rest were accommodated to the extent of 20–25 mol %.

In the cubic-Pn3m phase, most of the lipid additives brought about an increase in lattice constant. The exceptions were DOPE and oleamide, which effected a drop in unit cell size.

Beyond a certain concentration limit, the lipid additives either triggered one or a series of phase transitions or saturated the phase and separated out as a crystalline solid.

The sequence of phases induced by the assorted lipid additives used in this study is consistent with expectations in terms of interfacial curvature changes as follows: inverted hexagonal, inverted cubic, lamellar, and normal hexagonal. The normal cubic phase, which sits between the lamellar and normal hexagonal phases, was not observed in this study. However, it was not looked for carefully either.

The changes in type and microstructure of the phases formed by hydrated monoolein when combined with the different lipid additives have been rationalized on the basis of lipid molecular shape, interfacial curvature, and chain-packing energy.

TABLE 2 Effect of lipid additives on the phase properties of hydrated monoolein at 20°C

Lipid additive*	Phase sequence induced by lipid additive†	Phase sequence induced by lipid additive arranged by interfacial curvature‡
DOPE	$P \rightarrow H_{II}$	H_{II} **P**
DOPC	$P \rightarrow L$	**P** L
DOPS	$P \rightarrow I \rightarrow P \rightarrow L$	**P** I P L
Cardiolipin	$P \rightarrow I \rightarrow P \rightarrow L$	**P** I P L
Lyso-PC	$P \rightarrow L \rightarrow H_I$	**P** L H_I
DMPE-mPEG550	$P \rightarrow I \rightarrow L \rightarrow H_I$	**P** I L H_I
2-MO	$P \rightarrow I$	**P** I
Oleamide	$P \rightarrow (P + Lc)$	(**P**+Lc)
Cholesterol	$P \rightarrow I \rightarrow (I+Lc)$	**P** I (I+Lc)

All samples were prepared with 60% (w/w) water and 40% (w/w) total lipid. The reference phase is of the cubic-Pn3m type.

*Oleamide and cholesterol have been placed in a category of their own because, unlike the other lipid additives, above a certain concentration limit they are no longer soluble in the lipid mix and separate out as crystals.

†Phase designation key is as follows: P, cubic-Pn3m; L, L_α; I, cubic-Im3m; H_I, normal hexagonal; H_{II}, inverted hexagonal; Lc, lamellar crystal.

‡Phases observed are arranged from left to right in order of increasing interfacial curvature. Thus, the inverted and normal hexagonal phases occupy the left and right extremities in the column of phases. The original cubic-Pn3m phase is emboldened to distinguish it from the modification that appears at higher DOPS and cardiolipin concentrations.

The quantitative data collected regarding phase identity and microstructure characteristics for the mixed lipid systems studied can be used as a guide for designing cubic matrices composed of different lipid types. In turn, these will hopefully allow for the stable reconstitution and ultimate crystallization of different membrane proteins having disparate needs in terms of the character of the supporting lipid membrane. In contrast, the microstructure of the cubic-Pn3m phase can be fine-tuned over a wide range by adjusting the proportions of 1- and 2-monoolein without dramatically altering the chemical composition of the membrane.

The systems studied are simple models consisting of hydrated binary lipid mixtures. Under real crystallization conditions, potential phase perturbants, such as proteins, native membrane lipids, detergents, and precipitants, will be present. The results should be interpreted with this in mind.

The observed effects of lipid additives on phase identity and microstructure were relatively insensitive to temperature in the range studied.

The quantitative data collected regarding phase identity and microstructure characteristics for the mixed lipid systems studied should also prove useful in studies of how protein activity is influenced by membrane stress. The range of lipid systems studied and the variety of effects seen should facilitate quantitative interpretation of how specific lipid combinations alter the stress profile of the membrane and thus protein activity in reconstituted systems.

Although the current study was limited in scope to combinations of single lipid species with hydrated monoolein, it

is interesting to speculate as to the outcome of using several lipid additives in combination. For example, it might be possible to procure a cubic-Pn3m phase consisting of 10 mol % DOPC, 10 mol % DOPE, and 80 mol % monoolein (in the usual ratio of 40% (w/w) total lipid and 60% (w/w) water) with a lattice constant similar to that of the unadulterated, or reference, monoolein system (106 Å at 20°C). DOPC and DOPE have opposite effects of approximately equal magnitude on the microstructure of the cubic-Pn3m phase (Fig. 5, *A* and *B*). In a 1/1 combination, their individual effects may cancel. Such a mixture may optimally suit certain membrane proteins that demand lipid type heterogeneity in the hosting mesophase and a cubic-Pn3m phase of defined microstructure for reconstitution and subsequent crystallization.

Data deposition

Relevant data reported in this paper has been deposited in the Lipid Data Bank (http://www.lipidat.chemistry.ohio-state.edu/mo-lipids/mo-lipids.pdf).

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