Membrane Protein Crystallization in Lipidic Mesophases: Detergent Effects

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ABSTRACT The "cubic phase method" for growing crystals of membrane proteins uses a complex mixture of water, lipid, protein, and other components. The current view is that the cubic phase is integral to the process. Thus additives from whatever source introduce the possibility of destabilizing the phase, thereby compromising the crystallization process. Detergents are used to solubilize membrane proteins and are likely to be ported into the cubic medium with the target protein. Depending on the identity and concentration of the detergent, the cubic phase, which itself is membranous, may be solubilized or destabilized in such a way as to render it unsuitable as a crystal growing system. The nonionic detergent *n*-dodecyl-β-D-maltopyranoside is commonly used in membrane protein work. In this study, we evaluate its effect on the cubic mesophase of hydrated monoolein. X-ray diffraction was used for phase identification and mesophase microstructure characterization. The results show that while low levels of the detergent are tolerated, increasing concentrations trigger a cubic-to-lamellar phase transition in a temperature-dependent manner. This finding is rationalized in the context of complementary molecular shapes of the lipid and the detergent and has implications for the mechanism of crystallization in lipidic mesophases as discussed.

INTRODUCTION

The medium-chain-length alkyl glycosides are high-solubility, nonionic detergents (Warr et al., 1986). Because of their mild nature, they have found extensive use in the solubilization of membrane proteins for subsequent structure characterization, reconstitution, and crystallization studies. The alkyl glycoside dodecyl maltoside (DM) has been used in such applications and is currently being evaluated for its impact on membrane protein crystallization using lipidic mesophases, hereafter referred to as the *in meso* method (Caffrey, 2000). The latter makes use of a multicomponent system in which hydrated monoolein, as a cubic mesophase, figures prominently (Rummel et al., 1998). While the exact role of the cubic phase in the crystallization process is still a mystery, it exists as the major phase before and subsequent to protein crystal formation. Thus it would appear that it is an essential feature of the method. Accordingly, adventitious materials introduced into the system along with the protein or from whatever source have the potential of destabilizing the host cubic phase. Given the assumption that the cubic phase serves an essential role in the process, such a destabilizing effect might be considered detrimental to the overall objective. By the same token, because we do not understand the very mechanism of *in meso* crystallization, it is possible that such additives might well facilitate the process (Caffrey, 2000).

The purpose of the current study is to evaluate the effect that DM has on the phase behavior and microstructure of hydrated monoolein under conditions similar to those used in a typical *in meso* crystallization trial. This takes the form of monoolein dispersed with 40% (w/w) water, which exists in the cubic phase at 20°C (Hyde et al., 1984; Briggs et al., 1996; Qiu and Caffrey, 2000). The detergent would normally accompany the protein in the *in meso* crystallization mix because it is a common membrane-solubilizing agent. The actual concentration of DM that ends up in the mixture depends on the protein and the particulars of the purification process. Accordingly, in this study we examine the effect of DM over a range of concentrations.

The original *in meso* method was developed with bacteriorhodopsin as the test membrane protein. Crystallization was performed at 20°C in a 3:2 (w/w) mixture of monoolein and aqueous medium. With reference to the temperature-composition phase diagram of the monoolein/water system, the phase expected to form under such conditions of hydration and temperature is of the cubic type (Fig. 1; Qiu and Caffrey, 2000). In the case of other membrane proteins, a temperature other than 20°C may be preferred. Accordingly, we have examined phase behavior above and below 20°C in the range from 0°C to 40°C in this study.

The equilibrium phase diagram of the monoolein/water system shows a solid (as opposed to a liquid) crystal phase, prevailing at temperatures below 17°C (Fig. 1). The former is referred to as the lamellar crystal or L_c phase. It consists of lipid bilayer sheets stacked one atop the other with rigid chains oriented normal or tilted with respect to the bilayer plane. The L_c phase is most unlikely to support reconstitution and crystallization of membrane proteins, and at this point it is considered undesirable as far as *in meso* crystallization is concerned. The question arises, then, of how to

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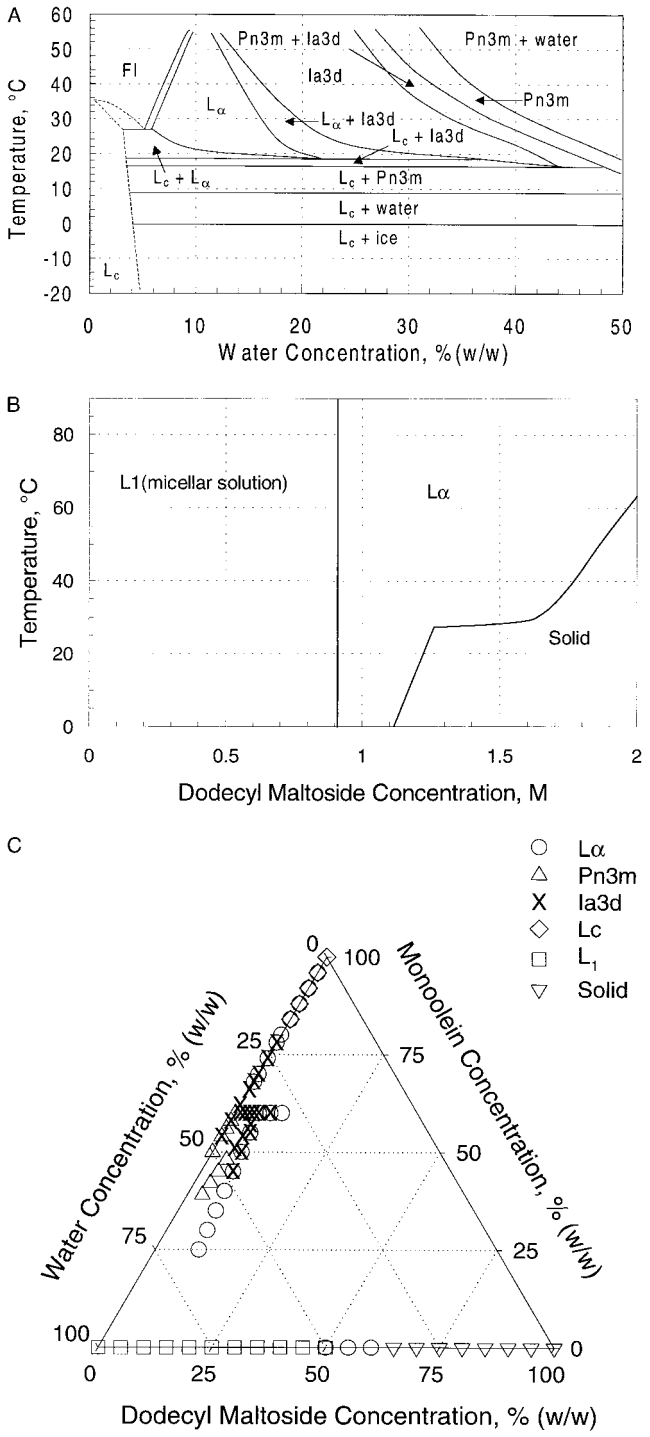

FIGURE 1 Phase behavior of monoolein, dodecyl maltoside, and water in various combinations: temperature-composition phase diagrams of the monoolein/water (*A*, redrawn from Qiu and Caffrey, 2000) and dodecyl maltoside/water systems (*B*, redrawn from Warr et al., 1986). The isothermal phase diagram for the monoolein/dodecyl maltoside/water system at 20°C is shown in *C*. It was constructed based on the data in Figs. 1, *A* and *B*, 2, and 4.

access the pure cubic phase below 17°C if a coexisting solid L_c phase represents the equilibrium state for the system. The answer lies in the ability of liquid crystal phases to undercool in the same way that water remains liquid when cooled appropriately to below 0°C. Indeed, it has been shown that special care must be taken to ensure the expression of equilibrium behavior in lipidic systems and that without it, metastability or undercooling prevails (Qiu and Caffrey, 2000). In the current study, the response of the monoolein/water system to increasing concentrations of DM was examined under both equilibrium and metastable conditions.

In what follows, we show how DM modifies the phase behavior of the monoolein/water system over a range of temperatures under equilibrium and metastable conditions. The phases formed are identified and characterized structurally by means of low- and wide-angle x-ray diffraction. At sufficiently high concentrations, DM completely destabilizes the cubic phase and triggers formation of the lamellar liquid crystal (Lα) phase. This effect is rationalized in the context of the detergent and the lipid as amphiphiles having complementary molecular shapes.

MATERIALS AND METHODS

Monoolein (356.54 g/mol) was purchased from Nu Chek Prep (Elysian, MN) with better than 99% purity, as determined by thin-layer chromatography (Qiu and Caffrey, 1998) and was used without further purification. *n*-Dodecyl-β-D-maltopyranoside (510.6 g/mol) was from Anatrace (Maumee, OH). It had a reported purity in excess of 99%, as determined by high-performance liquid chromatography, and was used without further purification. Dextrose (glucose) and D-(+)-maltose were from Fisher Scientific (Pittsburgh, PA). Ultrapure water was obtained from a Milli-Q Water System consisting of a carbon filter cartridge, two ion exchange filter cartridges, and an organic removal cartridge.

Sample preparation

Dry solid monoolein (~20 mg) was mechanically mixed with appropriate amounts of water or DM solution (~13 mg) in a syringe-based mixing device as described (Cheng et al., 1998) to achieve the desired sample composition. The preparations were made at room temperature (~20°C). For most of the samples, the aqueous component represented 40% (w/w) of the sample. The remainder of the sample consisted of monoolein. The DM solutions used covered the range from 0 to 0.5 M, corresponding to a final DM concentration in the overall mixture ranging from 0 to 0.2 M. The homogeneously mixed samples were transferred to 1-mm diameter quartz capillaries (Charles Supper, Natick, MA), flame-sealed and glued with 5-min epoxy (Hardman, Belleville, NJ), and were stored before data collection at either 4°C (equilibrium measurements) or at room temperature (metastable measurements) for anywhere from one to several days before use in x-ray diffraction measurements. The actual water content of the samples was determined gravimetrically with a microbalance (M3P-000V001; Sartorius Corp., Edgewood, NY) (Cheng et al., 1998).

In a separate study, samples were prepared as above with a weight ratio of DM (0.25 M aqueous solution) to monoolein from 2:3 to 3:1. In another study, samples were prepared in which the DM/monoolein weight ratio was fixed at 1:12 (corresponding to a sample with 60% (w/w) monoolein and 40% (w/w) 0.25 M DM solution), while the water content of the sample was increased from 35% to 58% (w/w).

X-ray diffraction

Copper K_α x-rays (1.5418 Å, nickel (0.025 mm thick) filtered) for use in diffraction measurements were produced using a two-beam port rotating anode x-ray generator (18 kW, RU-300; Rigaku U.S.A., Danvers, MA) as described (Qiu and Caffrey, 2000). The sample-to-detector distance (~250 mm) was calculated using silver behenate (d_{001} = 58.376 Å; Blanton et al., 1995).

Equilibrium phase x-ray diffraction patterns were collected in the temperature range from −15°C to 40°C in increments of 5°C in the heating direction. Up to eight samples were accommodated at one time in the temperature-controlled beryllium sample holder. Eight diffraction patterns (covering a real space range from ~2 Å to 160 Å at a sample-to-detector distance of 200 to 250 mm) were collected side by side behind a 25.4-mm-wide lead slit on a 200 mm × 250 mm image plate (Fuji HR-IIIN; Fuji Medical Systems, USA, Stamford, CT). Before diffraction measurements were taken, samples were incubated at −15°C for at least 2 h to fully develop the equilibrium L_c phase. This is what we refer to as the standard "subzero degree" (Celsius) incubation. Subsequently, the temperature was increased to and samples were incubated at a particular measurement temperature for 5–10 h before the 30-min x-ray exposure was made.

The metastable phase diffraction measurements were performed in the temperature range from 0°C to 40°C. In this case, samples were divided into two sets. The first set was incubated initially at 20°C for 5 h, followed by a 30-min exposure. The temperature was then dropped to 0°C in steps of 5°C with a 5–10-h incubation at each intermediate temperature, followed by the 30-min exposure. The second set of samples was also incubated at 20°C for 5 h, followed by a 30-min data collection period. Subsequently, the temperature was increased to 40°C in increments of 5°C, with the same thermal equilibration and data collection protocols as above.

All other measurements were performed at 20°C with a preincubation period of 5 h and a 30-min exposure as above.

Image analysis

A phosphorimage scanner (Storm 840; Molecular Dynamics, Sunnyvale, CA) operating at a resolution of 100 μm/pixel and a dynamic range of 10^5 was used to read images recorded on the image plates. The radial integration of diffracted intensity was performed on all images with the FIT2D program (Hammersley, 1997), the output of which was intensity versus scattering angle plots for each frame. Diffraction peaks were fitted by Gaussians, using the FIT2D and PEAKFIT programs (Jandel Scientific).

RESULTS

Temperature-composition phase diagrams

Two types of phase diagrams were constructed in the course of this study. The first represents equilibrium behavior, while the second incorporates metastability and the natural tendency of liquid crystal phases to undercool.

Equilibrium behavior

The equilibrium phase diagram for the three-component system consisting of monoolein, DM, and water constructed in the range from −15°C to 40°C is shown in Fig. 2 *A*. In reality, this is a partial phase diagram wherein the concentration of monoolein is held constant at 60% (w/w), while the two other components are varied one against the other in the remaining 40% (w/w) of the sample. For purposes of mapping out the phase diagram, an aqueous solution was used in which the final DM concentration was increased from 0 to 0.2 M. To ensure equilibrium conditions, i.e., to set the system in the equilibrium L_c phase, all samples were

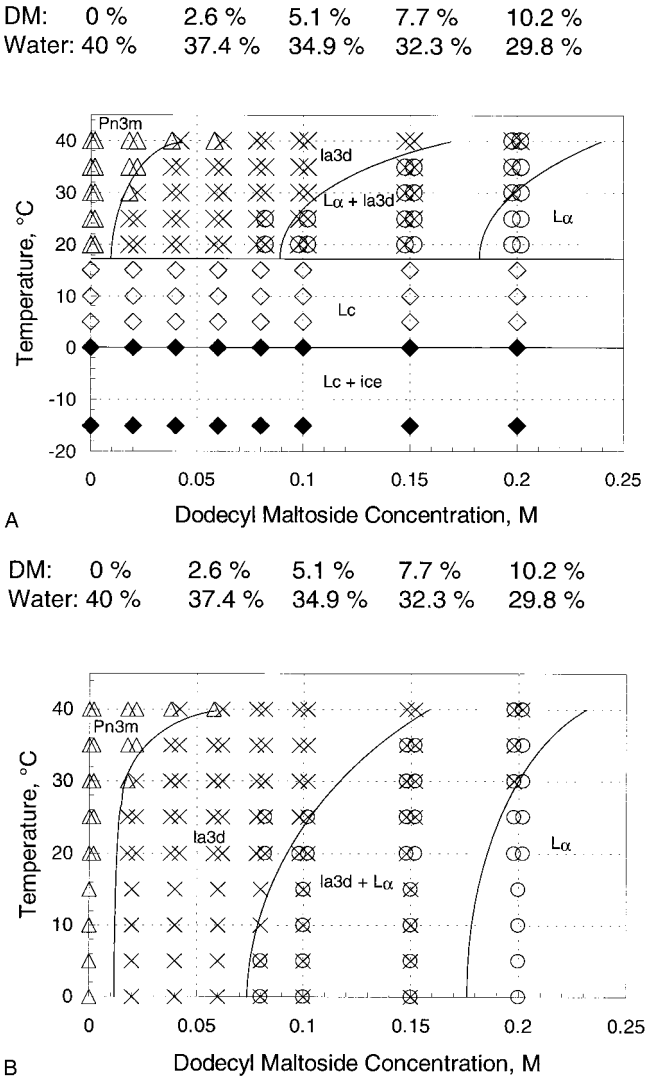

FIGURE 2 Identity and location in temperature-composition space of each phase and coexisting phases in the monoolein/dodecyl maltoside/water system as determined by x-ray diffraction. Samples were prepared with 60% (w/w) monoolein and a 40% (w/w) aqueous solution of dodecyl maltoside. The molar concentration of DM on the lower abscissa as well as the DM and water % (w/w) concentration on the upper abscissa represent the final concentration in the overall mixture (lipid + water + DM). The diffraction measurements were made in the heating direction from −15°C to 40°C for the equilibrium phase diagram (*A*) and in the heating direction from 20°C to 40°C and in the cooling direction from 20°C to 0°C for the metastable phase diagram (*B*). The exposure time was 30 min, and sample incubation at each temperature was for a minimum of 5 h. The solid lines represent phase boundaries and are drawn to guide the eye. Phases: ◇, L_c; ○, Lα; △, cubic-Pn3m; ×, cubic-Ia3d. The solid symbols indicate the presence of ice. The high-temperature part of the phase diagram was repeated once, and the data points for the two sets of measurements are offset from one another slightly on the abscissa for clarity.

preincubated at −15°C for at least 2 h before data collection. The L_c phase was identified by its characteristic diffraction pattern (Qiu and Caffrey, 2000). It consists of a series of equally spaced powder rings in the low-angle region and a group of sharp wide-angle reflections.

The equilibrium phase diagram is dominated by the L_c phase at temperatures below 20°C, regardless of DM concentration (Fig. 2 *A*). Furthermore, the lamellar repeat of the L_c phase is relatively insensitive to added DM in that it holds steady at ~49.5 Å in the range studied (Fig. 3 *B*, Appendix A). At and above 20°C, the solid L_c phase no longer exists. It is in this region that the assorted liquid crystal phases emerge, the identity and characteristics of which depend on DM concentration and temperature. Thus, in the absence of detergent, the cubic-Pn3m phase exists in what amounts to a sample consisting of 60% (w/w) monoolein and 40% (w/w) water in the 20–40°C range. As the DM concentration increases, the cubic-Pn3m phase gives way to the cubic-Ia3d and finally to the Lα phase. Regions of pure cubic-Pn3m, cubic-Ia3d, and Lα phase are observed in the 20–40°C range, and phase coexistence is found between the pure phases. The cubic-Pn3m/cubic-Ia3d and the cubic-Ia3d/Lα boundaries shift to higher temperatures with increasing DM concentration.

Metastable behavior

To allow for the full expression of metastable phase behavior, all samples examined in this part of the study were prepared at 20°C. Under this condition, the cubic-Pn3m, cubic-Ia3d, and Lα liquid crystal phases are observed over the range of DM concentrations used (Fig. 2 *B*). This is the same behavior that was reported on in the equilibrium phase diagram above (Fig. 2 *A*). However, in contrast to the equilibrium conditions, these samples were cooled slowly and in a stepwise manner to 0°C. As expected, this treatment allowed the liquid crystal phases formed at 20°C to undercool and to do so down to 0°C. Thus, along the 0°C isotherm the same series of phases extending from the cubic-Pn3m to the cubic-Ia3d and Lα phases is observed with increasing DM concentration as was seen at 20°C. Furthermore, the cubic-Pn3m/cubic-Ia3d and cubic-Ia3d/Lα boundaries that existed above 20°C extend smoothly down to 0°C. Comparing the phase diagrams in Fig. 2, *A* and *B*, we see that metastability prevails in the latter in the temperature range from 0°C to 20°C. Where the L_c phase represents equilibrium phase behavior below 20°C, we now find it replaced by one or another of three different liquid crystal phases under metastable conditions. Thus what characterizes the metastable phase diagram is a complete absence of the solid L_c phase and the persistence of liquid crystal phases down to the lowest temperature examined.

Lattice parameter temperature and composition dependence

The temperature and composition dependence of the lattice parameters of the solid and the different mesophases in the equilibrium and metastable phase diagrams are shown in Fig. 3. Both systems exhibit typical liquid crystal phase thermal expansivities within the limits of measurement accuracy (Briggs et al., 1996; Qiu and Caffrey, 2000). Thus, for example, we see that with increasing temperature the lattice parameters of the liquid crystal phases tend to fall (Fig. 3, *A* and *C*). The effect is more pronounced in the cubic phases and less so for the Lα phase. The L_c phase is essentially temperature insensitive in the range studied, as expected for the solid state.

The lattice parameters of both the L_c and the liquid crystal phases are relatively insensitive to DM concentration up to 0.2 M (Fig. 3, *B* and *D*). This is true under both equilibrium and metastable conditions. Nonetheless, the detergent can induce dramatic changes in phase behavior, depending on temperature and on whether equilibrium or metastability prevails.

In the studies just described, the concentration of monoolein in the system remained constant, while the relative amounts of the two other components, DM and water, varied in opposite directions. We have also examined the effect of holding the relative amounts of monoolein and DM constant while changing the overall water content of the sample at 20°C. The starting point for this study was the system corresponding to 60% (w/w) monoolein and 40% (w/w) 0.25 M DM solution (final DM concentration in the overall mixture, 0.1 M). According to the phase diagrams in Fig. 2, this should place the system in the cubic-Ia3d or the cubic-Ia3d plus Lα coexistence region. The experiment was performed twice. On one occasion the cubic-Ia3d phase alone was observed. On the other, cubic-Ia3d plus Lα phase coexistence was found (Fig. 4 *A*). The effect of increasing the water concentration to 58% (w/w) under these conditions was to induce the formation of the cubic-Pn3m phase.

In a separate study, we examined the effect of holding constant the relative amounts of DM and water while adjusting the concentration of monoolein at 20°C. Practically, this was carried out by dispersing monoolein with increasing amounts of a DM solution of fixed concentration, in this case, 0.25 M. As in the previous study, the starting point for the study corresponded to a sample with 60% (w/w) monoolein and 40% (w/w) 0.25 M DM solution. The effect of increasing the relative amount of DM solution was to destabilize the cubic-Ia3d plus Lα phase coexistence and to trigger Lα phase formation (Fig. 4 *B*). The latter existed pure at a final concentration of 8–10%(w/w) DM.

DISCUSSION

The *in meso* method for growing crystals of membrane proteins makes use of hydrated monoolein in the cubic

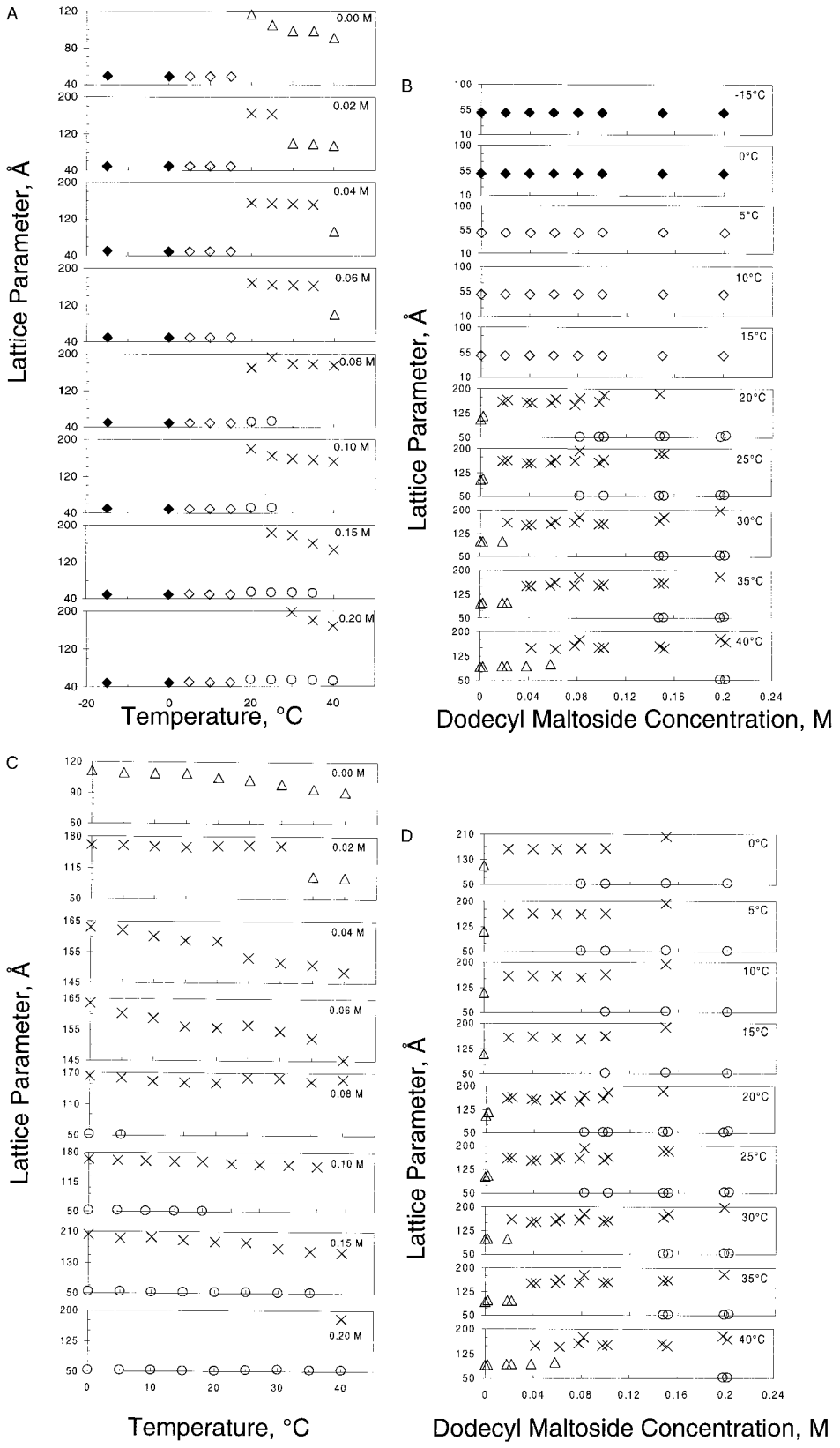

FIGURE 3 Temperature (*A* and *C*) and composition (*B* and *D*) dependence of the lattice parameters of the phases found in the monoolein/dodecyl maltoside/water system at the indicated temperatures and sample composition in units of molar dodecyl maltoside. Measurements were made under equilibrium (*A* and *B*) and metastable (*C* and *D*) conditions. The phases and other conditions are as described in the legend to Fig. 2.

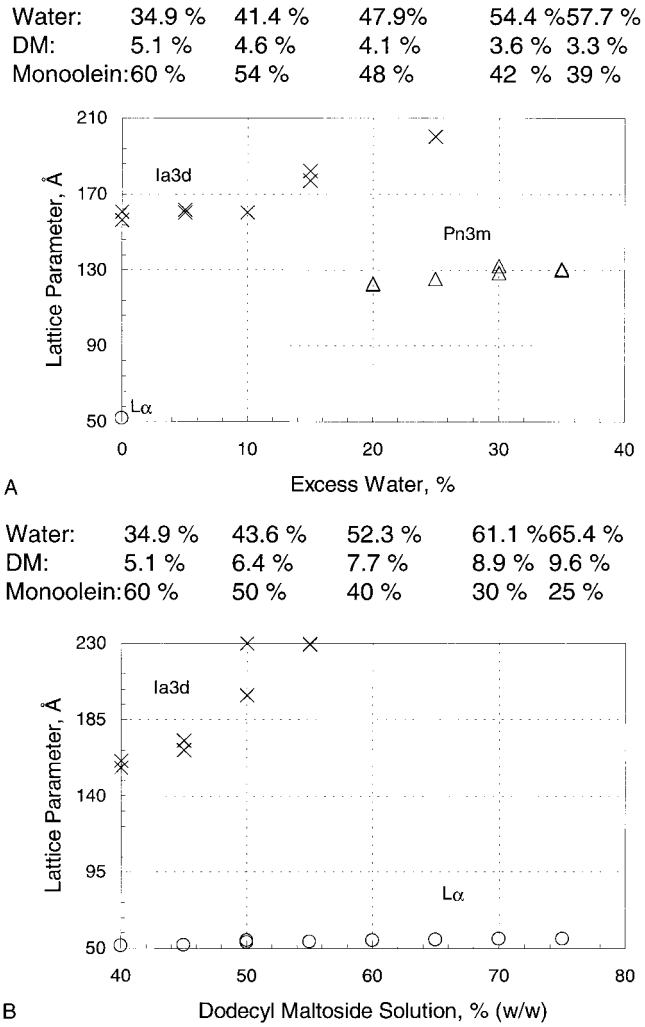

FIGURE 4 Identity and lattice parameter of each phase and coexisting phases in the monoolein/dodecyl maltoside/water system as determined by x-ray diffraction in control experiments. (*A*) The molar ratio of monoolein/dodecyl maltoside was fixed at 17:1 (12:/1 w/w) corresponding to 40% (w/w) 0.25 M dodecyl maltoside and 60% (w/w) monoolein, while the water content of the sample was increased. The relative amounts of all three components in the system are shown along the upper abscissa. (*B*) The dodecyl maltoside concentration in the aqueous solution was fixed at 0.25 M, while the percentage aqueous medium in the overall mix was raised from 40% to 75%. The relative amounts of all three components in the system are shown along the upper abscissa. In this experiment, the dodecyl maltoside/water ratio was fixed. All measurements were performed at 20°C after samples were incubated for at least 5 h.

mesophase (Rummel et al., 1998; Luecke et al., 1999). Typically, the protein is stripped from the native biomembrane by solubilizing with a detergent. DM is commonly used for this purpose. The solubilized protein is dispersed and presumably reconstituted into the membranes of the cubic mesophase before crystal formation (Caffrey, 2000). On its journey into the monoolein-containing mixture, the protein is accompanied by the detergent and possibly by native membrane lipid. It is conceivable that the detergent plays a role in crystal growth. In the case of bacteriorhodopsin, the detergent used was octyl glucoside, an alkyl and a saccharide homolog of DM. We are in the process of attempting membrane protein crystallization using proteins solubilized in DM. Accordingly, the purpose of this study was to determine first and foremost if the cubic phase formed by hydrated monoolein could tolerate DM at the levels expected to be present during a typical crystallization study. For this purpose, the partial phase diagrams in Fig. 2 were constructed. A second objective was to evaluate the effect that temperature had on phase stability and the nature of the interaction between DM and the cubic phase. A final objective was to determine if metastability, which has been documented in the simple, two-component monoolein/water system (Qiu and Caffrey, 2000), would persist in the presence of DM. If so, then the range of temperatures in which the monoolein/DM/water system might be used for crystallization was to be determined. In all of these studies, x-ray diffraction was used. Thus, in addition to providing for an unequivocal identification of phase type, the method allowed for quantitation of phase microstructure.

Phase behavior

We begin our discussion of the effect that DM has on the phase behavior of monoolein/water by examining the equilibrium phase diagram of the latter two-component system (Fig. 1). The *in meso* method makes use of a system consisting of monoolein at 40% (w/w) water. With reference to Fig. 1 *A*, we see that this produces the cubic-Ia3d phase at 20°C. However, it is important to note that the phase boundary lines drawn in Fig. 1 *A* are approximate and are a best visual fit to a set of phase identity coordinates in temperature-composition space (Qiu and Caffrey, 2000). Thus, in our experience a monoolein sample prepared with 40% (w/w) water nominally will result in the cubic-Pn3m phase and/or the cubic-Ia3d phase. This uncertainty has many origins. They include 1) errors in sample preparation involving relatively small (mg) quantities of lipid and water, 2) slight changes in temperature encountered during sample preparation, 3) the fact that phase behavior is very sensitive to temperature and composition in the vicinity of 20°C and 40% (w/w) water (see Fig. 1 *A*), 4) the notorious capacity of the cubic phase to undercool, and 5) the minuscule differences in energy between the two cubic phases. Thus the sense is that reproducible behavior is expected only when extraordinary care is taken to ensure a high degree of accuracy in sample composition and a fixed thermal history during sample preparation. Many of these issues have been addressed (Hyde et al., 1984; Cheng et al., 1998; Qiu and Caffrey, 2000). Suffice it to say that samples prepared to a target composition of 40% (w/w) water can give rise to one and/or the other of the two cubic phases when prepared under standard conditions at 20°C.

The phase diagram in Fig. 2 *A* shows how the detergent, DM, alters hydrated monoolein phase behavior as a function of temperature. At zero DM concentration, the phase change with temperature corresponds to the 40% (w/w) water isopleth (line of constant composition) in Fig. 1 *A*. This is essentially what is observed. Specifically, the L_c phase persists up to 15°C at least and transforms to the cubic phase, in this case the cubic-Pn3m phase, with increasing temperature at and above 20°C. The cubic-Pn3m phase is stable up to 40°C. As DM is added to the aqueous phase, it has little impact on L_c phase behavior below 20°C. At and above 20°C, DM triggers a series of phase transformations. The first happens in the low millimolar DM concentration range and is of the cubic-Pn3m-to-cubic-Ia3d phase type. The second involves a cubic-Ia3d-to-Lα phase transition, where the phase boundary rises from 0.1 M to 0.2 M DM with increasing temperature. To some degree, this behavior mimics what is happening in the simple two-component monoolein/water phase diagram as the water content of the sample is reduced isothermally (Fig. 1 *A*). We will revisit this point later.

The bulk of the *in meso* crystallization studies performed to date have been carried out at 20°C. Thus, we proceed to the question of the impact that DM has on cubic phase stability under such conditions and to its possible effects on membrane protein crystallization. The data in Fig. 2 *B* show that the cubic phase persists up to a concentration of ~0.1 M DM in the aqueous medium at 20°C and to ~0.2 M at 40°C. This is in excess, by a factor of 10, of the concentration of DM expected to be introduced along with protein to the *in meso* system. Our first conclusion therefore is that DM is likely to be compatible with membrane protein crystallization *in meso* in that it does not destabilize the cubic phase at low concentrations. Furthermore, the tolerance of the system for DM increases with increasing temperature, up to 40°C at least.

While the cubic phase is stable in the presence of low concentrations of DM, the system converts to the solid L_c phase below 20°C under equilibrium conditions. Thus, if equilibrium conditions were to prevail, the monoolein/water system could not be used for *in meso* crystallization below room temperature, simply because the cubic phase does not form there. This is where the phenomenon of metastability or undercooling becomes evident. We know from previous studies that the liquid crystal phases observed in the monoolein/water system undercool when treated appropriately (Qiu and Caffrey, 2000). This involves sample preparation at 20°C and subsequent slow cooling. The undercooled phases so formed have been known to persist for years. However, the equilibrium L_c phase can be accessed by a low-temperature incubation at −15°C for as little as 2 h. Thus, by undercooling the cubic phase, we have an opportunity to carry out *in meso* crystallization all the way down to 0°C. The question that concerned us in the current study had to do with how DM might affect such undercooling behavior. The data in Fig. 2 *B* show clearly that undercooling persists over the entire DM concentration range examined and all the way down to 0°C. Thus, we proceed to our second conclusion that the monoolein/water system should prove useful in membrane protein crystallization over a relatively wide range of DM concentrations and down to 0°C.

Rationalizing the effects of DM

At low concentrations, DM has little effect on the phase stability of hydrated monoolein other than to induce a cubic-Pn3m-to-cubic-Ia3d phase transformation. At higher concentrations, however, DM destabilizes the cubic phase and triggers formation of the planar Lα phase. The concentrations of DM at which these effects occur are temperature dependent. However, temperature effects will be neglected for the moment.

Qualitatively, we can understand the tendency of monoolein to form the highly curved cubic mesophase in light of its molecular shape (Fig. 5 *A*). The molecule has a relatively small polar headgroup consisting of glycerol and a single ester linkage. In contrast, the chain to which it is attached is long (18 carbons to be exact), with a kink in the middle of the chain arising from a *cis* double bond between the 9th and 10th carbon atoms. In the liquid crystal phase, the chain is considered "fluid," with an abundance of *trans*/*gauche* isomers along the length of the chain. Thus the dynamically averaged shape of the molecule brings to mind a cone-shaped object with the polar headgroup at the pointed end of the cone and the methyl terminus of the acyl chain at the wider end. However, close packing of cones leads to a spherical object. The cubic phases have curved hydrocarbon/water interfaces that approximately parallel a minimal surface where the termini from adjacent monolayers in a bilayer meet (Hyde et al., 1984). Thus we should envision

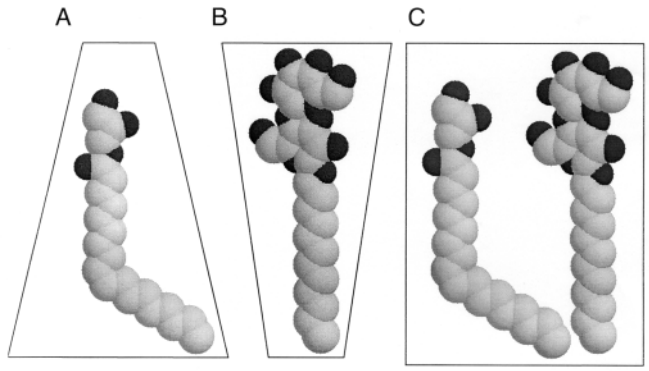

FIGURE 5 Space-filling models of monoolein (*A*) and dodecyl maltoside (*B*) in isolation and together (*C*). The continuous line surrounding the models was drawn to give a sense of the dynamically averaged molecular volume occupied in the different arrangements. Light and dark spheres represent carbon and oxygen atoms, respectively.

the dynamically averaged shape of an individual monoolein molecule more as a differentially curved wedge than as a cone. And now we proceed to the effect that DM has on the cubic mesophase of monoolein. If we examine the molecular structure of DM for a moment (Fig. 5 *B*), we immediately note a shape that is complementary to that of monoolein. DM, in contrast to monoolein, has a large polar headgroup consisting of two glucose moieties in a 1–4 glycosidic linkage, which in turn is glycosidically linked to a relatively short (12 carbon atoms long) alkyl chain. The corresponding dynamically averaged molecular shape that this conjures up is an inverted cone or wedge. Thus, if DM partitions into the lipid compartment of the monoolein mesophase, as is likely, given its amphipathic nature, one might expect that the tendency of the mixed system to create a curved hydrocarbon/water interface will be lessened as DM is titrated in. In the limit where curvature is completely removed, the bilayers flatten and a lamellar phase emerges. This is precisely the effect seen as DM is added to the cubic mesophase of hydrated monoolein (Fig. 2).

There is an additional effect to consider as we attempt to rationalize DM's effect on cubic phase stability as expressed in the phase diagrams of Fig. 2. This has to do with the fact that the measurements were made under conditions where the concentration of monoolein remained constant while the relative amounts of DM and water varied in opposite directions. Thus, as the DM concentration was increased, the concentration of water in the system decreased. Inspection of Fig. 1 shows that lowering the water concentration destabilizes the cubic phase in favor of the lamellar phase. Thus it is possible that the effect of increasing DM concentration, as revealed in Fig. 2, corresponds simply to a lowering of water concentration. However, while this effect may play some role, it cannot be the only one, for the following reason. At the highest concentration of DM used in the study, viz. 0.2 M DM in the aqueous medium (which constitutes 40% (w/w) of the overall sample, i.e., monoolein, water, and DM), the detergent accounts for 10% (w/w) of the overall sample, with water representing the remaining 30% (w/w). If we neglect the contribution of DM to the system, this corresponds to a monoolein/water mix with close to 33% (w/w) water. Referring back to the monoolein/water phase diagram in Fig. 1, we see that a shift from 40% to 33% (w/w) water has little effect on phase behavior.

The foregoing assumes that there is no interaction between the detergent and water. In reality, we expect a large interaction because of the carbohydrate and, thus, water-loving nature of the DM headgroup. Accordingly, the addition of DM to the system will exert an osmotic or water-withdrawing effect as water is entrained by noncovalent interactions in the immediate vicinity of the carbohydrate moiety and sequestered away from the water-loving glycerol headgroup of monoolein. Indeed, the effect may be to exaggerate the inverted wedge shape of the DM molecule and to enhance its ability to "unfurl" the bilayers that make up the cubic mesophase.

Indirectly, we have examined the effect that the carbohydrate portion of DM might have on the phase behavior of the monoolein system at 40% (w/w) water in the cubic phase at 20°C as follows. The lipid was dispersed with a solution containing increasing amounts of either maltose, a disaccharide, or glucose, a monosaccharide, to mimic the sugar end of DM. The results in Fig. 6 show that neither sugar destabilizes the cubic-Pn3m phase, which persists in the presence of 0.5 M maltose and 1.2 M glucose. In both cases, the sugar lowers the lattice parameter of the cubic phase, suggesting a water-withdrawing effect. It is interesting to note that the potency of the disaccharide, maltose, in this regard is approximately twice that of the monosaccharide, glucose. These data suggest therefore that the effect in which DM triggers the cubic-to-lamellar transition in hydrated monoolein is attributable, at least in part, to its amphipathic nature and to the complementarity of its molecular shape to that of the host lipid, monoolein.

In addition to mapping out the phase diagrams in Fig. 2, the nature of the interaction between DM, monoolein, and water was examined in a system where a 0.25 M DM solution was mixed in increasing proportions with a fixed amount of monoolein. In this study, which was carried out at 20°C, the DM/water ratio was fixed (1:7 w/w), while the relative amounts of the aqueous solution and the lipid changed. The data are shown in Fig. 4 *B*, along with the relative amounts of all three components in the system. Moving from left to right in the figure, we find that the cubic-to-lamellar phase transition is induced. This happens despite the fact that the water content of the system has surpassed 60% (w/w). If the concentration of water were the

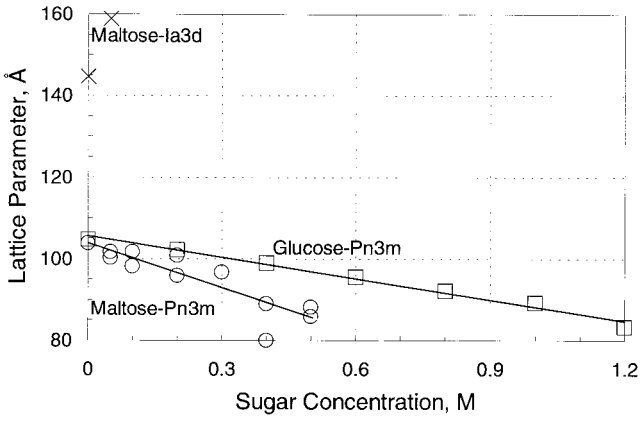

FIGURE 6 Dependence of the cubic phase lattice parameter on glucose and maltose concentration in hydrated monoolein at 20°C. Concentration refers to the final sugar content of the overall mixture (lipid + water + sugar). The maltose study was repeated once, and data from both data sets are shown. In one of the data sets the cubic-Ia3d phase was observed under low sugar concentration. This fickle feature of cubic phase behavior is commented on in the text.

only factor dictating phase behavior, such a large degree of hydration would have converted the system into the fully hydrated cubic-Pn3m (see Fig. 1). Obviously, this is not the case, because the water carries detergent with it, which, at the end of the addition, has reached a concentration of 10% (w/w) in the overall sample. Thus the system responds by adjusting phase more to the minor component, in this case the detergent, than it does to added water.

In a second and related study, the detergent/lipid ratio was fixed (at 1:12 w/w, 1:17 mol/mol) while the water content of the system was raised. The measurements were made at 20°C, and the results (Fig. 4 *A*) show that in this case increasing the water content caused a conversion to the cubic-Pn3m phase. By the end of the addition, the water content of the system is 58% (w/w) and the DM concentration is 3.3% (w/w) overall. Given the statement in the preceding paragraph that the detergent essentially dictates phase behavior, one might expect that a concentration of 3.3% (w/w) DM would stabilize the cubic-Ia3d phase or cubic-Ia3d plus Lα phase coexistence as opposed to the cubic-Pn3m phase (see Fig. 2). We explain this apparent disparity by noting that the highest water content sample in Fig. 4 *A* has 58% (w/w) water. The detergent has reasonable water solubility (cmc = 0.2 mM, 0.01% (w/w)) and will partition into it. As a result, less is available for distributing into the lipid mesophase. In essence, having excess water in the system lowers the effectiveness of the detergent in altering lipid phase behavior.

The literature includes reports on the effects other additives have on the phase behavior of hydrated monoolein. In one from this group, NaCl was shown to profoundly stabilize the inverted hexagonal phase at the expense of the cubic-Pn3m phase (Caffrey, 1987). The effect continued up to 5 M NaCl. In a separate study, oleic acid was found to induce a cubic-Pn3m-to-cubic-Ia3d phase transition that could be reversed at high salt concentration (Aota-Nakano et al., 1999). Protonic equilibrium was examined in the mixed monoolein/oleic acid system with the cubic-Ia3d, cubic-Pn3m, and inverted hexagonal phases stabilized at pH 6–7, 5.5–6, and <5.5, respectively.

Implications for membrane protein crystallization

As noted, our objective was to evaluate the effects that the detergent, DM, has on the phase properties of the system used for *in meso* crystallization of membrane proteins. What we find is that at low concentrations, and in the range expected for typical crystallization trials, DM does not have an impact on cubic phase stability. With increasing concentration, DM stabilizes the cubic-Ia3d over the cubic-Pn3m phase. It is not clear at this juncture whether this will affect crystallizability, because a careful study of the cubic phase type preference for crystallization has not yet been made. At sufficiently high concentrations, however, the detergent triggers a cubic-to-lamellar transition. It has been postulated that a lamellar phase represents the conduit from the bulk cubic phase to the crystal surface (Caffrey, 2000). The view is that the bulk cubic phase in which the protein is reconstituted gives way to a lamellar phase of the Lα type in the immediate environment of the crystal. Under conditions where crystal formation is favored, the protein migrates through the tortuosity of the cubic phase into a locally formed lamellar phase that serves as an epitaxial launch pad into the crystal. If indeed the lamellar phase figures in the crystallization process, then the presence in the *in meso* mix of a detergent such as DM, which ultimately favors Lα phase formation, may facilitate the process. But we have just shown that a relatively high concentration of DM is required in the monoolein/water system to induce Lα phase formation. It may be that such concentrations are achieved locally and transiently in a system with a low overall detergent content and/or that the conditions prevailing at the time of crystallization lower the concentration of detergent needed to induce lamellar phase formation.

CONCLUSIONS

A partial temperature-composition phase diagram of the monoolein/water/DM system has been constructed in the temperature range from −15°C to 40°C under conditions of equilibrium (Figs. 1 and 2). The cubic mesophase at 40% aqueous phase remains stable up to ~0.1 M DM at 20°C. At higher concentrations, the cubic phase is destroyed and a lamellar liquid crystal phase emerges. These effects have been explained on the basis of monoolein and DM having complementary molecular shapes. The postulated involvement of a local lamellar phase in the crystal growth process would suggest a beneficial role for the detergent in the *in meso* method of membrane protein crystallization. While the measurements reported herein refer to DM, it is likely that the results and conclusions apply to related detergents and have some generality. Similar measurements have been conducted under conditions that allow mesophase undercooling to occur. In this case, the cubic and lamellar phases persist all the way down to 0°C. This result suggests that phase metastability can be exploited to effect crystallization *in meso* with proteins that require low temperatures for stability.

APPENDIX: PHASE LATTICE PARAMETER AS A FUNCTION OF TEMPERATURE AND COMPOSITION

Below is a tabulation of the data in Figs. 3, 4, and 6. Average values are reported where duplicate measurements were made.

TABLE A1 Equilibrium data (Fig. 3)

Temperature (°C)	Final concentration of dodecyl maltoside (M)	Phase lattice parameter (Å)				Temperature (°C)	Final concentration of dodecyl maltoside (M)	Phase lattice parameter (Å)			
		L_c	L_α	Pn3m	Ia3d			L_c	L_α	Pn3m	Ia3d
−15	0.00	49.5				20	0.00			116.7	
−15	0.02	49.6				20	0.02				164.5
−15	0.04	49.7				20	0.04				155.3
−15	0.06	49.5				20	0.06				167.6
−15	0.08	49.6				20	0.08		51.0		169.9
−15	0.10	49.7				20	0.10		51.7		179.9
−15	0.15	49.6				20	0.15		53.9		
−15	0.20	49.2				20	0.20		54.9		
0	0.00	49.3				25	0.00			105.4	
0	0.02	49.3				25	0.02				163.1
0	0.04	49.5				25	0.04				154.2
0	0.06	49.3				25	0.06				164.8
0	0.08	49.4				25	0.08		52.5		192.9
0	0.10	49.3				25	0.10		51.0		164.8
0	0.15	49.4				25	0.15		52.3		183.0
0	0.20	49.4				25	0.20		54.3		
5	0.00	49.2				30	0.00			99.2	
5	0.02	49.4				30	0.02			99.3	
5	0.04	49.4				30	0.04				152.6
5	0.06	49.3				30	0.06				163.1
5	0.08	49.4				30	0.08				178.1
5	0.10	49.5				30	0.10				157.3
5	0.15	49.7				30	0.15		52.4		178.0
5	0.20	49.4				30	0.20		53.3		197.6
10	0.00	49.3				35	0.00			98.9	
10	0.02	49.4				35	0.02			97.7	
10	0.04	49.4				35	0.04				150.9
10	0.06	49.3				35	0.06				161.8
10	0.08	49.3				35	0.08				176.9
10	0.10	49.2				35	0.10				155.0
10	0.15	49.5				35	0.15		51.2		159.7
10	0.20	49.2				35	0.20		52.6		180.3
15	0.00	49.3				40	0.06			99.0	
15	0.02	49.4				40	0.08				173.9
15	0.04	49.4				40	0.10				151.3
15	0.06	49.3				40	0.15				146.8
15	0.08	49.4				40	0.20		51.7		166.9
15	0.10	49.4									
15	0.15	48.9									
15	0.20	49.2									

TABLE A2 Metastable data (Fig. 3)

Temperature (°C)	Final concentration of dodecyl maltoside (M)	Phase lattice parameter (Å)			Temperature (°C)	Final concentration of dodecyl maltoside (M)	Phase lattice parameter (Å)		
		L_α	Pn3m	Ia3d			L_α	Pn3m	Ia3d
0	0.00		111.2		20	0.08			150.5
0	0.02			162.5	20	0.10	51.7		160.5
0	0.04			163.0	20	0.15	52.9		183.7
0	0.06			163.7	20	0.20	51.4		
0	0.08	51.8		164.1	25	0.00		102.6	
0	0.10	52.6		165.5	25	0.02			161.1
0	0.15	54.4		202.2	25	0.04			153.2
0	0.20	53.2			25	0.06			156.4
5	0.00		109.7		25	0.08			161.0
5	0.02			161.0	25	0.10			155.0
5	0.04			162.2	25	0.15	52.7		182.5
5	0.06			160.4	25	0.20	53.3		
5	0.08	51.2		161.3	30	0.00		98.7	
5	0.10	52.1		163.5	30	0.02			160.5
5	0.15	54.1		193.7	30	0.04			151.6
5	0.20	52.4			30	0.06			154.6
10	0.00		108.8		30	0.08			159.5
10	0.02			159.0	30	0.10			153.9
10	0.04			160.2	30	0.15	51.7		166.5
10	0.06			158.8	30	0.20	53.7		
10	0.08			153.7	35	0.00		93.8	
10	0.10	51.7		162.8	35	0.02		97.6	
10	0.15	53.67		195.4	35	0.04			150.8
10	0.20	52.2			35	0.06			152.2
15	0.00		109.0		35	0.08			152.4
15	0.02			157.9	35	0.10			152.0
15	0.04			158.7	35	0.15	51.0		158.4
15	0.06			156.0	35	0.20	53.4		
15	0.08			151.7	40	0.00		91.1	
15	0.10	51.7		160.4	40	0.02		94.0	
15	0.15	53.4		188.2	40	0.04			148.5
15	0.20	51.7			40	0.06			145.2
20	0.00		104.9		40	0.08			157.0
20	0.02			160.1	40	0.10			150.2
20	0.04			158.5	40	0.15			155.0
20	0.06			155.7	40	0.20	52.6		179.1

TABLE A3 DM solution effect (Fig. 4)

Temperature (°C)	Composition of dodecyl maltoside (%, w/w)	Phase lattice parameter (Å)	
		L_α	Ia3d
20	40.00		156.4
20	40.00	51.7	160.5
20	45.00	51.8	172.4
20	45.00	51.8	166.8
20	50.00	53.5	199.1
20	50.00	54.6	229.9
20	55.00	54.1	229.4
20	55.00	54.0	229.6
20	60.00	54.9	
20	60.00	55.0	
20	65.00	55.2	
20	65.00	55.3	
20	70.00	55.4	
20	70.00	55.5	
20	75.00	55.7	
20	75.00	55.7	

TABLE A4 Excess water effect (Fig. 4)

Temperature (°C)	Excess water (%, w/w)	Phase lattice parameter (Å)		
		L_α	Pn3m	Ia3d
20	0.00			156.4
20	0.00	51.7		160.5
20	5.00			162.0
20	5.00			160.3
20	10.00			160.5
20	10.00			160.7
20	15.00			182.0
20	15.00			177.1
20	20.00		122.7	
20	20.00		123.0	
20	25.00		125.4	
20	25.00			200.3
20	30.00		128.5	
20	30.00		132.0	
20	35.00		130.0	
20	35.00		130.6	

TABLE A5 Glucose effects (Fig. 6)

Temperature (°C)	Final concentration of glucose (M)	Phase lattice parameter (Å) Pn3m
20	0.00	104.8
20	0.20	102.1
20	0.40	99.0
20	0.60	95.7
20	0.80	92.2
20	1.00	89.3
20	1.20	83.4

TABLE A6 Maltose effects (Fig. 6)

Temperature (°C)	Final concentration of maltose (M)	Phase lattice parameter (Å)	
		Pn3m	Ia3d
20	0.00	144.6	
20	0.00		103.8
20	0.05	158.9	100.3
20	0.05		101.7
20	0.10		98.1
20	0.10		101.6
20	0.20		95.8
20	0.20		100.8
20	0.30		96.7
20	0.30		96.7
20	0.40		89.0
20	0.40		80.0
20	0.50		85.9
20	0.50		88.1

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Data deposition

Relevant data reported in this paper have been deposited in the Lipid Data Bank (http://www.ldb.chemistry.ohio-state.edu).

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