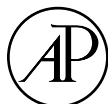

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Membrane protein crystallization

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Abstract

The need for high-resolution structure information on membrane proteins is immediate and growing. Currently, the only reliable way to get it is crystallographically. The rate-limiting step from protein to structure is crystal production. An overview of the current ideas and experimental approaches prevailing in the area of membrane protein crystallization is presented. The long-established surfactant-based method has been reviewed extensively and is not examined in detail here. The focus instead is on the latest methods, all of which exploit the spontaneous self-assembling properties of lipids and detergent as vesicles (vesicle-fusion method), discoidal micelles (bicelle method), and liquid crystals or mesophases (in meso or cubic-phase method). In the belief that a knowledge of the underlying phase science is integral to understanding the molecular basis of these assorted crystallization strategies, the article begins with a brief primer on lipids, mesophases, and phase science, and the related issue of form and function as applied to lipids is addressed. The experimental challenges associated with and the solutions for procuring adequate amounts of homogeneous membrane proteins, or parts thereof, are examined. The cubic-phase method is described from the following perspectives: how it is done in practice, its general applicability and successes to date, and the nature of the mesophases integral to the process. Practical aspects of the method are examined with regard to salt, detergent, and screen solution effects; crystallization at low temperatures; tailoring the cubic phase to suit the target protein; different cubic-phase types; dealing with low-protein samples, colorless proteins, microcrystals, and radiation damage; transport within the cubic phase for drug design, cofactor retention, and phasing; using spectroscopy for quality control; harvesting crystals; and miniaturization and robotization for high-throughput screening. The section ends with a hypothesis for nucleation and growth of membrane protein crystals in meso. Thus far, the bicelle and vesicle-fusion methods have produced crystals of one membrane protein, bacteriorhodopsin. The experimental details of both methods are reviewed and their general applicability in the future is commented on. The three new methods are rationalized by analogy to crystallization in microgravity and with respect to epitaxy. A list of Web resources in the area of membrane protein crystallogenesis is included.

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Keywords: Bicelle method; Crystallization mechanism; Cubic phase; Hexagonal phase; In cubo; In meso; In surfo; Lamellar phase; Liquid crystal; Microgravity; Nucleation and growth; Small-angle scattering; Structure–function; Vesicle-fusion method; X-ray diffraction

1. Introduction

A little over 2 decades ago, the sense in the community was that membrane proteins were refractory to crystallization and, thus, to high-resolution structure determination by crystallographic means. However, as the new millennium unfolds we find ourselves relatively "awash" in membrane protein structures. Depending on how they are enumerated, the number of unique membrane protein structures posted in the Protein Data Bank ranges from 40 to 60 (see Web resources at end of article). The breakthrough came in the early 1980s with the fabrication of detergent-based micelles rationally designed to solubilize membrane proteins. These aqueous dispersions lent themselves nicely to the more standard vapor diffusion and microdialysis methods of crystallization, and the rush to reveal membrane protein structure was on. However, it is probably fair to say that the pace of structure determination in the intervening years has been steady, but slow, with little in the way of dramatic innovation characterizing the field, until recently. Some 5 to 6 years ago, two completely new methods, one using bilayer vesicles (Takeda et al., 1998)

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and the other a lipidic mesophase (Landau and Rosenbusch, 1997), were introduced. They were accompanied by great hopes for major advances in the field. But perhaps in part because of the perceived difficulties in handling such dispersions, and thus reticence on the part of the community to try them, these two methods between them have produced on average just one structure annually. Another lipid/detergent approach was introduced a year ago. While it has been shown to produce diffraction-quality crystals of bacteriorhodopsin (bR),[1] it has not advanced beyond the test stage.

Why do membrane proteins, in contrast to their water-soluble counterparts, represent such a challenge? The answer can be found when it is realized that one of the primary impasses on the route that eventually leads to protein structure is encountered at the crystal production stage. Diffraction-quality crystals are particularly difficult to prepare currently when a membrane source is used. The reason for this is our limited ability to manipulate proteins bearing hydrophobic/amphiphilic surfaces that are usually enveloped with membrane lipid. More often than not, the protein gets trapped as an intractable aggregate in its watery course from membrane to crystal. As a result, access to the structure, and thus function, of tens of thousands of membrane proteins (Ostermeier and Michel, 1997; Wallin and von Heijne, 1998) is limited. In contrast, a veritable cornucopia of soluble proteins have offered up their structure (Protein Data Bank, 2003; http://www.rcsb.org/pdb), and valuable insight into function, reflecting the relative ease with which they are procured and crystallized. There exists therefore a great need for new ways of producing crystals of membrane proteins. In parallel, current methods must be refined and "industrialized" for high-throughput application. They should also be made user friendly for more aggressive and routine use by a wider segment of the community on proteins whose form and molecular functioning in signaling, transport, and metabolism continue to remain a mystery and thus beyond rational manipulation.

One of the purposes of this article is to present an overview of the current ideas and experimental approaches prevailing in the area of membrane protein crystallization. The long-established detergent- or surfactant-based ("in surfo") method has been reviewed extensively in the past (Hunte and Michel, 2003; Michel, 1983) and is not examined in any detail in this paper. The focus instead is on the latest methods, all of which in one way or another exploit the spontaneous self-assembling properties of lipids and detergent as vesicles, discoidal micelles (bicelles), and liquid crystals or mesophases. The article begins with a brief primer on lipids and mesophases by way of introducing the reader to the lexicon and concepts in the area of phase science. It is the author's firm belief that knowledge of the underlying phase behavior is integral to understanding the molecular basis and thus the full exploitation of these assorted crystallization strategies.

The structure of a handful of membrane proteins has been solved at close to atomic resolution by electron microscopy of two-dimensional crystals. As important as the method is, it is beyond the scope of the current review. An excellent overview of the crystallization aspects of the technique has just appeared and is strongly recommended (Kuhlbrandt, 2003).

2. Phase science: A primer

2.1. Liquid crystals and mesophases

In the context of the living cell, the lipid bilayer can be viewed as accommodating proteins and other molecules for the purpose of generating a selectively permeable, signal-transducing supramolecular complex which we recognize as the biological membrane. It is here that the lead characters in this article, membrane proteins, reside. The lipid component of membranes has the inherent capacity to adopt a variety of so-called liquid crystalline states, also referred to as mesophases, more ordered than a liquid but less so than a solid (Fig. 1). The planar lipid bilayer represents one such state—the lamellar phase. The remainder is grouped together in the nonlamellar-phase category. This includes the inverted hexagonal and cubic phases. The particular mesophase accessed depends, among other things, on temperature, pressure, lipid molecular structure and concentration, and composition of the aqueous dispersing medium (Caffrey, 1986a; Luzzati, 1968; Shipley, 1973).

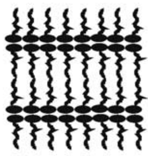
Mesophases come about as a result of spontaneous self-assembly. The process is spontaneous in the sense that an external source of energy, such as ATP, is not needed, nor indeed does it require a genetic blueprint. Spontaneous self-assembly arises in part as a result of the amphiphilic nature of the lipid molecule with its contiguous polar and apolar parts. When combined with water, supramolecular self-assembly and phase separation of the lipid are driven by the hydrophobic effect, which has its origins in the "narcissistic properties of water." Water likes itself!

2.2. Miscibility and phase diagrams

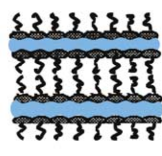
Lipid-phase behavior, which will figure prominently in the discussion of membrane protein crystallization

[1] *Abbreviations used:* AG, alkyl glycoside; bR, bacteriorhodopsin from *Halobacterium salinarium*; CFTR, cystic fibrosis transmembrane conductance regulator; L_α, lamellar liquid crystal phase; LHC2, light-harvesting complex 2 from *Rhodopseudomonas acidophila*; OG, octyl glucoside; PC, phosphatidylcholine; RCV, photosynthetic reaction center from *Rhodopseudomonas viridis.*

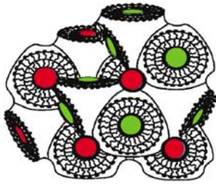
Lamellar crystal - Lc



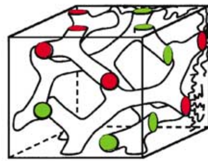
Lamellar liquid crystal - L_α



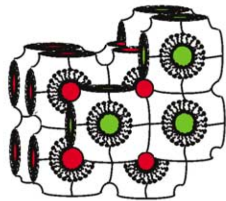
Cubic - Pn3m



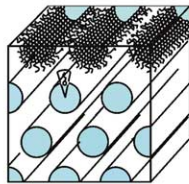
Cubic - Ia3d



Cubic - Im3m



Inverted hexagonal - H_{II}



Fluid isotropic - FI

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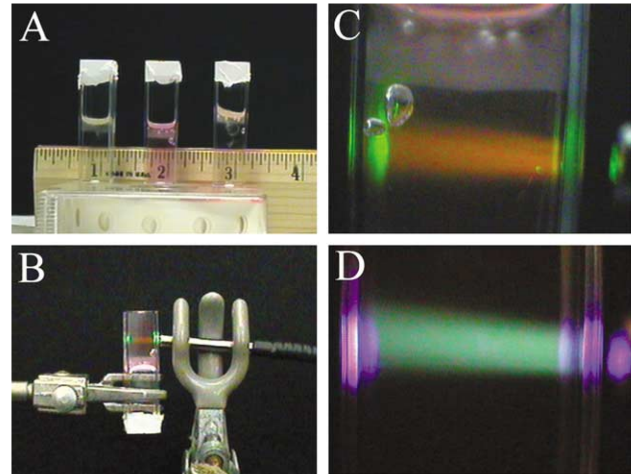
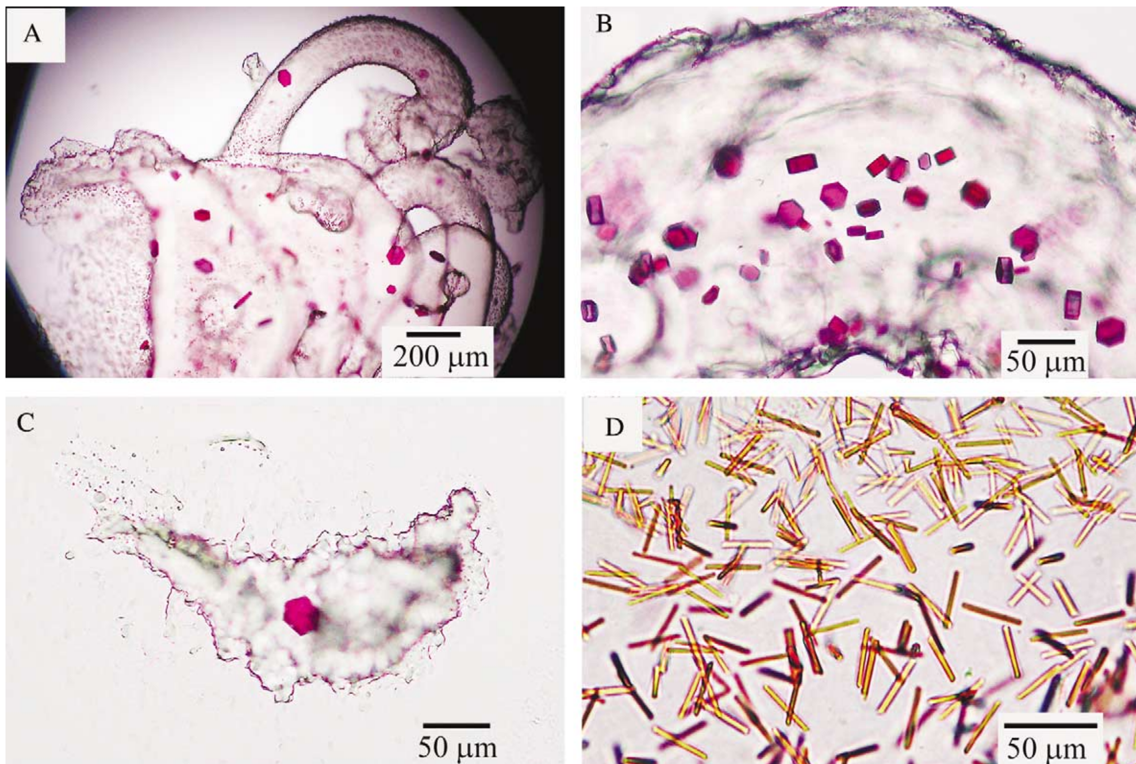


Fig. 11. Optical transparency and viscosity of the cubic phase. Shown are cuvettes filled with optically transparent cubic phase with and without added fluorophores. (A) Three cuvettes containing cubic phase. The transparency of the phase is apparent since it is possible to read the markings on the ruler behind the cuvettes. (B) Rhodamine B-doped cubic phase in a cuvette held upside down while being illuminated by green (530 nm) light incident from the right-hand side of the cell. The reddish-orange fluorescent band in the cubic phase marks the track of the beam through the sample. Transmitted light is seen as it scatters from the left-hand window of the cuvette. The viscous nature of the cubic phase is apparent since the cubic phase remains in the cuvette even though it is inverted. (C) Detailed, right-side-up view of B. (D) As in C, where the fluorophore is fluorescein. The incident light is blue (405 nm), fluorescence is green.



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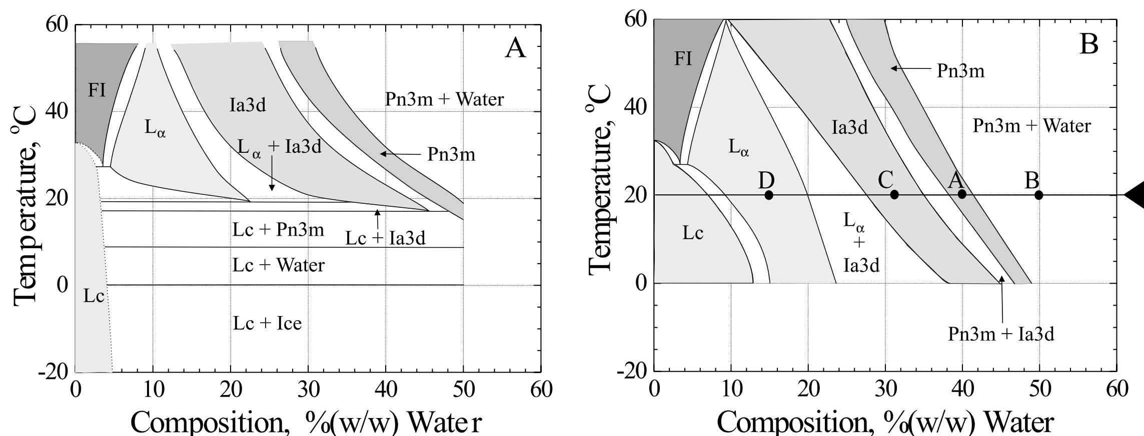

Fig. 2. Temperature–composition phase diagram for the monoolein/water system. (A) Equilibrium-phase diagram (Qiu and Caffrey, 2000). (B) Metastable-phase diagram (Briggs et al., 1996). In B, points along the 20 °C isotherm identified by capital letters are referred to in the text.

below, is conveniently, concisely, and quantitatively described in the form of an equilibrium temperature–composition (T–C) phase diagram (Caffrey, 1986b; Luzzati, 1968; Shipley, 1973). The simplest of these are of two components, for example, consisting of a single lipid and water for which phase behavior is presented as a function of water concentration and temperature. A typical T–C phase diagram is shown in Fig. 2A (Briggs et al., 1996; Qiu and Caffrey, 1999). Such diagrams should obey the Gibbs phase rule. Thus, in regions of two-phase coexistence, the composition of the phases remains fixed at a known value while overall sample composition and the relative amounts of the two phases change isothermally.

3. Form and function

3.1. Lipid form, function, and rational design

My interest in the more recent methods for membrane protein crystallization arose because they relate to a long-term project in my lab which has to do with form and function as applied to lipidic systems (Fig. 3). The view taken is that form, as in molecular structure, determines function. By function is meant the assorted activities ascribed to the lipid component of biomembranes through to their signaling role and to the performance of lipid additives in pharmaceutical and food products. However, it is not structure alone that determines function. Rather it is structure, in concert with composition and environmental factors (temperature, pressure), that gives rise to a well-defined mesophase and phase microstructure (lipid layer thickness, aqueous compartment dimension, etc.). These in turn dictate performance and functionality. The challenge is to understand the relationship between form and function such that the principles of rational design can be deciphered and used. The link between the two is phase behavior (as in the corresponding T–C phase diagram) and phase microstructure and rheology (flow, viscosity). Integral to realizing the principles of rational design is the need to decipher the rules that tie together the triad

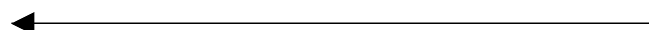

Fig. 1. Lipid phases. Cartoon representation of the various solid (lamellar crystal phase), mesophase (lamellar liquid crystal phase, cubic-Pn3m phase (space group 224), cubic-Ia3d phase (space group 230), cubic-Im3m phase (space group 229), inverted hexagonal phase), and liquid (fluid isotropic phase (Larsson, 1994)) states adopted by lipids. Individual lipids are shown as lollipop figures with the pop and stick parts representing the polar head group and the apolar acyl chain, respectively. The colored regions represent water.

Fig. 8. A sampling of membrane protein crystals grown in meso. (A–C) Bacteriorhodopsin. (D) Light-harvesting complex 2. In C the amount of cubic phase from which the crystal grew is estimated to be ~5 nl corresponding to ~20 ng protein. Images were provided by V. Cherezov and Y. Misquitta.

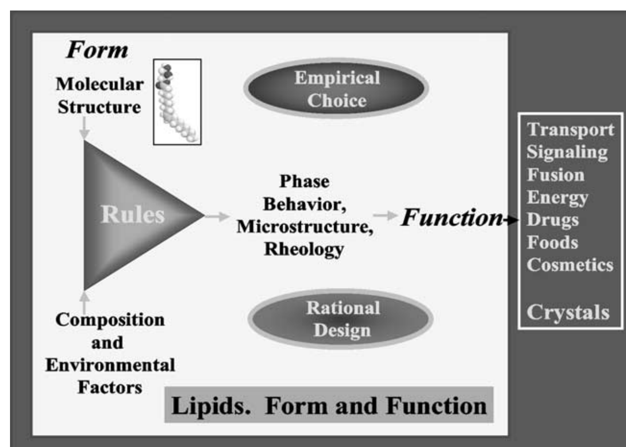

Fig. 3. The form and function concept as applied to lipids. A space-filling molecular model of monoolein is shown in the upper left hand corner. See text for details.

of molecular structure, composition and environmental factors, and phase behavior (Fig. 3). The final step involves establishing how phase properties impact on function.

With a view to uncovering the rules referred to above, it is useful to work with a lipid whose molecular structure can be altered easily in a way that impacts sensibly on phase behavior. The lipid of choice is one that has a simple molecular structure and that can access the full range of lyotropic (water-induced) and thermotropic (temperature-induced) mesophases in a biologically relevant composition (water content) and temperature range. The *cis* monounsaturated monoacylglycerols fit the bill reasonably well. Accordingly, the task of mapping the T–C phase diagrams for a host of monoacylglycerols for which molecular form is varied systematically is in progress (Briggs and Caffrey, 1994a,b; Briggs et al., 1996; Misquitta and Caffrey, 2001; Qiu and Caffrey, 1999, 2000). In this way, we are establishing the rules that relate form and phase behavior and, ultimately, function.

Monoolein is one such monoacylglycerol and its phase diagram has been mapped out in great detail (Fig. 2) (Qiu and Caffrey, 2000, and references therein). The cubic-phase method for crystallizing membrane proteins introduced below is based on this monoolein/water system (Rummel et al., 1998). The original objective of the method was to effect protein crystallization from within a cubic phase at 20 °C. Conditions for doing so in the monoolein/water system were arrived at by inspecting the 20 °C isotherm at which the cubic-Pn3m phase is accessed at an overall composition of 40% water and 60% monoolein (A in Fig. 2B). At this temperature, additional water creates a fully hydrated cubic phase in equilibrium with bulk water (B in Fig. 2B). Lesser amounts of water cause the cubic-Pn3m phase to transform to a cubic-Ia3d phase (C in Fig. 2B). The lamellar liquid crystal (L_α) phase forms at even lower hydration levels (D in Fig. 2B). Within any single (as opposed to two)-phase region, the lattice size, which scales with water-layer thickness in the mesophase, increases as hydration level rises. Lattice size (as well as phase identity) is determined accurately by low-angle X-ray diffraction (see below; Caffrey, 1986a; Qiu and Caffrey, 2000).

3.2. Protein form, function, and rational design

Given the audience to which this special issue is directed, little need be said to justify the relationship between protein structure and function and its relevance to rational design. It is obvious that the absolute and relative dispositions of the atoms within a protein play a major role in determining its functionality. Once protein structure is defined, so too are its activities and, by extension, function, although a protein's environmental context may play a role too. This is particularly true in the case of membrane proteins for which vectoral orientation across the lipid layer is important. How form enlightens function for a series of membrane proteins whose crystal structures have been determined recently has just been reviewed (Caffrey, 2002, and references therein).

Having introduced the concept of form and function as applied to both lipids and proteins, it seems reasonable to ask the question, "Does 'form' mean the same thing in the context of lipid and protein?" The answer is "No." With regard to lipid, form refers to molecular structure as illustrated in Fig. 3. As applied to protein, however, form refers to the 3-D structure of the folded macromolecule. Thus, protein form is post-self-assembly, while lipid form is pre-self-assembly. In this context then, a self-assembled mesophase and a folded protein are akin on the structure hierarchy, in the same way that the individual lipid molecule is on an approximate par with the amino acids of a protein.

4. X-ray diffraction from 3-D crystals

"There is no perfect method, but if there were, it would be crystallography." This tongue-in-cheek statement has often been used by the author by way of introducing the need for crystallography, X-ray crystallography in particular, as a means for the high-resolution structure determination of membrane proteins. Of the membrane proteins for which we have atomic resolution structures, X-ray crystallography is the only method that works. It is likely that this will remain the principal structural tool in the area for some time. Assuming the protein is in hand, the rate-limiting step in the overall process of structure determination is usually crystal production. Serious problems arise because of the amphiphilic nature of membrane proteins that have at once a polar and an apolar part. The standard route to crystallization involves first removing the protein from a membrane environment. The challenge then is to find a suitable temporary hosting environment for the excised protein and a means for ratcheting it into the crystal lattice in its native conformation.

4.1. The task ahead: Much to be done

The scale of the charge to the structural biology community can be appreciated when we consider that about a third of the genome codes for proteins that have at least one transmembrane segment. Given that fewer than 50 distinct membrane proteins have yielded to high-resolution structure determination, there remains a vast amount of work to be done in the area. While protein production is one of the major stumbling blocks,

as noted, getting membrane proteins to crystallize is another. That several tens of membrane protein structures have been solved points to the fact that crystals of diffraction size and quality can be grown. However, the yield to date is negligible compared to that for soluble proteins, indicating that existing crystallization methods must be refined and recast for high throughput and that the development of new crystallization strategies is to be encouraged.

4.2. Starting material

Although the focus of this article is on crystallization, a word about the proteinaceous ingredient of the crystal is in order. As noted, suitable starting material is often in short supply. Much effort is currently devoted to increasing the yield of membrane proteins in a crystallizable form. Sources include cellular membranes that the protein calls home. Under the best of circumstances, the membrane will come enriched naturally in the target protein, as in the case of bacteriorhodopsin and the purple membrane. At the other extreme are proteins that are not at all plentiful, and enormous amounts of biomaterials, effort, and time must be devoted to procuring mere microgram quantities. The cystic fibrosis transmembrane conductance regulator (CFTR) is one such protein (Hanrahan et al., 2002). Overexpression in a host organism can be used to boost yield. However, it is not unusual for the membrane protein to weaken or kill the host cell when overproduced. One way around this is to bypass the membrane altogether and to express the protein as an insoluble cytoplasmic inclusion body (Buchanan, 1999; Padan et al., 2003). This route requires that the protein be solubilized in concentrated solutions of urea or guanidine hydrochloride and then refolded in the presence of detergents. However, the fact that such proteins never encounter a natural membrane raises questions regarding the fidelity of the structure so determined. When cloning is an option, advantage can be taken of the ability to engineer in sequences (affinity tags) and/or fusion proteins that facilitate purification as well as amino acid analogs, such as selenomethionine for phasing purposes. Including in the recombinant protein a protease site for removal of the tag or fusion protein prior to crystallization is worth considering. The recombinant approach also affords the opportunity to modify the target should the native protein prove refractory to crystallization. Such modifications include N- and/or C-terminal as well as internal sequence trimming or extension and removal of troublesome co- and post-translational modification sites. Exploring the crystallizability of homologues of the target protein in other organisms is a strategy that has proved successful (Bass et al., 2002). Often, where the perceived business end of the molecule is not in the membrane, the membrane-anchoring part of the protein is summarily dismissed and removed. This reduces the task to crystallizing a soluble polypeptide whose structure, it is hoped, will faithfully represent that of the intact membrane-associated protein. Neuraminidase is one such example (Laver et al., 1999).

This same "pruning" approach can be taken as a last resort in pursuit of at least some structural information on the more complex, multidomain membrane proteins, such as the CFTR. Domains that are not likely to be buried in the membrane can be expressed separately or excised from the intact protein and used in crystallization trials. However, functional insights gleaned from structural information so derived must be evaluated with caution.

As with soluble proteins, every effort must be made to ensure that the membrane protein used in crystallization trials is of the highest possible biochemical and conformational purity and homogeneity. Assessments of purity based on electrophoresis and light scattering are used routinely to advantage here. More recently, mass spectrometry (MS) has emerged as an important alternative and/or supplement to the more traditional analytical techniques given that it offers picomole sensitivity, high-mass accuracy, high-throughput capability, and speed, all at very low cost (Cohen and Chait, 2001). Furthermore, mass spectrometers are ubiquitous and most institutions now provide MS facilities on a routine service basis.

5. An overview of methods for crystallizing membrane proteins

There are several methods by which 3-D crystals for X-ray diffraction can be produced. These are divided here into two major categories. The first and most successful, hereafter referred to as the in surfo method, was introduced some 2 decades ago. It uses surfactants to produce mixed micelles that incorporate the target protein, residual lipid if present, and detergent. These water-soluble dispersions, with or without added small amphiphiles such as heptane-1,2,3-triol, are treated in essentially the same way as soluble proteins for the production of crystals by vapor diffusion or microdialysis. The target protein can originate from native membranes or from the membranes or inclusion bodies of recombinant organisms.

Difficulties in getting membrane proteins to crystallize by the in surfo approach have been attributed to inherent protein flexibility and to conformational inhomogeneity. At fault too can be the relatively diminutive polar surface that is simply too small to extend beyond the surfactant swath and to make molecular handshakes with neighboring proteins in the crystal. A relatively new antibody fragment method has been introduced which makes good these deficits (Hunte and Michel, 2002).

The fragments can be tailored, to a degree, to fill the aforementioned gaps and to create stable protein–protein polar contacts within the crystal. Furthermore, by using high-affinity recombinant antibodies raised against a discontinuous epitope on the native protein surface, flexibility in the protein–antibody fragment cocrystal is minimized and conformational homogeneity is favored. All contribute to producing a well-diffracting crystal. This technically challenging approach has been used successfully in structure studies of cytochrome *c* oxidase, the cytochrome bc_1 complex (with and without cytochrome *c*), and the KcsA potassium channel (Hunte and Michel, 2002).

The second category of membrane protein crystallization methods includes those that make use of a lipidic cubic phase, a discoidal lipid/detergent mixed micelle, or vesicle fusion. In all three cases, an extended bilayer composed of lipid, detergent, and target protein is presumed to form. For this reason, these will be referred to collectively here as the bilayer methods of membrane protein crystallization. Unfortunately, we know little of the molecular mechanism whereby crystals form by the assorted in surfo protocols. Accordingly, the possibility cannot be excluded that a bilayered structure also forms as an intermediate in the crystallization pathway that begins with a solution of mixed micelles in the in surfo method. The focus of the rest of this article is on the bilayer methods, with a particular emphasis on that which employs a lipidic cubic phase.

5.1. The cubic-phase method

The Rosenbusch group at the Biozentrum in Basel has been active in the area of membrane protein crystallization from the beginning and has contributed many membrane protein structures through the years (Cowan et al., 1995; Dutzler et al., 1999; Garavito and Rosenbusch, 1986). It was one of the first groups to seek an understanding of the role that detergents, and phase science in general, play in producing crystals from membrane-bound proteins by the in surfo method. Along the way, they designed and synthesized a number of now popular detergents, mapped out miscibility, and sought to exploit the corresponding multicomponent phase diagrams where micelles, mesophases, and phase separations abound (Garavito and Rosenbusch, 1986; Rosenbusch et al., 2001; Zulaof and Rosenbusch, 1983). These efforts contributed in no small measure to the general activity of the times, which led eventually to the structure determination at close to atomic resolution of the first membrane protein, the photosynthetic reaction center, by Michel, Diesenhofer, and Huber in 1985 (Deisenhofer et al., 1985). Meanwhile, the Rosenbusch team explored alternate tacks by crossing the boundary from normal to inverted phases where reversed micelles, in a bulk organic solvent, were used with the membrane protein porin (Rosenbusch, 1990; Rosenbusch et al., 2001; Wirz and Rosenbusch, 1984). Little wonder then that it was this same group that introduced yet another method for growing crystals of membrane proteins which uses the lipidic mesophase as an incubator (Landau and Rosenbusch, 1996). From a phase science point of view, it represents an extension of their previous work.

The cubic mesophase (Fig. 1) figures prominently in this, what is sometimes called the "in cubo," method. We know very little about how and why the method works (Caffrey, 2000; Nollert et al., 2001) nor indeed do we know the identity of the structure or phase that feeds *directly* the growing crystal surface. Accordingly, I prefer to refer to it as the "in meso" method, where "meso" stands for the more general, noncommittal middle or liquid crystal phase. Mesophase and liquid crystal are used synonymously.

The basic recipe for growing crystals in meso follows (Landau and Rosenbusch, 1996): (1) Mix two parts protein solution/dispersion with three parts lipid (usually monoolein). The cubic phase forms spontaneously. (2) Add precipitant/salt and incubate, and crystals form in hours to weeks. All operations are at 20 °C. It is that simple, and it works with soluble and membrane proteins (Landau and Rosenbusch, 1996; Landau et al., 1997). With appropriate modifications, it can be used to grow crystals of small organic molecules and inorganic salts (Landau et al., 1997). The basic recipe has been modified for use in high-throughput applications, as described below.

The method involves combining protein with lipid and the formation of cubic phases. Luzzati and coworkers have been doing this for some time now (Luzzati, 1997; Mariani et al., 1988), and several Swedish groups have had an abiding interest in the area, particularly from a food technology perspective (Larsson, 1994). As mammals, we likely do this naturally in the course of masticating and digesting foods (Patton and Carey, 1979).

5.2. Successes to date: Generality

The utility of in meso crystallization rests on its ability to support the production of diffraction-quality crystals. In this regard, the method has fared reasonably well. Thus far, crystals of bR, halorhodopsin, light-harvesting complex 2 (LHC2), the reaction centers from *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* (RCV), sensory rhodopsin II (SR II), and, most recently, an SR II/transducer complex have been grown in meso (Chiu, personal communication; Gordeliy et al., 2002; Kolbe et al., 2000; Luecke et al., 1998, 2001). bR, halorhodopsin, SR II, and the SR II/transducer complex represent new, high-resolution structures. Unfortunately, in meso-grown crystals of RCV and LHC2

diffracted to only 3.7 and 25 Å, respectively (M. Chiu, personal communication). The best published resolution has been obtained with bR at 1.55 Å (Luecke et al., 1998; 1.38 Å, unpublished data from H. Luecke, personal communication). In the case of bR and halorhodopsin, the process of deciphering the structure of photocycle intermediates is in progress (G. Bueldt (PDB 1CWQ) personal communication; Kolbe et al., 2000; Lanyi and Luecke, 2001; Matsui et al., 2002). As to the generality of the method, the community awaits with bated breadth for the in meso crystallization of non-bacterial proteins and of proteins that are other than compact and robust bundles of transmembranal helices. Advances along these lines will be facilitated by an increased understanding of the fundamental mechanism of crystal nucleation and growth in meso and by making it a more user-friendly and accessible method.

5.3. In meso or in cubo? Not just semantics

As noted, the cubic-phase method is based on the assumption that the protein to be crystallized is initially reconstituted into the lipid bilayer of the cubic phase. This issue will be discussed in more detail below. However, a question has been raised as to the identity of the phase that feeds the face of the growing crystal (Caffrey, 2002; Nollert et al., 2002). Further, while the monoolein/water phase diagram upon which the method is based indicates that the cubic phase is stable under conditions that approximate those used in crystallization, there are other ingredients in the crystallization mix that could potentially destabilize the cubic phase. It is for this reason that we sought to determine the identity of the bulk mesophase(s) present before and during crystal growth. Small-angle X-ray diffraction measurements, of the type shown in Fig. 4, were used for purposes of phase identification. For the most part, the prevailing mesophase was found to be indeed of the cubic type. However, this is not always the case and the phases present vary with the concentration of protein and the identity and concentration of the precipitants used (Cherezov et al., 2001). Temperature is another factor and this will be dealt with separately below. Thus, we have found that at high concentrations of added protein—bR is our test membrane protein—coexisting lamellar (L_α) and cubic phases form initially. Upon incubation with the Na^+/K^+ phosphate salt "precipitant," the L_α phase converts to the cubic phase such that crystallization happens from a bulk cubic-phase medium (unpublished observations). However, the cubic phase does not necessarily remain stable throughout the crystal growing period and can transform with time to a birefringent or a liquid phase, depending on the precipitant used.

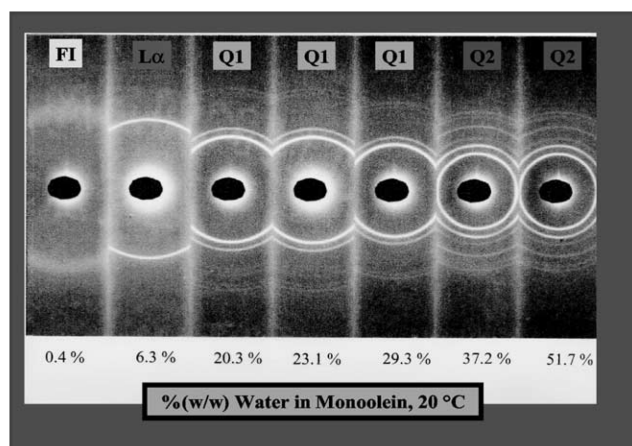

Fig. 4. Low-angle X-ray diffraction patterns of the various mesophases identified in the monoolein/water system at 20 °C. Key: FI, fluid isotropic phase; L_α, lamellar liquid crystal phase; Q1, cubic-Ia3d phase; Q2, cubic-Pn3m phase.

From this discussion, it is apparent that the exclusivity of the cubic phase as the hosting and portal medium is not cast in stone and that other mesophases, or structures reminiscent of them, may play a role. It is for this reason that the less limiting in meso descriptor will be used henceforth in this article.

5.4. Practical aspects of the in meso method

5.4.1. Salts

It has been proposed that the initial extreme and local salt-induced dehydration provides the impetus and driving force for nucleation and subsequent crystal growth in meso (Caffrey, 2002). In support of this, we have shown that the lattice parameter of the cubic phase drops upon addition of precipitant salt to the crystallization mix (Nollert et al., 2002). In addition to the lipid and detergent, the protein too must presumably give up some of its bound water during crystal formation. Protein–protein and other types of contacts may be favored to compensate for the lost water. These serve to recruit more proteins to the growing crystal face from within the mesophase. Further, since the protein is designed to span a bilayer that normally does not encounter regions of high curvature, the natural inclination is for the accreting domain to grow laterally to produce a layered structure (see A working hypothesis for how crystals grow from within the cubic phase). If the same is happening in adjacent layers, the proteins will establish contacts between lamellae and, in so doing, set up the three-dimensional lattice of the crystal. The stacking of sheets is likely favored by local dehydration since it frees up additional water for sequestration by the salt.

While much of our early work presented a consistent view of a salt- or polymer-induced dehydration, contraction of the lattice, and elevated curvature at the lipid/water interface, we have found subsequently that crystallization of bR can also occur from an expanded

cubic-phase lattice. This represents work in progress and will be reported on separately.

As will be shown below, the cubic phase has limited stability and undergoes a phase change when environmental and/or compositional conditions are adjusted suitably. Thus, it is possible that certain manipulations of the hosting mesophase in preparation for screening have the effect of destabilizing the cubic phase. It is important to note that this may not signal the end of the experiment because, as commented on previously, the cubic phase can recover when the lipid/protein dispersion is incubated with the salt or precipitant solution. This must be evaluated on a case-by-case basis.

5.4.2. Detergents

Nonionic detergents have found extensive use in solubilizing membrane proteins for subsequent characterization, reconstitution, and crystallization studies (Hunte et al., 2003; Michel, 1991). They are likely to accompany the protein into the in meso crystallization mix. As surfactants, they can wreak havoc on the lipidic mesophase which we assume is integral to growing crystals. Molecular geometry considerations suggest that the complementary molecular shapes of the popular alkyl glycoside (AG) detergents and monoolein should lead to a destabilization of the highly curved cubic phase in favor of a planar lamellar type structure in the presence of the detergent (Ai and Caffrey, 2000). Using X-ray diffraction, we have shown that relatively high concentrations of the AG dodecyl maltoside are tolerated by the cubic phase (Fig. 5). However, further additions bring about a complete transformation from the cubic to the lamellar phase (Ai and Caffrey, 2000). As will be described below, this is relevant to the mechanism of crystallization in the presence of detergents.

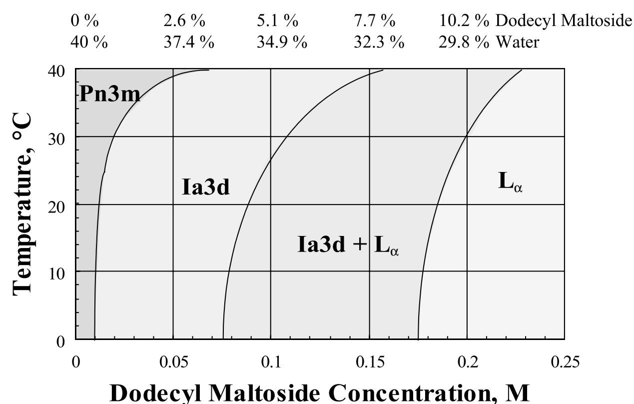

Fig. 5. Partial phase diagram of the monoolein/dodecyl maltoside/water system. The diagram was constructed using X-ray diffraction (Ai and Caffrey, 2000). Concentrations expressed in % units refer to %(w/w). Measurements were made in the heating and in the cooling direction from 20 °C. Key: Pn3m, cubic-Pn3m phase; Ia3d, cubic-Ia3d phase; L_α, lamellar liquid crystal phase.

To date, we have characterized the phase behavior of the monoolein/water system in the presence of several other commonly used AG. These include hexyl, octyl, nonyl, and decyl glucoside. The effects seen are qualitatively similar to those noted above with dodecyl maltoside in that high concentrations destabilize the cubic phase in favor of the lamellar phase (Misquitta et al., unpublished observations). The survey is being extended to include other detergents such as C8E4, C12E9, Triton X-100, 8-HESO, decyl and undecyl maltoside, sodium dodecyl sulfate, sodium cholate, sodium deoxycholate, and LDAO.

Where detergents are not a part of the in meso crystallization protocol, as in the case of the purple membrane (Nollert et al., 1999), the monoacylglycerol (in concert with membrane lipids) may serve in this capacity. Thus, for example, monoolein, which has finite water solubility and is amphiphilic, may facilitate the shuttling between the purple membrane rafts and the mesophase and eventually to the protein crystal, via transient, soluble intermediates.

Before finishing up the discussion of detergents, a word is in order regarding their quantitation. If we are to understand the molecular mechanism of membrane protein crystallization by the in meso or any other method, it is important that the exact composition of the crystallization mix be known. Most of the components are readily quantifiable and, thus, can be assayed for before addition or subsequent to their inclusion in the mix. Until recently the detergents represented an exception. These end up in the crystallization brew typically ferried in along with the protein in amounts that can vary over a wide range depending on the purification protocol and subsequent treatments. An FTIR-based assay has just appeared which would appear to make good the deficit (DaCosta and Baenziger, 2003). An alternative approach, one that is based on low-angle X-ray diffraction, is under development in the author's lab.

5.4.3. Screen compatibility

Typically, a precipitant is added to trigger nucleation and growth of membrane protein crystals in the in meso method. The commercially available screen solution series are convenient for use in such crystallization trails (McPherson, 1999). However, they contain an array of components, many of which could destroy the cubic phase. We have determined which of the Hampton Screen (50 solutions) and Hampton Screen 2 (48 solutions) series of solutions support cubic-phase formation (Cherezov et al., 2001). Small-angle X-ray diffraction was used for phase identification and for microstructure characterization. The study was done at 4 and at 20 °C. Two types of sample preparations were examined to match the conditions employed in typical crystallization screening trials. One used an excess of half-strength

screen solution (Prep. 1). The other used a limiting quantity of undiluted screen solution (Prep. 2). At 20 °C, over 90% of the screen solutions produced the cubic phase with Prep. 1. This figure dropped to 50% with Prep. 2. In contrast, 50–60% of the screens were cubic-phase compatible at 4 °C under Prep. 1 conditions. The figure fell to 25% with Prep. 2. The mode of action of the diverse screen components has been explained on the basis of the phase properties of the monoolein/water system (Cherezov et al., 2001). An evaluation of the compatibility of the other popular screen solution series is under way and will be reported on separately.

5.4.4. Other additives

As noted, urea and guanidine hydrochloride are commonly used to solubilize membrane proteins from inclusion bodies. While they are usually gotten rid of ahead of crystallization screening, it was of interest to know how the standard monoolein/water cubic-phase mix used in in meso crystallization would respond to high levels of such additives. Using small-angle X-ray diffraction, we have demonstrated that both additives can expand considerably the lattice of the cubic phase without inducing a phase transformation (Fig. 6). Further, the process is reversible. As will be described below, the cubic phase is porous and supports transport in its aqueous and apolar compartments. In separate studies, we have shown that the cubic phase loaded with urea and highly expanded readily loses the denaturant and contracts when equilibrated with water. This suggests a route to simultaneous renaturation and reconstitution into the cubic phase of membrane proteins for direct use in in meso crystallization. This approach eliminates steps in which the protein is exposed to potentially damaging detergent prior to and during crystallization.

5.5. Cubic-phase metastability, crystallization at 4 °C, and lipid rational design

The liquid crystalline phases are notorious in their capacity to undercool—in the same way that bulk water remains liquid below 0 °C when cooled appropriately. This property is responsible for the differences seen below ~17 °C between the two monoolein/water phase diagrams in Fig. 2, in which one represents equilibrium (Fig. 2A) and the other (Fig. 2B), metastable behavior. In the latter, the metastable cubic and lamellar phases can persist down to 0 °C (Fig. 2B) for years. What makes long-lived metastability potentially attractive from the point of view of crystallization in meso is the possibility of retaining the cubic phase to low temperatures. The proteins crystallized in meso thus far are robust and were crystallized at 20 °C. For more labile proteins, it might be desirable to work at lower temperatures. With the monoolein system, it should be possible to perform in meso crystallization in the 0–4 °C range at which the cubic phase persists under metastable conditions (Fig. 2B). However, this is not reliable because the system is thermodynamically unstable and can convert at any time to the equilibrium solid or lamellar crystalline (Lc) phase, which is unlikely to support protein crystal growth. Indeed, protein crystal formation in the metastable cubic phase may itself trigger lipid solidification and terminate the crystallization process. There exists therefore a need for a lipid system that produces the cubic phase that is at equilibrium under conditions of full hydration and that will support in

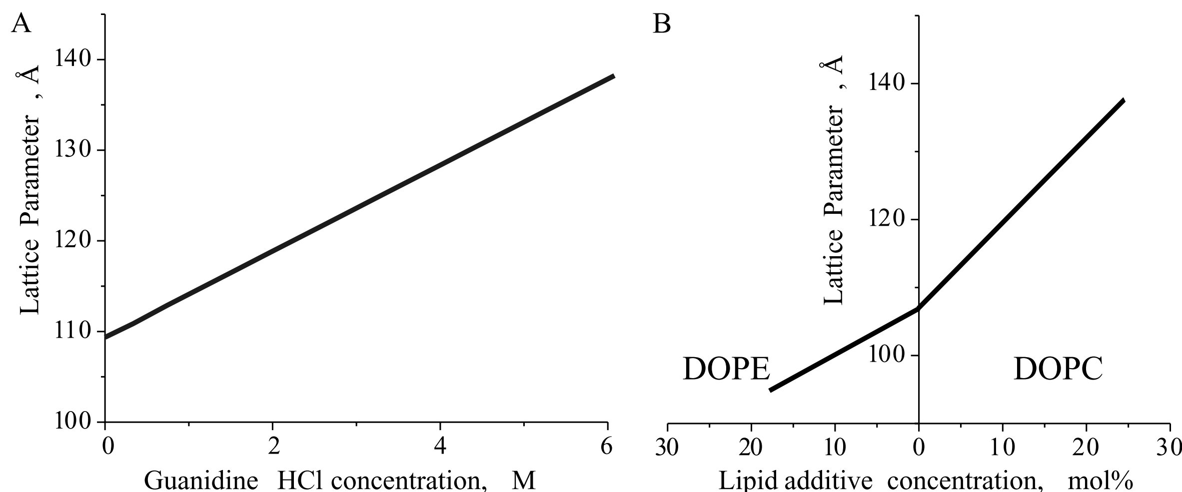

Fig. 6. Dependence of the lattice parameter of the cubic-Pn3m phase of hydrated monoolein on denaturant (A) and lipid (B) additive concentration. The dependence shown in A is for guanidine hydrochloride. A very similar dependence has been recorded for urea (unpublished observations). The dependence shown in B for the lipid additives dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) is approximate and is based on literature data (Cherezov et al., 2002).

meso crystallization at temperatures below ambient, 4 °C for example.

As noted, we have a program under way the objective of which is to establish how lipid molecular structure conspires with sample composition and environmental factors to define phase properties and thus, functionality (Fig. 3). A small but growing database of phase behavior for a homologous series of monoacylglycerols is in hand from which we hope to glean the rules for use in rational lipid design. The need identified in the last paragraph provides us with the opportunity to put these emerging principles to work. Accordingly, we have rationally designed a monoacylglycerol with a suitably long acyl chain and an aptly positioned double bond in the chain that should fit the bill. The lipid has been synthesized and purified and its phase behavior characterized. In agreement with the design specifications, it forms an equilibrium cubic phase that is stable at 4 °C under conditions of full hydration. Trials are in progress to determine the lowest temperature at which crystals of membrane proteins will grow in this rationally designed lipid.

5.6. Engineering cubic-phase microstructure to match the target protein

In the context of in meso crystallization, it is important to appreciate that the microstructure of the phase can change with temperature, sample composition, and lipid identity (Luzzati, 1968; Qiu and Caffrey, 2000). By microstructure is meant the lattice parameter of the phase and how it is constituted. Thus, the lamellar phase, for example, consists of planar sheets of lipid bilayers each separated by a layer of water (Fig. 1). As temperature, composition, and lipid identity change, the thickness of the lipid bilayer as well as that of the water layer can change. The same holds for the other mesophases, including the cubic phase (Fig. 7).

Hydrated monoolein in the cubic phase at 20 °C may provide a suitable matrix in which to grow membrane protein crystals. However, dropping temperature to 4 °C, while preserving the cubic phase as a result of metastability, will cause the lattice parameter to change and, along with it, the dimensions of the lipid bilayer (Fig. 7A) and the water channels (Fig. 7B) of the cubic phase (Briggs et al., 1996; Qiu and Caffrey, 2000). It is possible that such changes may no longer ensure retention of protein activity or support crystal growth for a host of reasons. By the same token, it may well provide an even more stabilizing and a better crystal-growing environment. We are currently quantifying the effects that lipid and water compartment sizes of the cubic phase have on the stability and crystallizability of several membrane proteins.

Reference has just been made to the sensitivity of phase microstructure to lipid identity. Support for this statement is based on X-ray diffraction measurements performed on the cubic phase of a homologous series of monoacylglycerols (Fig. 7). The data show expected behavior in that as chain length decreases so too does the thickness of the lipid layer that creates the apolar fabric of the cubic phase, when evaluated at a single temperature (Fig. 7A). Less intuitive perhaps is the finding that the aqueous channel diameter drops as chain length increases. This is consistent with a "flattening" and an attenuating curvature at the polar/apolar interface with the longer chained lipids.

While lipid identity can be used to tailor phase microstructure, it is possible that the desired microstructure might not be accessible with a single lipid species in

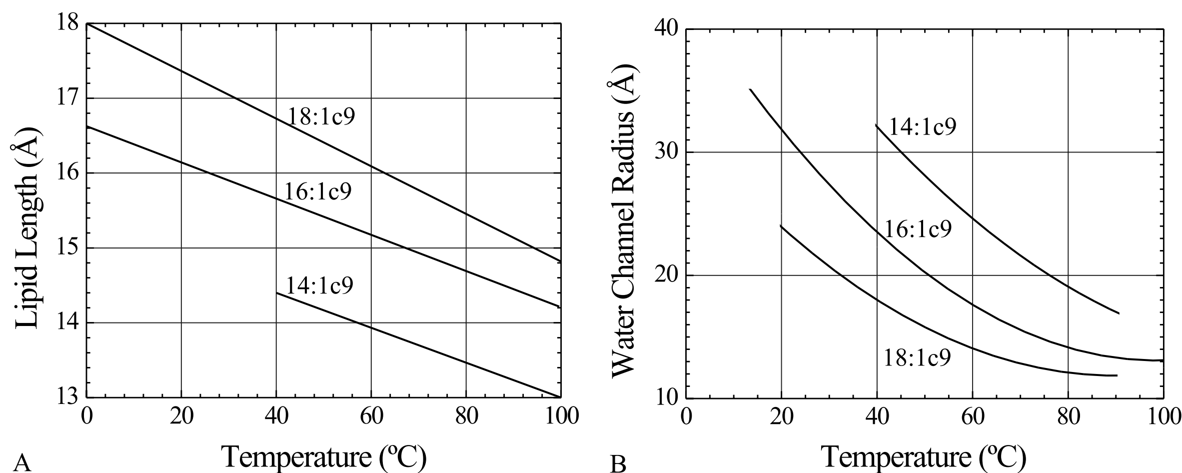

Fig. 7. Temperature-induced changes in the lipid and aqueous channel dimensions of the cubic phase. Temperature dependence of the lipid length in the cubic phase (A) and of fully hydrated, cubic-Pn3m phase water channel radius (B) for three monoacylglycerols. Lipid identity is given in shorthand, in which the first two digits indicate fatty acyl chain length in number of carbon atoms and the last digit refers to the position of the *cis* double bond along the chain in which the carbonyl carbon is No. 1. Monoolein is represented by 18:1c9. Data are from Briggs (1994), Briggs and Caffrey (1994a), and Briggs et al. (1996).

the temperature range of interest. In this case, it is possible to fine-tune by using mixtures of monoacylglycerols with different acyl chain lengths in which the mole ratio is adjusted to set microstructure at the desired intermediate value.

It is apparent from the data just presented that it is possible to engineer the microstructure of the mesophase over relatively wide limits by manipulating temperature and/or lipid identity and composition. However, it is also important to note that the two metrics of the cubic phase—the polar and apolar compartment dimensions—are not independently adjustable and indeed are tightly coupled, as indicated in Fig. 7. Nonetheless, this feature of tunability is an important tool that is available to the crystallographer in search of a suitable lipid matrix in which to grow crystals. Thus, proteins with extra-membranal domains that come in a variety of sizes can be accommodated as can those that originate from native membranes with different hydrophobic thicknesses (Munro, 1998).

5.7. Creating a more natural membrane setting

Hydrated *cis* monounsaturated monoacylglycerols form the cubic mesophase that has been used for in meso crystallization of membrane proteins. To date, monoolein (C18:c9), monopalmitolein (C16:9) (Landau and Rosenbusch, 1996), and monovacennin (C18:c7) (Gordeliy et al., 2002) have served in this capacity. It is possible that the hosting cubic phase created by monoacylglycerol alone, which itself is a most uncommon 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 and to making the hosting cubic phase more "familiar" to its guest protein, the degree to which the reference, cubic-Pn3m phase formed by hydrated monoolein can be modified by other lipid types was examined by X-ray diffraction (Cherezov et al., 2002). These included phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine, cardiolipin, lyso-PC, a polyethylene glycol-lipid, 2-monoolein, oleamide, and cholesterol. The study showed that all nine lipids were accommodated in the cubic phase of (1-)monoolein 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 data showed that some lipids caused the cubic-phase unit cell to expand while others induced a contraction (Fig. 6B). The changes in phase type and microstructure were rationalized on the basis of lipid molecular shape, interfacial curvature, and chain packing energy. These results should prove useful in rationally designing cubic-phase crystallization matrices with different lipid profiles that match the needs of a greater range of membrane proteins. It is important to realize too that while a lipid additive may destabilize the cubic phase, the latter can be recovered upon incubation with certain precipitant solutions, as noted.

5.8. Different cubic-phase types

There are several different cubic-phase types as indicated in Fig. 1. Each has its own characteristic low-angle diffraction pattern (Fig. 4) which is used to index and to identify the corresponding space group (Pn3m, Ia3d, Im3m, etc.). It is not clear which of the bicontinuous cubic phases is required for crystal growth. Certainly, the cubic-Pn3m phase predominates under conditions under which crystals grow (Nollert et al., 2001). However, we have seen systems in which the initial phase is of the cubic-Ia3d, cubic-Im3m, or cubic-Pn3m phase type. Indeed, mixtures of these phases and mixtures that include the lamellar phase have been observed that transform into the cubic-Pn3m phase upon incubation with precipitant and subsequent to crystal growth. One of our goals is to establish which of the assorted cubic phases is/are compatible with crystal growth and if compatibility is membrane protein specific.

5.9. Dealing with samples having a low protein concentration

The driving force for nucleation is greater the more supersaturated is the protein solution. Thus, a common strategy in crystallization is to work at the highest possible protein concentration to favor nucleation and to lower its concentration subsequently to just above the solubility limit for slow, orderly growth of a few good-quality crystals. It is likely that the same principles apply to crystallization in meso in which initially, the highest possible protein concentration should be used to favor nucleation. There are at least two issues that must be dealt with in this context that apply to membrane proteins. First, most membrane proteins are prepared and purified in combination with detergents. Thus, the detergent is carried along with the protein into the crystallization mix. It follows then that as the protein concentration increases, the detergent concentration will rise in parallel. This may work against crystallization because high levels of detergent destabilize the hosting mesophase (Fig. 5). Of course, the sensitivity to added detergent will depend, among other things, on the detergent type. Completely removing the detergent before folding the protein into the crystallization mix is not an option because it is needed to keep the protein soluble as a mixed micelle. One alternative is to reduce the

detergent load to an acceptable level before combining the protein with the hosting lipid, using Bio-Beads (Rigaud et al., 1997) for example. Another involves depleting the mesophase of its acquired detergent after the reconstitution step. Both strategies are currently under investigation in the author's lab.

The second issue has to do with raising the concentration of protein in the lipid bilayer of the cubic phase to facilitate nucleation. Two approaches come to mind that are quite different but that achieve this same end. The first exploits the water-carrying capacity of the cubic phase. This is a property that varies with lipid identity (Briggs and Caffrey, 1994a,b; Briggs et al., 1996; Misquitta and Caffrey, 2001; Qiu and Caffrey, 1999, 2000). Thus, the reconstituted protein will be more concentrated in the bilayer of the cubic phase prepared with a lipid of high water-carrying capacity than would obtain for a less hydrating lipid. The second approach involves sequential reconstitutions in which the protein concentration in the bilayer rises with each round. Both strategies are under investigation in the author's lab.

5.10. Exploiting transport in the cubic phase for cocrystallization, drug design, phasing, and cofactor retention

The cubic phase consists of a highly convoluted, continuous lipid bilayer that separates two interpenetrating but noncontacting aqueous channels (Fig. 1). It can be viewed as a molecular sponge. As with the familiar household sponge, it can be used equally well for absorption and release. The cubic phase has two compartment types. One is apolar, defined by the hydrocarbon chains of the lipid. The other is polar and water-filled. We are interested in the transport properties of the cubic phase from three perspectives. The first concerns release with applications in the area of controlled drug delivery. The second has to do with movement within the cubic phase as occurs during the crystallization process. Thus, what starts out as a uniformly pink/purple cubic phase prior to bR crystallization ends up as dark purple-colored crystals dispersed in a relatively colorless cubic phase (Fig. 8). This bears witness to the ability of the cubic phase to support directed movement of the protein, presumably in the lipid bilayer of the bulk cubic phase, to the face of the crystal. Another aspect of transport of relevance to crystallization is the diffusion of small molecules and ions between the cubic-phase bolus and the reservoir of precipitant solution. The inherent porosity of the cubic phase facilitates the process, allowing for gradients to form that drive nucleation and crystal growth. The final aspect of transport that impacts on crystal production concerns the use of the cubic phase as a medium for porting in small molecules as in drug discovery applications or heavy atoms for use in preparing isomorphous derivatives. Because the cubic phase has polar and apolar compartments with transport possible in both, it should be possible to diffuse in small- to medium-sized molecules covering the full range of polarities, including amphiphiles, for use in co- or complex crystal production. It should be noted that the aqueous channel size of the cubic phase of hydrated monoolein at 20 °C is sufficiently large to accommodate reasonably sized proteins. For illustrative purposes, a scaled drawing is presented in Fig. 9, in which lysozyme is seen to fit nicely into the aqueous channel. Ongoing studies in the author's lab are concerned with characterizing the diffusion of such proteins in and out of the cubic phase. These features indicate that it should be possible to use the in meso approach for the cocrystallization and structure determination of multicomponent complexes as suggested (Caffrey, 2002). Such complexes might include transmembranal signaling proteins in association with their soluble transducer counterparts.

Membrane proteins can lose covalently and noncovalently bound cofactors in the process of isolation, purification, and crystallization. The bacterial photosynthetic reaction center is an example of the latter, in which the secondary ubiquinone site appears partially filled in the crystal structure unless special precautions are implemented (Fritzsch et al., 2002). The cubic phase offers the possibility of minimizing, or perhaps even reversing, such losses when the cofactor is incorporated into the cubic phase in molar excess before and/or during the protein reconstitution step. The beauty of the cubic phase is that it can accommodate polar, apolar,

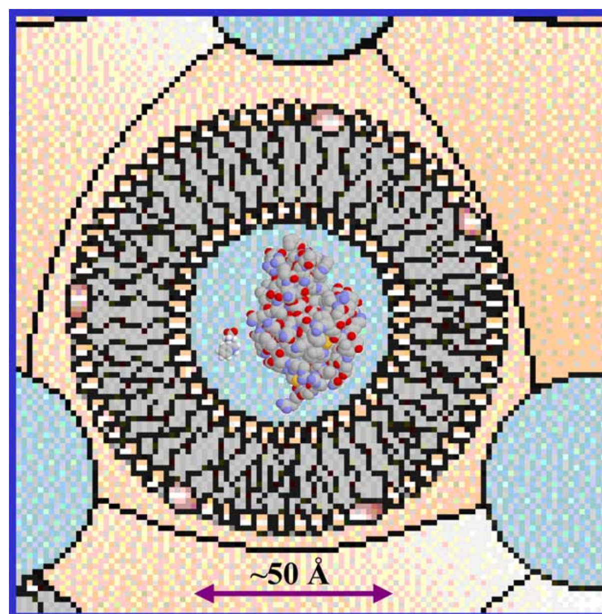

Fig. 9. Expanded view of one of the aqueous channels (light blue) in the cubic phase accommodating lysozyme (right) and tryptophan (left) drawn to scale. Lipid molecules forming the bilayer surrounding the aqueous channel are shown with round polar head groups (light brown) and squiggly hydrocarbon tails (black).

and amphiphilic cofactors. Further, the cubic phase is forgiving in that it is stable in the presence of sizable amounts of an assortment of additives (see Other additives, above; Ai and Caffrey, 2000; Cherezov et al., 2001, 2002).

5.11. Microcrystals

The original method for growing crystals in meso produced very small crystals. Indeed, it was necessary to use a microfocused X-ray beam for data collection, the crystals were that small. It is interesting to enquire as to what might limit the size of the crystal given that the cubic phase is composed of a single lipid bilayer and, thus, presumably a single protein reservoir. While it is true that a single continuous bilayer permeates a given domain within the cubic phase, the macroscopic sample itself is usually composed of multiple microdomains. Evidence in support of this statement comes from the observation that the cubic phase typically produces a powder diffraction pattern (Fig. 4). However, it is not uncommon for the same phase to produce a spotty diffraction pattern, the hallmark of large "crystallites" within the sample. Indeed, the crystallites can be so large as to produce just a few reflections, which can lead to problems identifying the space group of the cubic phase. It is not clear what form the lipid/water dispersion takes at the surface of a domain nor indeed the nature of the communication between domains. If movement of protein were confined to a given domain, then the domain size and its protein payload would ultimately limit crystal size. Back-of-the-envelope calculations show that a 10 mg/ml protein solution will produce a dispersion with $\sim$5 mg protein/ml of cubic phase. Further, if the in meso grown crystal is 50% protein by weight, then a 100-fold concentration of the protein is required in going from cubic phase to crystal. Thus, a spherical microdomain of cubic phase with a diameter of 100 μm is required to produce a single 4- to 5-μm diameter (spherical) crystal. These few model calculations demonstrate that relatively large domains and/or interdomain diffusion is needed to produce reasonably sized crystals. Currently, we are using microdiffraction to measure directly domain size and are exploring ways to increase domain size and interdomain diffusion with a view to producing larger, diffraction-quality crystals.

5.12. Radiation damage

The fact that small crystals are commonly produced by the in meso method (Fig. 8), and particularly when performed with nanoliter volumes of cubic phase (Fig. 8C) as described below, diffraction measurements must be made with synchrotron radiation. This naturally collimated source of X-rays often delivers an overabundance of photons (per unit time and area) that radiation damage even cryocooled crystals (Ravelli et al., 2002). A recently introduced method takes advantage of the damage-induced structural changes to aid in the phasing part of structure determination (Ravelli et al., 2003). The utility of this novel approach in membrane protein crystallography is under investigation using in meso-grown crystals.

5.13. Seeing without color!

The in meso approach is particularly suited to pigmented proteins. Their crystallization leads to obvious colored crystals in a colorless background. With colorless proteins the fear is that the crystals, and particularly microcrystals, would go undetected. This has been tested by crystallizing thaumatin, a "colorless" (water-soluble) protein, in meso. Clearly visible thaumatin crystals grew from the cubic phase (Fig. 10). The exercise shows that the in meso method is not limited to colored proteins. In this sense, it does not distinguish itself from the other crystallization methods as applied to membrane and soluble proteins.

As with standard crystallization techniques for soluble proteins and the in surfo method for membrane protein crystallization, there are additives that can be used and modifications made to render colorless crystals more visible in meso. As noted, the cubic phase is porous and dyes can be added to the surrounding medium for diffusion into the mesophase for crystal labeling. A range of conditions should be explored because the particular dye concentration for optimum contrast between crystal and background will vary with dye and protein type and with the prevailing conditions. If desired, proteins can be covalently tagged with highly absorbing or fluorescing tags prior to crystallization and can be engineered to include proteinaceous

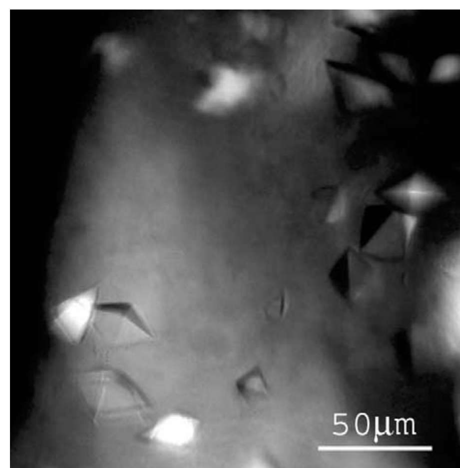

Fig. 10. Seeing "colorless" crystals grown in the cubic phase. A polarized light microscopic image of crystals of the colorless protein thaumatin, growing from the cubic phase of hydrated monoolein.

chromophores such as the assorted intrinsically fluorescent proteins (Zhang et al., 2002). In all cases, the effect of the modification on the structure and activity of the protein must be evaluated. The lactose permease from *Escherichia coli*, which to date has resisted high resolution structure determination, was rendered dark red by splicing in cytochrome b_{562}. In this case, the modification had little effect on permease activity (Prive and Kaback, 1996).

5.14. Quality control: Spectroscopy

Direct spectroscopic measurements on the cubic phase can be used to monitor the activity and conformation of the protein prior to and during crystallization in the spirit of quality control. This requires that homogeneous and optically clear samples of cubic phase be prepared in bulk in a cuvette (Hochkoeppler et al., 1995; Portman et al., 1991). That this is possible is demonstrated convincingly in Fig. 11. The transparency and optical isotropic properties of the cubic phase make it invaluable as a matrix in which to investigate the crystallization mechanism. UV–visible absorption, fluorescence, and circular dichroism are used on a routine basis in the author's lab by way of characterizing proteins in the cubic phase. Longer wavelength spectroscopies such as infrared are not compromised by light scattering and no special sample preparation protocols are needed in such applications.

5.15. High-throughput crystallization: Miniaturization and robotization

Crystallization trials usually involve vast numbers of samples. As a result, there is a need to miniaturize or scale down by way of reducing the amount of protein, precipitant, and space needed, all of which can come at a premium. Given the numbers involved, there is also an incentive to robotize the process. Added benefits of automation include sample-to-sample uniformity and the sanity of the crystal grower.

The biggest hurdle that must be overcome in robotizing the in meso method is the extreme viscosity of the cubic phase itself. It has the consistency of a thick toothpaste or Vaseline. It cannot be manipulated with standard liquid-handling devices of the type used in commercial crystallization robots. As part of our lipid-phase studies program, we developed an inexpensive mixing/delivery device, mostly from commercially available parts, that lends itself nicely to automating the in meso method (Cheng et al., 1998; Fig. 12). It was designed to work with milligram quantities of lipid with minimal waste, to facilitate the handling of viscous mesophases, and to have control over sample composition. The device is simple to build and to use and has been implemented in in meso crystallization trials and other applications in laboratories worldwide. Large-volume syringes can be employed to prepare hundreds of milligrams of cubic-phase/protein dispersions in a single step. The typical in meso crystallization screen performed in the laboratory currently uses 70 nl of protein-loaded cubic phase. The bolus is sealed, along with 1 μl of precipitant solution, on a glass slide, which greatly facilitates monitoring crystal growth by light microscopy. Seventy nanoliters of cubic phase represents ~300 ng of protein per screen. The samples are stored in temperature-controlled incubators for crystallization, which occurs, in the case of bR, in anywhere from half a day to several weeks. The crystals shown in Fig. 8C were grown using this system and ~5 nl of cubic phase representing ~20 ng of protein. Since the lipid mixing/delivery device was designed with a minimum of dead volume, 1 mg of protein can be used to perform comfortably many thousands of in meso crystallization screens. With automation, this can easily be done in a standard laboratory situation in a day. A robot to do

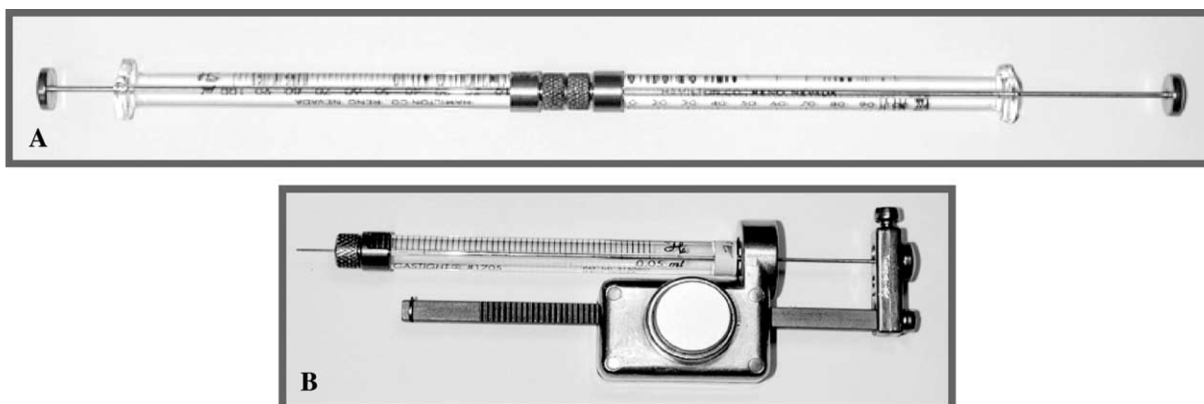

Fig. 12. Device for mixing and delivering viscous lipid/protein samples of the type used in in meso and other forms of membrane protein crystallization. (A) Coupled glass microsyringes are used for preparing homogeneous samples of defined composition. A complete description of the device and its construction has been reported (Cheng et al., 1998). (B) After homogenization, the lipid/protein sample is transferred to one of the syringes in A and is used in combination with a commercially available (Hamilton) mechanical dispenser for repeated delivery of small sample volumes.

just that is under construction (Muthusubramaniam, Zhang, Cherezov, and Caffrey, unpublished data).

5.16. Harvesting crystals grown in meso

How are in meso-grown crystals harvested? This is one of the most frequently asked questions about the in meso method. As noted, the cubic phase is notoriously viscous and the commonly held view is that it is virtually impossible to manipulate. It is true that it is viscous but with the right tools (Cheng et al., 1998) it can be handled with ease, as already noted. In a similar vein, harvesting crystals grown in meso is relatively straightforward—with the right tools. These consist of needles and spatulas of various size and shapes that are used to open up the bulk mesophase and to expose a crystal or to move a crystal to the surface. Once exposed, it can be picked up with a standard cryoloop and immediately flash cooled. Under these conditions, the lipid from the mesophase will solidify, and typically it produces continuous powder diffraction rings in the vicinity of 4 Å. This diffraction arises from the closely packed acyl chains within the lamellae of the lipid crystal. With a view to minimizing interference with diffraction data from the protein crystal, obviously the less mesophase that accompanies the crystal into the cryoloop the better. Because crystals grown in meso are often small, harvesting is best done with the aid of a reasonably high-power microscope.

Performing the harvesting manipulations in a humid atmosphere reduces the likelihood of the phase desiccating and triggering a phase transformation. This could lead to destruction of the crystal. An inexpensive household humidifier serves the purpose but a ring of moist paper towels around the microscope stage works just as well. If desiccation happens and the phase goes lamellar (L_α), as is expected with reference to the monoolein/water phase diagram (D in Fig. 2B), the crystal may no longer be visible, particularly if manipulations are being conducted under crossed polarizers. While the cubic phase is optically isotropic and usually transparent, the L_α phase is birefringent and can be highly scattering and opaque. It is also possible, if the L_α phase forms, that it remains transparent. In this case, it is easy to retrieve the crystal because the lamellar phase is considerably less viscous than the cubic phase. This feature can be used to advantage as indicated below.

It is possible to digest away the lipid and to fluidize the cubic phase by treating it with a commercially available lipase (Nollert and Landau, 1998). This makes the crystal easy to harvest. In the enzymatic hydrolysis, the monoacylglycerol is converted to a long-chain fatty acid and glycerol, itself a convenient cryoprotectant. bR crystals do well when subjected to this type of treatment. However, crystals of other proteins will require individual evaluation. For example, certain precipitants and other crystallization conditions may interfere with the lipase. Further, the alkali metal (sodium, potassium, etc.) salts of long-chain fatty acids are detergents and have the potential, if present, to destroy the crystal. In contrast, the alkali earth metal (calcium, magnesium, etc.) salts are insoluble waxes and create their own set of problems.

Luecke et al. (1999a,b) have used an excess of detergent (octyl glucoside) to assist in the harvesting of crystals grown in meso. In this case, the octyl glucoside (OG) triggers the expected transition to the less viscous lamellar phase (see Fig. 5) from which crystal retrieval is straightforward. The process should be expedited, as the crystals will dissolve if exposed to detergent for too long.

It is also possible to perform in meso crystallization in glass or quartz X-ray capillaries or in cryoloops. In either case, there is no need to isolate the crystal for separate mounting, and diffraction data can be recorded directly.

5.17. A working hypothesis for how crystals grow from within the cubic phase

We understand little about how in meso crystallization comes about. However, crystals of a host of materials have been grown from what starts out primarily as a protein-laden cubic phase. Given that we know the initial and end states, it is reasonable to speculate as to how we get from one to the other (Fig. 13). If we use bR as an example, the end state is a crystal with proteins arranged in sheets. The starting condition has bR dispersed in an aqueous micellar solution to which residual purple membrane (PM) lipid remains bound. The protein presumably is cummerbunded with solubilizing OG detergent around its hydrophobic midsection. The dispersion is then combined with lipid (monoolein) by mechanical mixing to produce a concoction with the following approximate overall composition: water/monoolein/OG/PM lipid/bR 300,000/15,000/750/10/1 by mole (Nollert et al., 2002).

Let us now contemplate the fate of the different components in the system (neglecting buffer, salt, etc.) up to crystallization. The detergent has high aqueous solubility. But it is also amphiphilic, with a proclivity for hydrophobic spaces and surfaces. At the ratio of monoolein-to-water used typically (40% (w/w) water), the cubic phase is stable (A in Fig. 2B). It incorporates a continuous, highly convoluted cubic membrane (a lipid bilayer) which separates two interpenetrating but noncontacting aqueous channel networks (Fig. 1). Both the polar aqueous and the apolar bilayer interior serve as compartments into which the detergent will partition. But the detergent is also drawn to the hydrophobic surface of the protein on which it piggybacked into the mix in the first place.

Typically, the aqueous protein/detergent mixed-micelle solution is combined with dry monoolein at 20 °C.

Upon initial contact, water will migrate from the solution into the monoolein. In so doing, it will establish a water activity gradient along which a series of phases will form. The sequence of phases is the same as is found along the 20 °C isotherm in the monoolein/water phase diagram (Fig. 2B). At low hydration levels, the first liquid crystal phase to form is of the lamellar type (D in Fig. 2B). This gives way to the cubic phase with increasing hydration (C in Fig. 2B).

As water leaves the aqueous protein solution for the dry lipid, there is a corresponding increase in the concentration of detergent and protein (etc.) in the residual aqueous solution. This will favor the formation of a lamellar phase by reference to the OG/water phase diagram (Warr et al., 1986). At the same time, the slightly soluble monoolein will partition into it, facilitated no doubt by the detergent. In this way, the mixed detergent/protein micelle will acquire monoolein. With reference to the monoolein/AG/water phase diagram (Fig. 5) and the section on detergents above (Section 5.4.2), we see that high concentrations of OG again favor the lamellar phase and it is likely that the protein now finds itself in a monoolein-enriched micelle with strong lamellar tendencies (Fig. 13). Close by, bulk monoolein is giving way to a local lamellar phase and it is possible that the two fuse. This produces a bilayer that is continuous into the bulk cubic phase and in which the protein is now reconstituted. Vectoral orientation and oligomerization of the protein within a given layer may be imposed at this stage in the process. As the system approaches equilibrium, defined by the temperature and the overall composition of the sample, the lamellar phase transforms into the bulk cubic phase. This condition prevails at low protein loads at which the concentration of adventitious detergent is also low. Since the lipid bilayer is continuous throughout, and the protein and detergent can diffuse within it, both are likely to distribute in the cubic phase. Consistent with this is the observation that in the case of bR, the phase adopts a homogeneous purple hue at this stage in the process.

In this state, the protein has been removed from the potentially hostile environment of a detergent micelle. It is stabilized to a degree in the lipid bilayer of the cubic phase with physical–chemical properties (lateral pressure, hydrophobic matching, etc.) more akin, but not identical, to that of the native membrane.

We now turn our attention to the protein crystallization process. For nuclei to form and for crystals to grow, it is required that the system be perturbed in some way. Not unlike crystallization protocols for soluble proteins, typically this involves adding salt (McPherson, 1999). What is it that salt does to trigger crystal nucleation and growth? Again, we have no hard answers here although a program is under way to get them, as noted (Cherezov et al., 2001; Nollert et al., 2002). We know that the salt will compete with the lipid (and the detergent and protein) in the cubic phase for available water. This is accompanied on occasion by deliquescence and the creation of a salt-saturated liquid (Caffrey, 2002). The water-withdrawing effect of the salt causes the cubic lattice to contract and for bilayer curvature to rise (Cherezov et al., 2001; Chung and Caffrey, 1994a,b; Nollert et al., 2002). This may be perturbation enough to herd the proteins into a lipidic corral where they crowd together, associate, and eventually organize into a crystal lattice. All the while, the salt dissolves in the aqueous medium. By shielding charges on the protein, the elevated ionic strength should also facilitate close protein contact, nucleation, and crystal growth. The dehydrating effect of the salt, if sufficient in degree, can induce transient and local lamellar phase formation (D in Fig. 2B; Fig. 13). Whether nucleation and growth take place directly in/from the cubic phase and without the involvement of any other, possibly local, intermediate structure or phase is not known. This is what I refer to as the postulated portal question. It is being examined in a number of ways, including diffraction with a nanometer-sized X-ray beam (Cherezov et al., 2000).

The original in meso method called for salt to be introduced by spinning (centrifuging) it as dry crystals through the viscous cubic phase. In its passage, the salt crystal leaves a track of variously dehydrated mesophase and salt. This multicomponent and multidirectional gradient in the mesophase(s) provides a vast and continuously varying range of conditions that evolve over time, serving to increase the probability of the protein experiencing just the right conditions for nucleation and crystal growth. The latest incarnation of the method has a bolus of protein-laden cubic phase injected into a droplet of precipitant solution which is then incubated in a sealed well for crystallization. In this case, the lower molecular weight components of the precipitant solution and the cubic-phase bolus will diffuse into one another. In so doing, spatial and temporal concentration gradients form within the bolus which, as above, serve to increase the likelihood of accessing conditions for crystallization.

As crystals grow, the mesophase loses color (Fig. 8). This is a process that happens over a period of hours to weeks in the case of the purple-colored bR. Presumably, during the course of growth the crystal is being fed by protein diffusing in the continuous lipid bilayer through the mesophase to be ratcheted into place at the growing face of the crystal. This suggests the existence of a tethering portal structure between the bulk mesophase and the crystal surface. We are actively pursuing the identity of such a conduit using, among other things, X-ray diffraction with nanometer-sized beams, as noted (Cherezov et al., 2000).

The hypothesis presented above is currently being tested and refined.

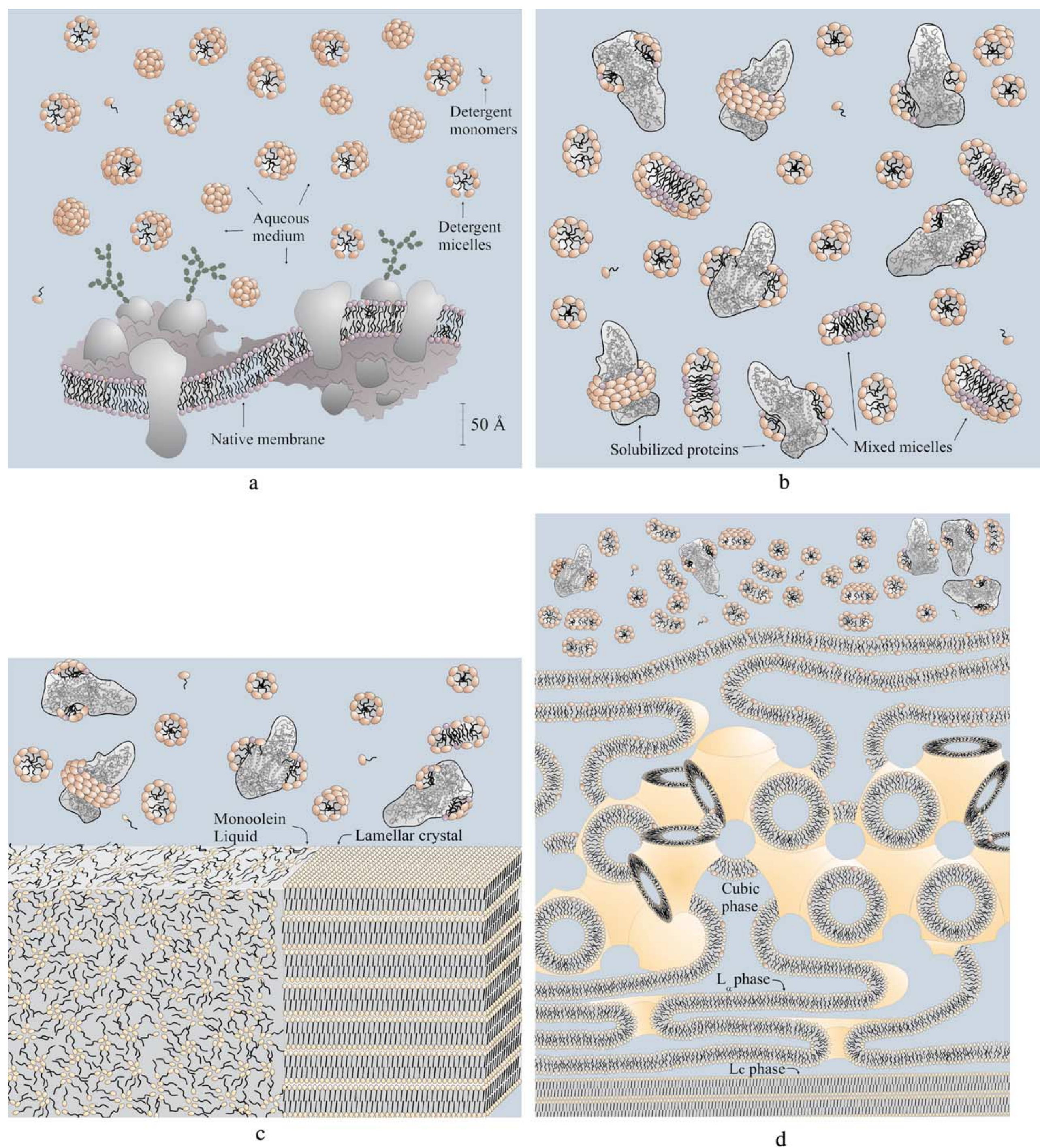

Fig. 13. Solubilization, reconstitution, and in meso crystallization of membrane proteins. Cartoon representation of the events taking place during solubilization (a, b), reconstitution (c–f), and in meso crystallization (g, h) of an integral membrane protein as outlined in the text. As much as possible, the dimensions of the monoolein (light brown), detergent (pink), native membrane lipid (purple), protein (gray; RCV, PDB 1PRC (Deisenhofer et al., 1995)), bilayer, and aqueous channels (blue) have been drawn to scale. (a) Isolated biological membrane in the presence of detergent micelle solution. (b) Solubilized membrane proteins exist as mixed micelles in solution. (c) Combining solubilized protein solution with monoolein in the fluid isotropic and lamellar crystal phases. (d) Hydration of monoolein to form contiguous L_α and cubic phases. Limited detergent and monoolein exchange occurs. (e) Protein reconstitution and dispersion in mesophases. (f) Reconstituted protein in bilayer of cubic phase. (g) Addition of precipitant (salt) to initiate crystallization by water-withdrawing and charge-screening effects. Bilayer curvature in cubic phase increases as water content drops. (h) Reversible crystallization of protein (and bound lipid, in the case of cocrystallization) from cubic phase through lamellar portal.

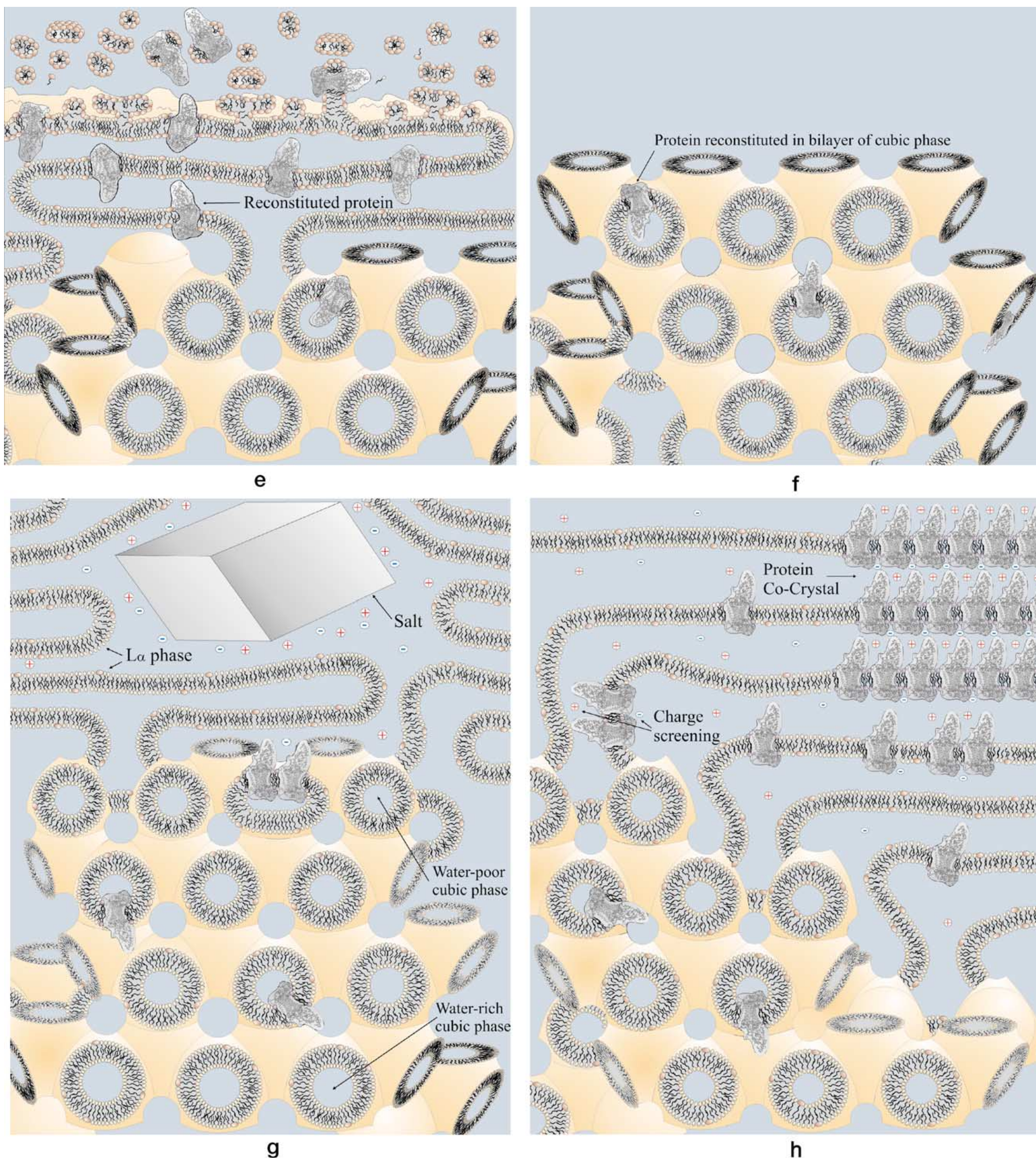

Fig. 13. (*continued*)

6. The bicelle method

This new approach for crystallizing membrane proteins was announced in February 2002 (Faham and Bowie, 2002). The test protein was bR. The method requires that the protein be reconstituted into flattened or discoidal micelles, also known as bicelles. Bicelles can be viewed as bilayered disks in which the perimeter of the disk is coated with detergent. The crystallization recipe, not unlike that for the in surfo and in meso methods, is relatively simple. A bicelle solution is prepared by mixing dimyristoyl phosphatidylcholine (DMPC) with the detergent Chapso (a zwitterionic sterol), in water. Bicellarization is facilitated by repeated vortex mixing and sonication and by heating and cooling the sample. At and below room temperature, the

bicelle solution is a clear, colorless liquid. Above room temperature (32–50 °C), it forms a clear, colorless viscous gel. The bicelle solution is next combined with the protein (in this case, bR as purple membrane) on ice in the liquid phase to facilitate mixing. This represents the protein reconstitution step. Crystallization is performed by standard vapor diffusion in hanging- or sitting-drop mode. This is achieved by combining the bicellarized protein solution with reservoir solution followed by incubation in a sealed well in equilibrium with the reservoir solution. Crystals were found to grow at room temperature and at 37 °C. The best diffracting crystals were obtained at the higher temperature. It is interesting to note that while crystals grew under the standard protocol conditions just described, larger crystals were obtained when a small amount of OG was added at the (hanging or sitting) drop preparation step. Further, since harvesting is usually done at room temperature at which the suspending medium is a true liquid, crystal retrieval is no more challenging than with the standard in surfo method.

It is difficult to evaluate this method in terms of the underlying mechanism by which nucleation and crystal growth occur, for the following reasons. (1) Bicelles are expected to form under conditions under which DMPC and Chapso are combined in the proper molar ratio at low temperatures. However, it is not clear what happens and if the bicelles are stable when the bR protein with its attendant purple membrane lipid and the precipitant (±detergent) are added to the mix. (2) The high-temperature gel has been described for a related bicelle system (consisting of DMPC and dihexanoyl PC, which produces small bR crystals by the bicelle method) as consisting of interconnected bicelles having a "perforated lamellar" structure. Here again, the question must be asked as to the nature and stability of the gel under conditions under which nucleation and crystal growth actually occur.

Let us accept for the moment that a gel of the type just described exists during crystallization at 37 °C and that a bicelle solution prevails during crystallization at room temperature. This suggests that there are in fact two distinct routes to crystallization in the current system. One happens by way of bicelles, which is analogous to the standard, detergent micelle-based in surfo method introduced at the beginning of this article. Indeed, the ingredients are not much different, especially when the in surfo method is carried out in the presence of exogenous lipids, PC for example, as in the case of the Ca^{+2}-ATPase from sarcoplasmic reticulum (Toyoshima et al., 2000). The other makes use of the so-called perforated lamellar structure. A perusal of the cubic phase in Figs. 1 and 13f could lead to a similar approximate description under the proper influence. It may be therefore that this latter route represents a minor variation on the in meso theme, as already intimated (Caffrey, 2002).

According to the authors of the bicelle method paper, the best quality bR crystals were obtained by the gel route. However, this required that the sample be incubated above room temperature—in that particular case at 37 °C. Improving the method will require finding conditions under which gelation occurs at a lower temperature because not all proteins will tolerate prolonged heating. This, in turn, will lead to the need to manipulate the gel, which is viscous and difficult to handle. But the tools developed and described above for working with what must be one of the most viscous materials, the cubic phase, will work very well in this application too (Cheng et al., 1998).

The bicelle method is in its infancy. To date, it has been shown to work with bR, where it produced a new crystal form with what appears to be extensive delipidation and enhanced protein–protein crystal contacts. We have reproduced the published results up to crystallization in the gel, and it works well (Cherezov and Caffrey, unpublished data). There is every reason to try it with other membrane proteins, which is what we are about. Prospective users might benefit from a Frequently Asked Questions Web site devoted to the method (http://www.doe-mbi.ucla.edu/~salem/bicelle_method_faq.html).

7. The vesicle-fusion method

This method appeared about the time that the in meso method was introduced (Takeda et al., 1998). It has been employed to grow diffraction-quality crystals of bR. These have provided information on the structure of photoreaction intermediates of bR and have highlighted the possibility that X-radiation damage has compromised previous structural work in the area (Matsui et al., 2002).

While there appear to be many variations, a representative vesicle-fusion protocol takes the following form. Purple membrane is pretreated with the detergent Tween 20, to remove protein "impurities." In the process, bR succumbs to a loss of a small amount of its native lipid. The detergent is washed away and spontaneous vesiculation occurs upon subsequent incubation for a week at 32 °C with a second detergent, octylthioglucoside (OTG). The vesiculation solution contains 1 M ammonium sulfate and, in one variation, 12% trehalose (Matsui et al., 2002). The bR is then crystallized from the vesicle dispersion by vapor diffusion over a reservoir of 2 M ammonium sulfate in the sitting-drop mode. Crystals form within 2 to 6 months at 10 °C. The lethargic nature of this crystallization is attributed to a proposed rate-limiting step involving the collapse and subsequent fusion of vesicles to form planar sheets. The alternating, head-to-tail alignment of bR molecules between adjacent lamellae is consistent with this mechanism.

It is not clear that the vesicle-fusion method is one that can be generalized to other membrane proteins. Vesicle formation, which is integral to the method, is a delicate process that begins with a very unique protein preparation, the purple membrane, and crystallization likely benefits from uniformly sized vesicles (~50 nm diameter) that are enriched in protein. Such may be difficult to procure with membrane proteins that do not naturally form ordered 2-D arrays of the purple membrane type. However, for proteins that do or that can be induced to, certainly this is a method worth exploring as a means for producing 3-D, diffraction-quality crystals. It might also be worth trying with proteins that can be reconstituted into lipid vesicles or liposomes. The CFTR is one such example. The sense, however, is that the protein concentration in the bilayer would need to be high. Nevertheless, vesicle fusion would give the preparation a 3-D aspect subsequent to which is the need for the proteins to register with one another between and within layers to form an ordered 3-D crystal. The other distinguishing feature of the current vesicle-fusion method is that the only amphiphiles added were detergents (Tween 20, OTG) and these were included in relatively small amounts. This serves to maintain the naturally high-protein concentration of the purple membrane, which may be critical to the vesicle-fusion and nucleation and crystal-growth processes. Choosing the right level and type of lipid (and other amphiphiles) for the preliminary reconstitution step therefore is likely to impact significantly on the outcome of the crystallization trial with proteins other than bR.

8. Why lipid mesophases should produce good crystals

8.1. The microgravity analogy

The pros and cons of growing crystals in microgravity are still under evaluation (Government and Policy Concentrates, 2000; Lorber et al., 2002). The mechanism responsible for the reported improvement in crystal and diffraction quality has not been established but much is made of the convection-free environment it affords (McPherson, 1999). In solution, when the depletion zone forms next to the growing crystal on earth, a gradient in protein concentration exists between the crystal surface and the bulk of the solution. The associated density gradient creates a continuous, gravity-driven convective current and the depletion zone is disturbed as the heavier protein-rich layers fall through the lighter ones onto the crystal surface. In a microgravity environment, the depletion zone is stabilized, which allows for the slow and orderly addition of protein to the growing crystal.

It is likely that growing crystals in meso is akin to growing them in microgravity for the following reasons. Upon nucleation and as the crystals begin to grow, a depletion zone is created in the surrounding lipid phase. However, in meso, the crystal presumably is tethered to and embedded in a highly viscous medium that will not support convection of the type described above for solution. Further, protein diffusion in the supporting phase is even slower than it is in solution (Cribier et al., 1993). Thus, a depletion zone is stabilized and the crystal face is fed by slowly diffusing protein from the bulk. This increases the probability of growing larger and more ordered crystals. It is likely too that impurities are "filtered out" by the lipid bilayer, producing a higher grade of crystal.

Settling of newly formed crystals onto others in the mix is commonly encountered in solution for earth-grown crystals (McPherson, 1999). It produces defects and limits crystal growth. Under conditions of microgravity and in meso, for obvious but different reasons, sedimentation-related impairments to crystal growth are not an issue.

The argument has been made that the in meso and the (high-temperature) bicelle methods are variations on the same theme in that crystal growth takes place from a continuous gel-like mesophase. For this reason, the analogy highlighted above between crystallization under conditions of microgravity and crystallization in meso extends to the bicelle method. If the vesicle-fusion method gives rise to extended bilayers in which proteins can diffuse as an intermediate in the crystallization process, then the analogy may extend to this method also.

8.2. Epitaxy

Epitaxial nucleation is a process whereby crystal seed formation is facilitated when proteins in solution align themselves on an exposed lattice the size of which approaches an integral multiple of the nascent crystal lattice. For protein crystals grown from solution, a variety of materials have served this purpose (Essen et al., 1998; McPherson, 1999). The growth of membrane proteins from a mesophase may represent a variation on this theme. Thus, in the case of membrane proteins crystallized in meso, and by the bicelle and vesicle-fusion methods, for which structures are available, the crystals consist of stacked planar sheets of protein (Type I membrane protein crystals (Michel, 1983)). The scheme presented above for how the lipidic phase feeds the face of the crystal invokes a planar stack of lipid bilayers that extends from the bulk cubic phase to the crystal surface. There may be an epitaxial relationship between the crystal lattice and the conducting lamellar phase. A nanometer-sized X-ray beam is being used currently to study this proposed portal structure in meso (Cherezov et al., 2000). A similar epitaxial relationship is possible in the case of crystals grown by the bicelle and vesicle-fusion methods.

9. Conclusions and perspectives

It is apparent that the need for high-resolution structures of membrane proteins is acute. As outlined in this review, the crystallographer now has available a host of strategies with which to tackle crystallization. These include the long-established surfactant-based or in surfo method and three relatively new techniques—the in meso or cubic-phase method, the vesicle-fusion method, and the bicelle method, collectively referred to here as the bilayer methods. The one to use is the one that works! Alas, at this stage this may involve eventually trying all and, of course, there is no guarantee of success.

The in surfo method has the most impressive track record to date. But it has the advantage of preceding the bilayer methods by almost 2 decades. Its versatility is enhanced when it is used in combination with protein sculpting, in which protein segments are removed or added, as in the antibody fragment approach, and with the use of imaginative precipitant and dispersing solution cocktails. The method has been shown to work with an array of protein types having widely disparate topologies and degrees of association with the membrane, both as single polypeptides and as large multisubunit/cofactor complexes, and from different phyla and membrane types. Further, because it is liquid solution-based and can be implemented by vapor diffusion and microdialysis as for soluble proteins, it is easy to use and to automate. It will continue to be the workhorse method. However, it is possible that some proteins will prove refractory to in surfo crystallization, in which case the bilayer methods should be tried.

Are there any indicators to suggest that the bilayer methods should be tried first? Perhaps. Given that all of the proteins whose structures have been solved to high resolution using crystals grown in meso have the descriptor "rhodopsin" in common, should the target protein be similarly named or constituted, the in meso method would be a good first bet.

The in meso or cubic-phase method is a relatively new technique. In its short lifetime, it has fared reasonably well in terms of the number of proteins that have yielded to it. The sense is that it should prove more generally applicable when it is more extensively tested. Thus, one of the thrusts in the area is to make the method accessible and user friendly.

The number of variables that can be adjusted to effect in meso crystallization include most of those availed of in the in surfo method plus those that relate to the hosting cubic-phase matrix. These include lipid type and the relative amount of each lipid in the case of mixed-lipid systems. An attractive feature of the cubic phase in this regard is that it is quite forgiving, retaining its original phase state in the presence of a wide array of polar, apolar, and amphiphilic additives (Ai and Caffrey, 2000; Cherezov et al., 2001, 2002). Further, the cubic-phase microstructure can be tailor-made to a degree to suit the requirements of the target protein. Unfortunately, these cannot be known for certain in advance. However, calculated hydropathy profiles and topology maps can be generated from sequence and source membrane information, and related biophysical studies, and used to advantage here. Because the cubic phase is constituted of a lipid bilayer, the protein is likely to feel more at home there and, thus, less prone to losing activity during in meso crystallization than if it is micellarized and in direct contact with an excess of detergent, as in the in surfo method. The in meso method is nicely suited to structural studies of complexes. The cubic phase itself is an open, porous, and bicontinuous structure that disposes the protein for interaction and complex formation with other membrane proteins and soluble components. Despite its attractive features, sight should not be lost of the fact that the bilayer of the cubic phase is highly curved and cannot be considered equivalent to the considerably more planar membrane from which the protein to be crystallized is typically derived. However, because the cubic phase is an intermediate host, any conformational changes induced by such a nonnative environment may be reversed as the protein passages to and into the crystal.

In contrast to the in surfo and in meso approaches, the bicelle and vesicle-fusion methods are in their infancy and still undergoing testing. To date, both have produced crystals of just one protein, bR. It is hard to accept that they will not work with other proteins. Accordingly, they should be included in the toolkit of the membrane protein crystallographer. As with any method, their range of applicability will emerge only after evaluation with a broad array of membrane protein types.

It is obvious that all three bilayer methods must undergo more extensive testing. For this to happen in the near future, they must be rendered user friendly. We are making progress in this area, as noted. Progress will benefit too from automation. This is relatively routine for the crystallization of soluble proteins for which liquid handling is required. Greater challenges are presented in attempting to robotize the bilayer methods primarily because of extreme viscosity problems. Again, these issues have been dealt with and a prototype robot has been built and tested in the author's lab, demonstrating proof of principle. The prospect for more general use of these bilayer methods therefore is good.

It has been noted that our level of understanding of how membrane proteins crystallize in any of these methods is meager. This highlights the need to decipher the underlying molecular mechanism so that each method can be applied to its full potential and modified

in a rational way to suit particular circumstances. The methods are distinct but, as alluded to, may represent a continuum. This is particularly so for the three bilayer methods, all of which share a bilayer motif. With regard to the in surfo method, a moment of reflection suggests that there is a world of physical chemistry taking place in a droplet of protein solution during the course of crystallization. As drop size reduces—during vapor diffusion for example—micellarized proteins come into intimate contact with one another and likely pass close to or possibly through the so-called consolute boundary (Garavito and Ferguson-Miller, 2001; Loll et al., 2001; Wiener, 2001). In so doing, extended, self-assembling structures that are reminiscent of those proposed to exist in the in meso, bicelle, and vesicle-fusion methods are likely to form, if only transiently. In this way then the in surfo and bilayer methods are related, and insights into the underlying mechanism of one should inform the workings of the others.

Significant progress is being made in the development and implementation of crystallization strategies for membrane proteins as outlined in this article. The rate-limiting step that defines the emergence of new membrane protein structures may now be poised to shift to the protein production stage.

10. Web resources

The following is a list of Web sites that the author finds useful with regard to membrane protein crystallization issues.

10.1. The Lipid Data Bank (http://www.ldb.chemistry.ohio-state.edu)

A suite of relational databases on the phase properties, miscibility, and molecular structure of lipids. It also has tables of critical micelle concentrations, aggregation numbers, etc., for an assortment of detergents. The Lipid Data Bank includes an Other Sites section with links to a host of useful membrane-, lipid-, and detergent-related resources on the Web.

10.2. The Protein Data Bank

As of this writing (March 2003), the Protein Data Bank (http://www.rcsb.org/pdb) hosts 20,473 structures, the majority of which were obtained by X-ray crystallographic means using soluble proteins.

10.3. Compilations of membrane proteins of known structure

Two compilations are particularly noteworthy. One is from Hartmut Michel's group (http://www.mpipb-frankfurt.mpg.de/michel/public/memprotstruct.html). The other is from Steve White's lab (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). The latter provides tools for hydropathy plot and other relevant analyses (http://blanco.biomol.uci.edu/mpex/).

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