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Strategies for Crystallizing Membrane Proteins[1]

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Crystallizing membrane proteins remains a challenging endeavor despite the increasing number of membrane protein structures solved by X-ray crystallography. The critical factors in determining the success of the crystallization experiments are the purification and preparation of membrane protein samples. Moreover, there is the added complication that the crystallization conditions must be optimized for use in the presence of detergents although the methods used to crystallize most membrane proteins are, in essence, straightforward applications of standard methodologies for soluble protein crystallization. The roles that detergents play in the stability and aggregation of membrane proteins as well as the colloidal properties of the protein–detergent complexes need to be appreciated and controlled *before and during* the crystallization trials. All X-ray quality crystals of membrane proteins were grown from preparations of detergent-solubilized protein, where the heterogeneous natural lipids from the membrane have been replaced by a *homogeneous* detergent environment. It is the preparation of such monodisperse, isotropic solutions of membrane proteins that has allowed the successful application of the standard crystallization methods routinely used on soluble proteins. In this review, the issues of protein purification and sample preparation are addressed as well as the new refinements in crystallization methodologies for membrane proteins. How the physical behavior of the detergent, in the form of micelles or protein–detergent aggregates, affects crystallization and the adaptation of published protocols to new membrane protein systems are also addressed. The general conclusion is that many integral membrane proteins could be crystallized if pure and monodisperse preparations in a suitable detergent system can be prepared.

KEY WORDS: Membrane proteins; crystallization; nonionic detergents; protein–detergent interactions; monodispersity.

INTRODUCTION

Over the past four decades, X-ray crystallography and the resulting atomic models of proteins and nucleic acids have contributed greatly to our understanding of the structural, molecular, and chemical aspects of biological phenomena. However, despite the ever increasing number of crystal structures of integral membrane proteins (Deisenhofer *et al.*, 1985; Chang *et al.*, 1986; Allen *et al.*, 1987; Weiss *et al.*, 1990, 1991; Cowan *et al.*, 1992; Krauss *et al.*, 1993; Kühlbrandt *et al.*, 1994; Picot *et al.*, 1994; McDermott *et al.*, 1995; Schirmer *et al.*, 1995), their crystallographic analysis still remains difficult for one simple reason: straightforward, reliable methodologies and strategies for obtaining X-ray quality crystals of integral membrane proteins do not yet exist. This sobering statement is not meant to be discouraging; as this review attempts to underscore, rudimentary methods and strategies for obtaining crystalline preparations of integral membrane proteins have yielded X-ray quality crystals in several circumstances. However, we are at a stage of

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technique development similar to protein crystallographers in the late 1960's and early 1970's: we are searching for easily crystallizable membrane proteins to develop and refine the "art" into a science. Several reviews (Michel, 1983; Garavito *et al.*, 1986; Kühlbrandt, 1988; Garavito and Picot, 1990; Michel, 1991; Reiss-Husson, 1992; Sowadski, 1994) have covered the field of membrane protein crystallization, more or less extensively, and many rules, dogmas, and bits of lore have arisen. Unfortunately, much of the interesting information about crystallization experiments with membrane proteins remains unpublished and anecdotal. However, we will discuss these "softer" pieces of evidence and casual observations we have made in our laboratory.

THE PROBLEM

The primary problem in dealing with membrane proteins is that they are designed to exist in the quite anisotropic, amphipathic environment of biological membranes. To remain integrated within a lipid bilayer, a significant portion of their surface is hydrophobic. Once removed from the bilayer, membrane proteins are not readily soluble in *either* aqueous or apolar environments and cannot alone form the isotropic, monodisperse solutions needed to grow crystals. Thus, straightforward techniques for handling membrane proteins, in a manner suitable for crystallization experiments, do not always exist. The poor choice of a solubilization system can often lead to metastable solubilization and subsequent, nonspecific aggregation. Moreover, the activity and/or physical stability of a membrane protein may be compromised by its removal from the lipid bilayer. Hence, finding solubilization conditions for integral membrane proteins, suitable for designing crystallization protocols, remains a major obstacle.

Several methods for crystallizing integral membrane proteins have been proposed over the years (see the reviews mentioned above for more discussion). First, the crystallization of membrane proteins solubilized in organic solvents was initially proposed; the hydrophobic seed protein crambin, which was crystallized from ethanol by slowly increasing the water content of the solvent (Teeter, 1984), was the paradigm for this technique. However, many membrane proteins are not stably solubilized in organic solvents (Steck and Fox, 1972) and this method is not now often considered.

Second, the application of limited proteolysis to form water-soluble species of membrane proteins has had much more success in yielding X-ray quality crystals. Hemagglutinin from the membrane layer of influenza virus (Wilson *et al.*, 1981) was crystallized in this manner and its 3-dimensional structure solved. The use of genetic engineering to create discrete protein fragments should also eliminate the primary drawbacks of proteolytic cleavage. The recent structure of the tyrosine kinase domain of the insulin receptor (Hubbard *et al.*, 1994) is an excellent example of this methodology. However, this approach will only work if there are *defined* extramembranous domains in the protein that are independent and stable folding units. While many hormone receptors like the insulin receptor fall into this category, many membrane proteins do not. Moreover, this approach does not allow the structural analysis of transmembrane phenomena.

Third, Roepe and Kaback (1989) have attempted to render "soluble" the lactose permease from *E. coli*, an extremely hydrophobic membrane protein, using a number of techniques. Unfortunately, no solvent system has yet been devised to solubilize the *native* protein. However, Kaback and colleagues have also attempted some genetic engineering to render an otherwise hydrophobic protein more water soluble, with some success (Privé *et al.*, 1994). While genetic and/or chemical modification of a membrane protein may make it appear more water-soluble, no evidence yet exists that suggests this would be a routine strategy for preparing membrane proteins for crystallization.

Finally, surfactant systems like nonionic detergents have provided a general and efficacious means to solubilize and manipulate membrane proteins, and methods were devised to crystallize these proteins from detergent-solubilized preparations. In essence, the successful methods for crystallizing membrane proteins apply conventional crystallization techniques (McPherson, 1982) directly to a preparation of detergent-solubilized membrane protein. In most cases, crystals were obtained with the addition of standard precipitation agents like ammonium sulfate (AS) or polyethylene glycol (PEG). The techniques so far developed (see the reviews noted above) have allowed the growth of large, X-ray quality crystals of integral membrane proteins from monodisperse, micellar solutions of detergent-solubilized protein.

As the protein-detergent aggregate is the species that crystallizes, its characteristics and behavior in solution become important to understand. In solution, the protein-bound detergent appears to be distributed

as a uniform band or torus of mass about the protein surface, presumably covering the hydrophobic, transmembrane region (Le Maire *et al.*, 1983). Crystallographic analyses of membrane protein crystals confirm the general features of this model (Fig. 1) and suggest that detergent–detergent interactions may play a critical but subtle role in membrane protein crystallization (Roth *et al.*, 1989, 1991; Pebay-Peyroula *et al.*, 1995). The amount of detergent bound to the protein surface varies depending on detergent characteristics (type, concentration, etc.), solvent environment (pH, ionic strength, etc.), and, of course, the protein. A "typical" membrane protein may bind between 40–200% of its weight in detergent (Garavito *et al.*, 1983; Harlan, 1993; Møller and Le Maire, 1993).

For example, when the membrane protein prostaglandin H synthase-1 (PGHS-1) is detergent solubilized, the protein binds a considerable amount of detergent and lipid (Harlan, 1993) as determined using radiolabeled detergent and size-exclusion chromatography (Andreu, 1985). The amount of β-D-octyl glucopyranoside (β-OG) found to bind to PGHS-1 was found to vary with detergent concentration (Harlan, 1993). At 10.3 mM (or 0.3% w/v) β-OG, PGHS-1 bound 0.15 g detergent per gram protein or about 40 molecules of detergent per monomer. At 23.9 mM (or 0.7% w/v) β-OG, the amount of β-OG bound to PGHS-1 roughly doubled to 0.34 g detergent per gram protein or about 80 molecules of detergent per monomer. As most, if not all, of this detergent mass is brought into the crystal, protein-bound detergent will then make up a significant proportion of the nonsolvent mass in the crystal. It seems obvious that the character and behavior of this detergent layer cannot be ignored when designing crystallization experiments.

In preparing membrane proteins for crystallization, the first major obstacles to crystal growth are encountered: lipid contamination, protein inactivation, and protein aggregation. The initial solubilization of a membrane protein results in a rather heterogeneous population of aggregates containing protein, lipid, and detergent. After the protein is purified, the preparation should ideally consist of a single, monodisperse species: a stably solubilized protein–detergent aggregate. Choosing detergent(s) for solubilization and manipulation of a membrane protein thus depends on: (1) its ability to maintain the native structure and function of the protein, (2) its effectiveness in delipidating the protein, and (3) its capacity for maintaining the protein in a stable solubilized state. The protein–detergent system should be characterized for its suitability for crystallization experiments by determination of enzymatic activity or native functionality, by determination of the degree of lipid contamination (using, for example, thin layer chromatography), and by determination of the size and stability of the observed protein–detergent aggregates (using ultracentrifugation, light-scattering, or molecular sieve chromatography). Selecting a detergent that also allows the crystallization of a particular membrane protein–detergent aggregate will place further constraints on the system (see below). As an ideal detergent for all circumstances does not yet exist, compromises are often made depending on the goal of the experiments. Extensive characterization of the detergent effects on a protein is critical in order to avoid inactivation, denaturation, or aggregation (De Grip, 1982).

DETERGENT BEHAVIOR

Many basic aspects of detergent physical chemistry have significant effects on crystallization. More comprehensive reviews (Helenius and Simons, 1975; Helenius *et al.*, 1979; Kühlbrandt, 1988; Zulauf, 1991) cover, from a biochemical viewpoint, the action and behavior of detergents, while monographs by Tanford (1980) and Rosen (1978) as well as a review by Wennerström and Lindman (1979) describe in detail the physical chemistry of detergents. Detergents are surface-active molecules that self-associate and bind to hydrophobic surfaces in a concentration-dependent manner (Wennerström and Lindman, 1979). The amphipathic character of detergents is evident in their structures, which consist of a polar (or charged) head group and a hydrophobic tail. Most detergents fall into three categories depending on the type of head group: ionic (cationic or anionic), nonionic, and zwitterionic. The behavior of a specific detergent is dependent on the character and stereochemistry of the head group and tail.

Detergent monomers in aqueous solutions are involved in two kinds of basic phase transitions. First, the monomers can crystallize in aqueous solution (Wennerström and Lindman, 1979), although many of the detergents used in protein crystallization experiments are not readily crystallizable (Garavito *et al.*, 1986). Second, detergent monomers self-associate to form structures called *micelles* (Wennerström and Lindman, 1979; Tanford, 1980) and the threshold monomer concentration at which micelles begin to form is called the *critical micellar concentration*

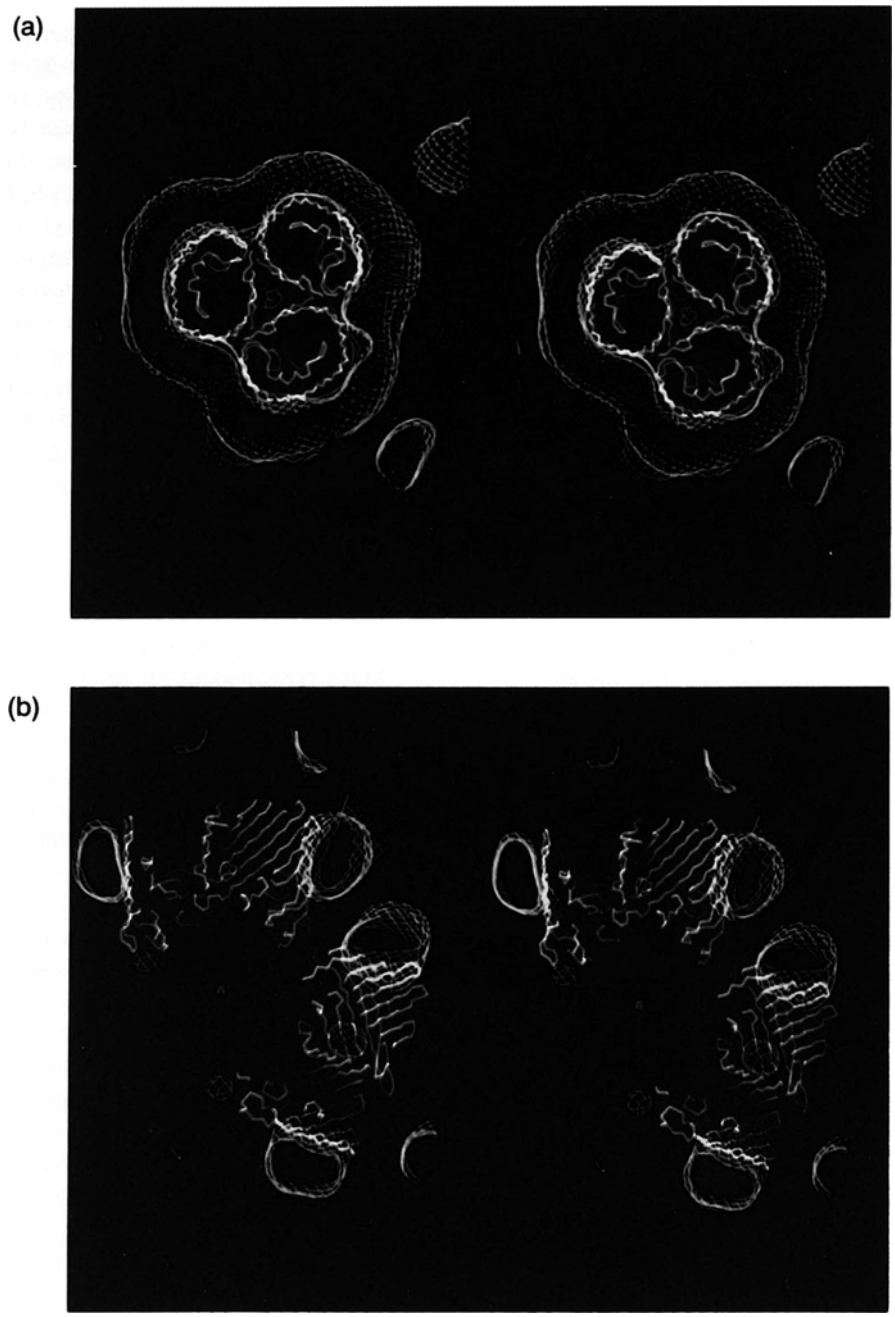

Fig. 1. A stereo view of the superimposed images of *E. coli* OmpF porin in two different detergents, as determined by single-crystal neutron diffraction (Pebay-Peyroula *et al.*, 1995). The blue contours are from the detergent ring created by N,N-dimethyldecylamine-*n*-oxide (DDAO) and the green contours are from β-OG. Under the conditions of the experiment, only the hydrophobic core of the detergent rings is well visualized while the detergent head groups are not well resolved. In panel (a), a porin trimer is viewed down its molecular 3-fold axis showing the detergent ring extending out from the protein surface (represented by the violet α-carbon skeleton superimposed on the map). In panel (b), the contact surfaces between two porin trimers in the crystal are shown. While the protein–protein contacts are made, the head groups of the detergents, which are not resolved here, abut against each other; it is obvious that changes in the detergent structure could disrupt these contact sites. Photographs are courtesy of Drs. P. Timmins and E. Pebay-Peyroula (ILL, Grenoble).

(CMC). When the detergent concentration exceeds the CMC, hydrophobic and amphipathic solutes can be solubilized into micelles. The complete and stable solubilization of most integral membrane proteins also occurs above the CMC. Detergent monomers associate with a hydrophobic protein surface, in similar manner as they associate in micelles, to create "micelle-like" protein-detergent aggregates (Le Maire *et al.*, 1983) that are freely soluble. Membrane protein crystallization is generally done at detergent concentrations above the CMC (Kühlbrandt, 1988; Garavito and Picot, 1990) where micelles co-exist with the protein–detergent aggregate.

The size and shape of an average micelle depends on the type, size, and stereochemistry of the detergent monomer (Rosen, 1978; Mitchell *et al.*, 1983; Zulauf, 1991) as well as the solvent environment. The size of a micelle can be described by its average molecular weight, hydrodynamic radius, or aggregation number (the average number of monomers per micelle). Several nonionic detergents used to crystallize membrane proteins form relatively small spherical or oblate micelles (Timmins *et al.*, 1988; Roxby and Mills, 1990; Kameyama and Takagi, 1990). Experimental evidence suggests that micelles are quite fluid and rapidly exchange monomer with the solvent (Rosen, 1978; Wennerström and Lindman, 1979). The more fluid the detergent monomers are within the micellar region, the more likely the detergent monomer can adapt uniformly to the protein surface. How well a detergent can solubilize a protein may in part be dependent on how effectively and uniformly the monomer binds to the convoluted protein surface.

Micelles are involved in additional phase transitions that can further affect crystal nucleation and growth (Zulauf, 1991). These phase changes, which involve micelle–micelle interactions, can create nonisotropic solutions and detergent mesophases with distinct structural properties (Rosen, 1978; Wennerström and Lindman, 1979; Mitchell *et al.*, 1983; Zulauf, 1991). For example, the micelles can deform and, under certain conditions, fuse together to form more complex macromolecular structures (Rosen, 1978; Wennerström and Lindman, 1979; Mitchell *et al.*, 1983). While these phase phenomena tend not to occur at low detergent concentrations (i.e., that used when handling membrane proteins), they may occur in membrane protein crystals where the local detergent concentration can reach 20–30% w/v concentrations (Garavito *et al.*, 1983; Pebay-Peyroula *et al.*, 1995; R. M. Garavito, unpublished observations).

The physical characteristics of a detergent that determine micelle size and shape would also determine the size and shape of the detergent layer on a protein. A detergent layer of large physical size can disturb the protein–protein interactions necessary for crystallization by acting as a physical barrier to the close approach of molecules. The detergent "rings" about the crystallized protein (Fig. 1) have been visualized by single-crystal neutron diffraction studies on *Rps. viridis* photosynthetic reaction center (RC) (Roth *et al.*, 1989) and the tetragonal crystal form of *E. coli* OmpF porin (Pebay-Peyroula *et al.*, 1995). In these cases, the detergent surfaces of adjacent protein–detergent aggregates abut against each other. Evidence also exists for the fusion of the protein-bound detergent layers with the crystal: Roth and co-workers observed micelle fusion in the crystals of *Rhb. spheroides* RC and Peter Timmins (ILL, Grenoble), in a lecture at the 1993 NIH Workshop on the Crystallization of Membrane Proteins, presented evidence that a bilayer-like detergent continuum is formed in the trigonal crystal form of the *Rps. capsulatus* porin-detergent complex. Whether manifested through direct micelle–micelle surface interactions or micelle fusion in the crystal lattice, it is obvious that the size, structure, and physical behavior of the detergent, as a monomer, in the micelle, and in the protein–detergent complex, must affect many aspects of the crystallization process.

The most commonly observed detergent-dependent phase phenomenon in crystallization experiments on membrane proteins is called the *cloud point* (see Zulauf, 1991) where an isotropic detergent solution turns turbid upon passing quickly through a consolute phase boundary. This phase transition results in the eventual separation of the solution into two immiscible solutions, a rather viscous micelle-rich phase and a thinner micelle-poor phase. This phase transition is easily induced by a number of solvent variables (e.g., detergent type, salt, temperature, and precipitant concentration), and Bordier (1981) used this phenomenon to isolate detergent-solubilized membrane proteins from soluble proteins. Phasing out of the detergent during crystallization experiments often affects nucleation, crystal growth, crystal stability, and, in many cases, crystal mounting. AS and other salts used in crystallization experiments affect the phase diagram in similar and predictable ways. For example, the alkyl-oligooxyethylene detergents like octyl-tetraoxyethylene (C_8E_4) display a *lower* consolute boundary (see Zulauf, 1991, for an excellent discussion), which can be considerably depressed in the presence of high salt.

The phasing-out phenomenon has been explained as the temperature-dependent or ionic strength-dependent dehydration of detergent micelles and their eventual aggregation into large clusters (Zulauf and Hayter, 1982; Zulauf and Rosenbusch, 1983). When the consolute boundary is crossed, the micelle clusters phase out and form a second aqueous, detergent-rich phase. Weckstrom (1985) has studied the relationship between phasing and the salt and precipitant concentrations for OmpF porin crystallization.

Some detergents, particularly the alkyl glycoside detergents like β-OG, decyl-β-D-maltoside ($C_{10}M$) have radically altered phase *behavior* in the presence of precipitants like PEG (Garavito *et al.*, 1986; Zulauf, 1991). Addition of PEG to a β-OG solution completely *inverts* the diagram and creates an *upper* consolute boundary. This unusual behavior with β-OG (and perhaps with other alkyl glycoside detergents) might be due to the nonideal behavior of β-OG in aqueous solutions (Kameyama and Takagi, 1990; Roxby and Mills, 1990). While micelle aggregation into clusters may again account for the phase transition, the molecular processes involved in the phase separation at an upper consolute boundary have not yet been fully explored. However, most of the crystallization experiments done in PEG and β-OG (see Garavito and Picot, 1990) approach this boundary to induce crystal growth. This raises the possibility that micelle–micelle interactions might be used to assist the crystallization process for membrane proteins by enhancing nucleation (Garavito and Picot, 1990).

CRYSTALLIZATION STRATEGIES

When planning crystallization experiments on an integral membrane protein, the gross structural characteristics of the protein and the molecular nature of a protein–detergent aggregate should also be considered before embarking on the project. Features of membrane protein structure could affect protein–detergent interactions and, therefore, influence the crystallization process. Circumstantial evidence suggests that the crystallization of a membrane protein will be less affected by the detergent if the *extramembranous surfaces or domains* of the protein are large and dominant. In other words, a more accessible protein surface provides more contact area for crystallization. Proteins or protein complexes with large extramembranous domains might be relatively "easy" to crystallize (i.e., they would crystallize more like soluble proteins) if stable protein–detergent complexes could be formed. On the other hand, a protein deeply embedded in the membrane (e.g., *E. coli* OmpF porin) might require more subtle manipulation of the detergent environment (Fig. 1). Thus, the large size of a protein complex would not necessarily be a disadvantage in crystallization experiments. A corollary to these assumptions is that *in vitro* protein complexes that increase and alter the surface area of a membrane protein (as well as perhaps stabilizing it) might thus improve the chances for crystallization. One manner in which this may be done is by creating antibody complexes, using monoclonal Fab or Fv fragments, with a target protein. As the capability now exists to engineer large amounts of Fab or Fv fragments (Kleymann *et al.*, 1995), this unproven technique might become a common tool in membrane protein crystallization.

The Primary Detergent

Evaluating successful crystallization experiments on membrane proteins (Garavito and Picot, 1990; Kühlbrandt, 1988) leads one to realize how similar the crystallization conditions are to those found for soluble proteins. Nonetheless, there are distinct aspects of the crystallization behavior of membrane proteins that demonstrate the important role of the solubilizing detergent. Table I lists a number of detergents frequently used in successful crystallization experiments. Choosing a detergent remains a trial-and-error process and is complicated by the fact that not all membrane proteins are stable in the best detergents for crystallization.

Once a protein has been prepared in a suitable detergent system, the "classical" methods for protein

Table I. Useful Detergents[a]

N,N-Dimethyldecylamine-*n*-oxide (DDAO)	N,N-Dimethyldodecylamine-*n*-oxide (LDAO)
Octyl-β-D-glucopyranoside (β-OG or C_8G)	Nonyl-β-D-glucopyranoside (β-NG or C_9G)
Decyl-β-D-maltoside ($C_{10}M$)	Dodecyl-β-D-maltoside ($C_{12}M$)
Octyl-β-D-thioglucopyranoside (C_8TG)	*n*-Octyl-2-hydroxyethylsulfoxide (HESO)
n-Octyl-tetraoxyethylene (C_8E_4)	*n*-Octyl-pentaoxyethylene (C_8E_5)

[a] Crystals of membrane proteins have been obtained by the authors or others using these detergents. Abbrevations are given in parentheses.

crystallization can then be used (McPherson, 1982) to induce the protein–protein interactions needed for crystallization. Both ammonium sulfate and PEG are effective crystallization agents in the presence of low concentrations (0.1–1.0% by volume or weight) of detergent. However, the presence of precipitants will modify the physical properties of the detergent in the crystallization system and precipitant-induced protein–detergent and detergent–detergent interactions will occur and must be taken into account. One can, therefore, expect that the crystallization process will be influenced by the phase transitions of detergents. For example, detergent phase separation, or even the approach toward a phase boundary, has been correlated with the crystallization of porin (Garavito and Picot, 1990), or with denaturation of the protein as is the case with bacteriorhodopsin (Michel, 1982b). The participation of protein–detergent aggregates in this phasing process has not been well studied, but it is clear that they partition into the micelle aggregates and, as shown by Bordier (1981), they will almost always partition into the detergent phase when phase separation occurs. Hence, the design of crystallization experiments is constrained by the phase behavior of the detergent as well as the protein.

While numerous aspects of detergent physical chemistry can influence the crystallization of membrane proteins, several characteristics of detergents stand out as important when attempting to grow X-ray quality crystals of membrane proteins. First, the detergents that are generally chemically well defined and nonionic (e.g., β-OG, N,N-dimethyldodecylamine-N-oxide (LDAO), $C_{10}M$, or octyl-pentaoxyethylene) and that have moderate to high CMCs are the best candidates for crystallization experiments. One important aspect that is often overlooked is that detergents having moderate to high CMCs allow better detergent exchange and much more accurate control over the detergent concentration. Second, the detergents form monodisperse aqueous solutions of micelles with a small *apparent* size (20–30 Å radius) and a fairly homogeneous spherical or oblate ellipsoidal shape; thus the resulting protein–detergent aggregate may not have a total hydrodynamic size much larger than expected for the protein alone. The aspect of homogeneous shape might also explain why the cholate class of detergents is much less successful in allowing membrane protein crystal growth: they seem to form large, physically heterogeneous micelle-like aggregates (Helenius and Simon, 1975; Helenius *et al.*, 1979). Third, the detergents' micelle size and shape are seemingly unaffected by detergent concentrations just above the CMC (Zulauf, 1991), which means these physical parameters are probably not changing significantly during the crystallization experiments. Finally, the phase behavior of the detergents is such that isotropic solutions can be obtained under a reasonably wide range of temperatures, salt concentrations, and precipitant concentrations (Garavito and Picot, 1990). Commonly used detergents for solubilization and for purification (e.g., Tween-20 or Triton X-100) may allow some crystal formation, but have not yet provided any crystals diffracting to high resolution. If such detergents are used for protein preparation, they should be exchanged, at the end of the preparation, with another detergent more suitable for crystallization. In some cases, however, detergent exchange may be difficult and may require the use of other detergents for solubilization.

A question arises about the effect of lipids on crystallization. While heterogeneous native lipids have adverse effects on crystallization (Garavito *et al.*, 1986), one might ask if crystallization could occur in the presence of pure lipids. As most aqueous systems of phospholipids exist not as monodisperse micellar solutions, but as bilayer structures, crystal formation probably would not occur unless detergents are added to create a micellar solution. However, Eisele and Rosenbusch (1989) have examined this hypothesis, using *E. coli* OmpF porin and short-chain lipids as a model system, and observed good crystal growth. Thus, the ability of pure surfactants, whether a detergent or lipid, to form a homogeneous micellar system may be an important physical criterion for crystallization.

A cursory glance at the list of detergents in Kühlbrandt (1988) or in Garavito *et al.*, (1986) suggests that a wide range of detergents have been explored and are well characterized. This is not the case as detergent screening is a time-consuming and exhausting process. A number of new detergents are available commercially or as custom syntheses that may be quite useful in membrane biochemistry such as dimethylalkylphosphine oxides (Kresheck, 1981), alkyl betaines (Hermann, 1966), phosphobetaines (Hermann *et al.*, 1966), alkyl phophorylcholines (Weltzien *et al.*, 1979), and lysolecithin analogues (Weltzien *et al.*, 1979). Weltzien and colleagues (Weltzien *et al.*, 1979; Timmins *et al.*, 1988) have studied the last category of detergents and suggest detergents having a lysolecithin-like structure are excellent candidates for membrane protein research. Anatrace, Inc. (Maumee,

Ohio; 1-800-252-1280) has recently introduced a series of alkyl-cyclohexyl maltoside detergents that can effectively solubilize membrane proteins and allow the crystallization of bacterial porins (H. Kim and R. M. Garavito, unpublished observations).

As a last caveat, the purity of a surfactant, whether a detergent or a lipid, is critical to its reproducible behavior. Lorber *et al.* (1990) have recently reported how detergent purity affects the stability and crystallizability of bacteriorhodopsin. *Very few commercially available detergents are truly pure* (despite the claims of the manufacturer) and the purity can vary markedly from batch to batch. It is recommended to keep records of the lot numbers used and to periodically determine the CMC of the detergents used. The CMC can be easily measured by several different methods (see Zulauf *et al.*, 1989). If commercially available detergents are not pure enough, the detergent will need to be further purified and then recharacterized.

Precipitant System

Large, X-ray quality crystals have grown in the presence of either ammonium sulfate or PEG, though crystals have occurred more often with PEG as the precipitant. Both AS and PEG should be explored in the first set of experiments, if the protein is stable in high concentrations of those compounds. An important criterion for choosing one precipitant system over another is whether the detergent/salt/additive system allows one to reach high enough precipitant concentrations to induce crystal growth in the absence of detergent phase separation. We recommend that one first determine at which precipitant concentrations phase transitions occur with the detergent system being used. This is important as some zwitterionic detergents will not only phase-separate at high precipitant concentrations, but also can form birefringent liquid crystal phases (J. A. Jenkins and R. M. Garavito, unpublished observations). Because of this phenomenon, one must view the appearance of "protein" microcrystals with a certain amount of skepticism until they are analyzed.

Additives or Small Amphiphiles

The behavior of the detergent is significantly affected by the addition of amphiphilic compounds. These co-solutes or co-surfactants interact directly with micelles and protein–detergent aggregates by partitioning into the detergent layers (Rosen, 1978; Wennerström and Lindman, 1979), creating mixed micelles, and these compounds can alter the apparent CMC, micelle size, and phase transitions of a detergent solution. While contamination by amphiphilic compounds (e.g., detergent impurities) is undesirable, judicious use of an additive can suppress detergent phase separation or select a particular crystal form. In the simplest system, a second well-defined detergent component is added to the crystallization experiments at a low molar or weight ratio with respect to the primary detergent (Garavito and Rosenbusch, 1986; Garavito *et al.*, 1984; Stauffer et al., 1990).

Amphiphilic compounds other than detergent (i.e., no observable CMC below 0.1 M) can also act as co-solutes and have allowed the growth of large X-ray quality crystals of certain membrane proteins (Michel, 1982a, b; Papiz *et al.*, 1989). Examples of such compounds are alcohols (Garavito et al., 1986), alkyl-diols, alkyl-hydroxyethanol (Garavito *et al.*, 1984), and the "small amphiphiles" like heptane-1,2,3-triol (Michel, 1982a, b; Michel, 1983). These amphiphilic compounds must be added at relatively high concentrations (1–5% w/v) to have an influence on the crystallization process and the detergent phase transitions. Recent studies have confirmed that heptane-1,2,3-triol addition can reduce the *apparent* micelle size of LDAO (Timmins *et al.*, 1991) and β-OG (Thiyagarajan and Tiede, 1994), but how these "small amphiphiles" affect membrane protein crystallization has not been adequately explained.

The crystallization experiments on protein complexes from photosynthetic bacteria still provide the best examples for the use (and drawbacks) of additives in crystallization protocols (Michel, 1982a, b; Papiz *et al.*, 1989; Schertler *et al.*, 1993; Buchanan *et al.*, 1993). Michel (1982) reported the first 3-dimensional crystals of *Rps. viridis* RC. Crystals were obtained by the vapor diffusion method in the presence of 2.2–2.4 M AS and the detergent LDAO. LDAO and the addition of heptane-1,2,3-triol were important in the crystallization of the *Rps. viridis* RC complex (Michel, 1982a, 1983). The crystals of RC complex from *Rhb. spheriodes* can also be obtained with heptane-1,2,3-triol (Allen *et al.*, 1987; Chang *et al.*, 1985; Buchanan *et al.*, 1993).

Their efficacy may also depend on the precipitant system: many small amphiphiles phase out or crystallize at high AS concentrations (Michel, 1982b; Papiz *et al.*, 1989; Schertler *et al.*, 1993). The drawbacks of small amphiphile use (protein denaturation, irrepro-

ducible nucleation, and crystal metastability) are a result of the high concentrations needed to induce crystallization. Often, supersaturating concentrations of the small amphiphiles are reached which results in amphiphile crystallization; if protein crystals have already grown, they sometimes degrade when the amphiphile crystallizes. Hartmut Michel, in a lecture at the 1993 NIH Workshop on the Crystallization of Membrane Proteins, detailed how his group's work on the crystal structure of the B850/800 light-harvesting complex from *Rhodospirillum molischianum* has been stymied because of unwanted amphiphile effects. The problems encountered when using small amphiphiles often affect attempts to prepared heavy atom derivatives. McDermott *et al.* (1995) successfully overcame these problems to determine the crystal structure of the light-harvesting LH2 complex from *Rhodopseudomonas acidophila* by employing a novel "back soak" technique to localize the heavy atom sites. Thus, additives can be very useful for obtaining crystals although they are not a panacea for membrane protein crystallization. As crystals are often obtained without resorting to adding such compounds, additives may be best used as a means to fine-tune existing crystallization conditions.

Crystallization System

Virtually all crystallization systems work on membrane proteins. Large, X-ray quality crystals have been grown using bulk methods, vapor diffusion (hanging and sitting drop), microdialysis, and free-interface diffusion. Microdialysis and large-scale vapor diffusion have yielded the largest crystals (Garavito and Picot, 1990; Michel, 1991; Reiss-Husson, 1992) although microscale methods of sitting or hanging drop vapor diffusion afford quick and economical ways to test different crystallization conditions. The free interface diffusion method (McPherson, 1982) has also been quite successful for screening crystallization conditions for membrane protein on a microscale (Eisele and Rosenbusch, 1989). However, some physical modifications to a crystallization system might be necessary to accommodate detergent-induced physical changes in the protein solution behavior. With the hanging drop method, it should be noted that the reduction in surface tension of the protein solution, due to the presence of detergent, limits the drop size to less than 10 μL.

Temperature

The phase transitions of detergents are very temperature sensitive (Rosen, 1978; Wennerström and Lindman, 1979; Zulauf, 1991) and membrane protein crystallization often displays similar temperature sensitivity (Garavito *et al.*, 1986; D. Picot and R. M. Garavito, unpublished observations). It is important to maintain adequate temperature control over the crystallization experiments for reproducible results. However, as temperature can be a critical variable in detergent phase behavior, the use of temperature as a crystallization variable is also possible (Garavito *et al.*, 1986).

Purity and Homogeneity of the Protein Preparation

Successful crystallization often depends on the preparation of pure, homogeneous protein (Bott *et al.*, 1982; Giege *et al.*, 1986) and thus all factors that create chemical heterogeneity must be minimized or eliminated. The improvement in protein purity for OmpF porin (Garavito *et al.*, 1983), bacterial RC (Deisenhofer *et al.*, 1985; Buchanan *et al.*, 1993), prostaglandin H synthase (Picot *et al.*, 1994), and bacterial LH2 complexes (McDermott *et al.*, 1995) was a critical factor in the growth of large, X-ray quality crystals. Heterogeneity due to incomplete delipidation and detergent exchange can be conveniently monitored by a number of techniques (Kates, 1986) such as thin layer chromatography (chloroform/methanol/ammonia 65:35:5 or water-saturated 2-butanone; visualization with iodine). *Genetic variability* should be dealt with by purifying protein from homozygous organisms or from genetically distinct expression systems. *Post-translational modifications* (e.g., glycosylation or phosphorylation) that create a heterogeneous population of protein must also be dealt with. While the chemical modifications themselves do not necessarily affect crystallization, the heterogeneity they introduce will. Thus, a wise choice of expression systems and expression conditions will dramatically improve the chances of successful crystallization.

A membrane protein must then be extracted from the membrane and solubilized before crystallization can be attempted. The solubilization step must keep the protein functional and provide a basis for creating a monodisperse, homogeneous protein–detergent complex that is suitable for biochemical and biophysical

characterization. Purification then becomes a most crucial step for crystallization: it must not only yield pure protein, but it must also reduce all factors that cause molecular heterogeneity. For example, native lipids are heterogeneous in composition and their removal is necessary in order to achieve a homogeneous protein–detergent preparation. The presence of excess lipid, as well as detergent contaminants, can be checked easily with thin layer chromatography.

Searching for Crystallization Conditions

The search for successful crystallization conditions is the most intimidating and exhausting part of the research. A careful look at the conditions where X-ray quality crystals of other membrane proteins grew demonstrates that while crystallization conditions for similar proteins are similar, they are also uniquely different. A systematic search of conditions will always be necessary and the number of variables in the system is large. The crystallization experiments on bacteriorhodopsin (Michel, 1982b; Schertler *et al.*, 1993) and *Rdb. spheroides* (Chang *et al.*, 1985; Allen *et al.*, 1987; Buchanan *et al.*, 1993) shows how subtle changes in conditions can cause dramatic changes in crystal quality. The porins from *E. coli* and photosynthetic bacteria also show how similar proteins crystallize under roughly similar conditions of salt and PEG (Garavito and Picot, 1990) but again the precise conditions for the growth of X-ray quality crystals differ distinctly from protein to protein.

The dilemma one faces in setting up crystallization trials is how one searches possible conditions. Comparing bacterial RC from *R. spheroides*, *E. coli* OmpF porin, and ovine prostaglandin H synthase, one sees that they can crystallize under nearly identical conditions although they differ markedly in their structure, function, and membrane source (Garavito and Picot, 1990). In most of the cases we have analyzed, crystallization often occurs near the phase separation boundary, regardless of the detergent used. While it is clear that the phase separation event is not important for crystallization, the molecular interactions that cause micelle aggregation and the eventual phase separation of the detergent might play a role in crystal nucleation and growth (Garavito and Picot, 1990). Zulauf and colleagues (Zulauf and Hayter, 1982; Zulauf, 1991) have shown that micelle aggregation occurs well in front of the phase separation boundary.

In our experience, the best results occur when detergent phase separation can be reduced or eliminated. The phase region open to crystallization experiments is thus dependent on where the phase separation boundary is and how the phase separation boundary and the crystallization boundary change as the crystallization conditions (i.e., salt, precipitant, and detergent) change. Hence, before crystallization experiments are set up, it is highly recommended to prescreen all possible buffer–detergent–precipitant conditions for undesirable phase behavior and to define the region open to crystallization experiments. This labor-intensive step need only be done once and can thus be used as a laboratory database. Such a strategy has recently yielded crystals of the plasma membrane H^+-ATPase from *Neurospora crassa* (Scarborough, 1994).

Once a set of conditions has been defined, searching this potential "crystallization" space is the next obstacle. A number of options are available as far as search strategies are concerned (Carter, 1990; Weber, 1990; Cudney *et al.*, 1994; D'Arcy, 1994; Kingston *et al.*, 1994; Stura *et al.*, 1994). The most intriguing method is the "sparse matrix" method (Jancarik and Kim, 1991) that has been modified by a number of groups (Cudney *et al.*, 1994; D'Arcy, 1994). The basis of the sparse matrix strategy is that only a limited set (50–100) of extreme crystallization conditions are searched for crystallization "potential" (e.g., microcrystal formation or formation of globular, birefringent material). It is not uncommon that the first screen with a combination of a sparse matrix and hanging-drop vapor diffusion yield small, well-formed crystals from soluble proteins (Jancarik and Kim, 1991; Cudney *et al.*, 1994; D'Arcy, 1994).

Recently, Gouaux and co-workers have adapted the sparse matrix method for use on membrane proteins (Song and Gouaux, 1995) and have successfully crystallized alpha-hemolysin (Gouaux *et al.*, 1994). What they did was to prescreen the buffer–detergent–precipitant combinations in the "standard" sparse matrix array (Jancarik and Kim, 1991) for undesirable phase behavior and then to alter some of the conditions to make them compatible with a chosen detergent (in this case, C_8E_4). We have utilized this adapted sparse matrix screen to identify crystallization conditions for the OmpA protein from the outer membrane of *E. coli* (H. Kim and R. M. Garavito, unpublished results) and human prostaglandin H synthase isoform 2 (B. Perman and R. M. Garavito, unpublished results). The sparse matrix strategy, when optimized for other detergents,

therefore may provide a powerful and adaptable means to screen potential crystallization conditions for membrane proteins.

A CASE HISTORY: THE CRYSTALLIZATION OF PROSTAGLANDIN H SYNTHASE-1

Crystallization

PGHS-1, a mammalian membrane-bound enzyme of the arachidonate cascade (Picot *et al.*, 1994), is a dimer of 144,000 Da and is homogeneously glycosylated. The purification protocol was designed to provide the virtually lipid-free enzyme with minimal loss of activity (Picot *et al.*, 1994; D. Picot, P. J. Loll, J. E. Harlan, and R. M. Garavito, unpublished data). As prolonged incubation at high detergent concentration inactivates the enzyme, the additional chromatographic steps, needed to increase specific activity, reduce lipid contamination, and reduce the inactivation of the enzyme by lipid peroxides, were done as rapidly as possible. The optimized purification protocol improves the reproducibility and growth characteristics of large crystals suitable for X-ray diffraction analysis. The apo-protein was concentrated to 12 mg/ml with a Centricon PM30 (Amicon, Danvers, Massachusetts). The holo-enzyme was then reconstituted with one to two equimolar amount of hemin from a freshly made 15 mM stock solution in dimethyl sulfoxide. The protein solution was then dialyzed (Spectrapor 4 cellulose dialysis tubing, MWCO 12-14000 Da) overnight against a buffer containing 20 mM sodium phosphate buffer, pH 6.7, 50 mM NaCl, 0.6% β-OG, 1 mM NaN_3, and 1 mM flurbiprofen (a PGHS-1 cyclooxygenase inhibitor).

The dialyzed protein was then aliquoted into 30 μL amounts, and concentrated solutions of NaCl (2 M) and PEG 4000 (40% w/v; EM Sciences) were added to obtain the desired starting concentrations. Typical starting concentrations ranged from 50–150 mM for NaCl and 2–4% (w/v) for PEG 4000. Drops of protein solution were then placed on *well-siliconized* cover slips and inverted over vapor diffusion reservoirs containing higher PEG concentrations. In contrast to the usual method of preparing hanging drop crystallization (McPherson, 1982), the drops were not mixed with the reservoir solutions. This modified method requires that the reservoir solutions contain concentrations of PEG, phosphate buffer, and NaCl that are multiples of the concentrations in the drop at the outset of the experiment. For example, a drop containing 2% PEG, 20 mM sodium phosphate, and 100 mM NaCl might be placed over a well containing 6% PEG, 60 mM sodium phosphate, and 300 mM NaCl. The usual range of reservoir PEG screened was 4–8% (w/v). The crystallization experiments were carried out at 19°C in an environmental room.

A protein precipitate is observed to form shortly after the hanging drops are set up. The time required for this precipitate to form varies from a few seconds to a few days, depending on the starting conditions. While small crystals may appear as soon as a few hours after hanging the drops, crystals suitable for X-ray diffraction studies usually require about two weeks to appear. The crystals grow mainly as long needles with typical dimensions of 0.2 × 0.2 × 1–3 mm; the long axis of the needle is parallel to the *a*-axis and the two faces normal to it are parallel to the [011] and [0*1*1] planes. The diffraction pattern showed that the crystal system is orthorhombic and the unit cell parameters are $a = 99.4$ Å, $b = 210.3$ Å, and $c = 233.1$ Å ($V = 4.87 \times 10^6$ Å^3). Solutions to difference Patterson functions calculated with isomorphous heavy atom derivatives are consistent with space group I222 but not with $I2_12_12_1$ (Picot *et al.*, 1994). As the unit cell contains 16 molecules, the resulting V_m is 4.4 Å^3 Da^{-1}, corresponding to a solvent and detergent content of about 70%. Large crystals of PGHS-1 have also been grown in the presence of other detergents (e.g., C_{10}M, decyl-dimethylamine oxide and octyl-pentaoxyethylene), and at least one other crystal form has been observed (D. Picot and R. M. Garavito, unpublished observations).

Detergents play important roles at different stages in the PGHS crystallization process, beyond the obvious one of maintaining the protein's solubility. Detergents used for the early solubilization and purification steps will influence the crystallization: Drastically better crystals were obtained with material solubilized and purified in C_{10}M rather than Tween 20, although the crystals were grown from solutions containing β-OG in both cases. Several factors play a role since not only is the detergent exchange more difficult from Tween 20 to β-OG than from C_{10}M to β-OG, but also the aggregation state of PGHS is dependent on the type and the concentration of detergent used. Enzyme prepared with C_{10}M has different properties than that prepared in Tween 20 (Harlan, 1993).

PGHS crystals grow under conditions very close to the detergent-mediated phase transition (Garavito and Picot, 1990). As this phase transition in pure deter-

gent solutions corresponds to the aggregation of micelles (see above), the phase transition observed in our crystallization experiments might proceed by a similar mechanism: the aggregation of protein–detergent complexes. It is tempting to speculate that PGHS crystals form in a regime where detergent–detergent interactions have assumed great importance and the molecular packing in the PGHS crystals (Fig. 2) is consistent with this hypothesis. The packing of the PGHS dimers in the crystal create hydrophobic channel along the *a*-axis, the wall of which is formed mainly by the hydrophobic surface of the membrane-binding domain in the PGHS. This channel and its symmetry mate run along the whole crystal and encompass a cylinder of 38 Å diameter. The hydrophobic surface of the channel must be coated with detergent molecules, which probably fill the whole channel: The PGHS monomer binds at least 70 molecules of β-OG

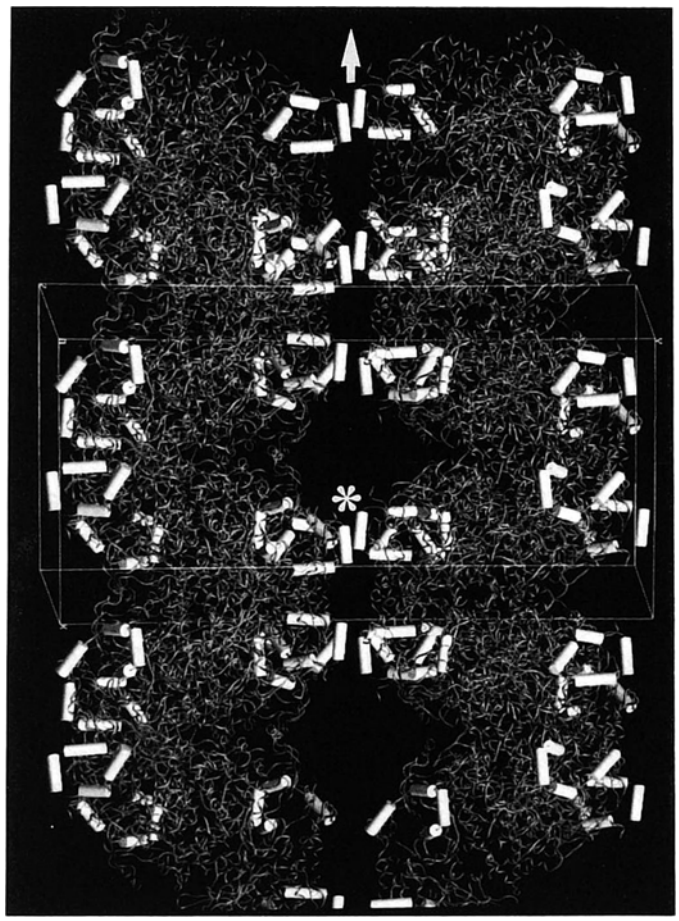

Fig. 2. A photograph of the pseudo tetragonal packing arrangement in PGHS-1 crystals. The protein backbone is represented by a gray rope while the amphiphilic helices of the membrane-binding domain are highlighted by white tubes; the unit cell is shown as a box. The crystal packing forms a hydrophobic channel, 35 Å in diameter, which runs parallel to the *a*-axis at $(x,0,0)$; the *a*-axis is shown vertical in this view and denoted by an arrow. The channel wall is mainly formed by the hydrophobic surfaces of the membrane-binding domain in the PGHS dimer. Two molecules of well-ordered detergent have been located at the interface between a pair of PGHS-1 dimers (noted with an asterisk; D. Picot and R. M. Garavito, unpublished observations). Another solvent channel, 80 Å in diameter, also runs through the whole crystal parallel to the *a*-axis but at $(x,{}^1/_2,0)$. However, the channel is formed by the hydrophilic surfaces of the protein and no detergent has been identified in this channel.

or 140 per dimer protein (Harlan, 1993) under the crystallization conditions. Moreover, it is in this region that two molecules of tightly bound molecules of β-OG have been found (D. Picot and R. M. Garavito, unpublished observations). Along this *c* axis channel, contacts between the PGHS dimers are minimal and are nearly exclusively formed by contacts between the membrane-binding motifs.

Crystal Characterization

The crystallization conditions for PGHS are highly reproducible from preparation to preparation but the yield of good crystals is never outstanding: most of the crystals are either too small or not single. Moreover, several physical and environmental factors resulted in a low success rate in mounting crystals for data collection, particularly in the early phase of the project. First, as the crystallization occurs near a detergent phase boundary, phase separation often occurs either during or after crystallization. Because the small drops contain precipitate and (owing to the detergent phase separation) two liquid phases, the drops contain little homogeneous mother liquor for crystal manipulation. Second, the crystals are quite fragile and often adhere strongly to the precipitate and/or the cover slip. These are not uncommon problems encountered when working with membrane protein crystals (Garavito *et al.*, 1983; Michel, 1982b; Kühlbrandt, 1988).

Far more difficult to control are the environmental factors which lead to disorder or nonisomorphism. The fact that good crystals grow over a relatively large range of PEG and salt concentrations accounts for slight nonisomorphism between crystals: changes in the salt and PEG concentrations can noticeably alter the quality of the diffraction data *in a unique and anisotropic manner* (D. Picot, unpublished observations). Compounding this problem, subtle changes in the detergent environment can influence the stability of the crystal, and the uncontrolled variation of the detergent concentration of the mother liquor can easily destroy PGHS-1 crystals. For example, soaking solutions with β-OG concentrations just above the CMC will slowly dissolve the crystal; the rate of crystal dissolution increases substantially as the β-OG concentrations exceeds the CMC. Increasing the PEG concentration retards but does not stop this process.

Two factors are at work here: the detergent is an integral part of the protein crystal, and a substantial amount of protein-bound detergent is brought into the crystals during crystal growth. In a hanging drop vapor diffusion experiment, even though the detergent concentration increases as crystallization proceeds, the protein–detergent ratio remains constant. Hence, the detergent concentration *in the mother liquor* may not increase as significantly. Adding artificial mother liquor with a detergent concentration equal to the total detergent concentration in the drop probably disturbs the detergent monomer–aggregate equilibrium and changes the amount of detergent around the protein. The packing arrangement in PGHS-1 crystals (Fig. 2) suggests that changes in the detergent interface could easily disrupt crystal contacts.

Similar observations have been made with other membrane proteins (Michel, 1982a, b; Garavito *et al.*, 1983; Kühlbrandt, 1988; Garavito and Picot, 1990), consistent with the hypothesis of an active role played by detergent interactions in the crystallization process. Figure 1 shows how detergent surfaces can approach each other in the crystal; slight changes in detergent behavior could radically alter these contract sites. Shifts of the CMC, induced, for example, by the increasing salt concentration in the crystallization drop (Zulauf, 1991, Lorber *et al.*, 1990), need to be taken into consideration. In the case of *E. coli* OmpF porin, this sensitivity to changing detergent concentration, particularly during mounting, was the major reason to change from vapor diffusion (Garavito *et al.*, 1983) to microdialysis methods (Garavito and Rosenbusch, 1986), where the ambient detergent concentration could be better controlled. Unfortunately, only hanging-drop vapor diffusion yielded the largest and best order crystals for PGHS-1, thus severely constraining how the detergent concentration could be altered post-crystallization.

It was necessary to develop controlled solvent exchange procedures and crystal mounting strategies to avoid any changes in the solvent environment which would dramatically reduce the diffraction quality of PGHS-1 crystals. Our studies on detergent binding by PGHS-1 and the behavior of the enzyme–detergent complex in solution (Harlan, 1993) yielded insights into the behavior of this complex in the crystal. One important parameter was that the concentration of the detergent in the artificial mother liquor should not exceed the detergent's CMC: Crystals could survive transfer into solutions matching the reservoir conditions when the detergent concentration was at or just below the "corrected" CMC, even though the total β-OG concentration in the drop was above 1.2% (w/v). The corrected CMC for β-OG (ca. 0.45% w/v), used

for the soaking buffer, corresponds approximately to the depression of the CMC from that in dilute buffer (ca. 0.67% w/v) to that observed in the crystallization medium containing salt and PEG. With this correction, the crystals could then be transferred, in three to five steps, into a standard soaking buffer: 10% (w/v) PEG 4000, 20 mM sodium phosphate, pH 6.7, 100 mM NaCl, and 1 mM flurbiprofen, 1 mM NaN_3, and 0.45% (w/v) β-OG. The transfer of the crystals into the soaking buffer should not proceed too quickly (about 5–15 min per step seems acceptable).

The crystal transfer and stabilization protocols kept the crystals stable in a soaking buffer for 1–2 weeks. As a result, more crystals were thus suitable for data collection and transferable into the soaking conditions necessary for reproducible heavy atom derivatization. After these advances in crystal stabilization and manipulation were made, heavy atom derivative screening progressed rapidly and resulted in the crystallographic determination of PGHS-1 structure (Picot *et al.*, 1994). Indeed, finding good stabilization conditions for the crystals was the rate-limiting step in searching for heavy atom derivatives. However, it should be pointed out that even in the best case, only one in ten mounted crystals exhibit reasonably high resolution diffraction. The take-home message is that poorly ordered, low-resolution diffraction from membrane protein crystals are not simply signs of crystal growth problems but may also indicate problems with crystal handling.

FINAL COMMENTS

There are no *a priori* reasons that preclude the crystallization of any membrane protein *if it can be obtained as a pure, stable preparation in a micellar detergent solution.* However, a substantial effort must be put into obtaining a protein preparation that is homogeneous and monodisperse by physical, chemical, and genetic criteria. Extending these methods to other membrane proteins might be straightforward but certainly not trivial. We needed to purify 1.0 g of active PGHS and set up over 15,000 crystallization experiments over 6 years to define crystallization systems and solve its structure (Picot *et al.*, 1994). Much of the effort was focused on dealing with the biochemical parameters related to crystallization: protein stability, homogeneity, and monodispersity. This underscores the often hidden, seemingly Sisyphean effort needed to bring such a project to fruition and stresses the need for improved methodologies for membrane protein expression, purification, and crystallization.

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