THE PRESENT STATE OF TWO-DIMENSIONAL CRYSTALLIZATION OF MEMBRANE PROTEINS

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Abstract—This review summarizes the present literature on two-dimensional crystallization of membrane proteins, with emphasis on the technical aspects. It includes all the intrinsic membrane proteins that have been crystallized after solubilization. Four general ways of making crystals are described in detail. Furthermore, suggestions for improving crystallization conditions are presented.

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I. INTRODUCTION

For the determination of the structure of intrinsic membrane proteins at high resolution, it is necessary to work on two-dimensional (2D) or three-dimensional (3D) crystals. Spectacular results have been obtained by X-ray diffraction studies on 3D crystals grown from photosynthetic reaction centres isolated from photosynthetic bacteria (Deisenhofer et al., 1985; Allen et al., 1987). Apart from these reaction centres, only a few proteins have been crystallized in a form suitable for high-resolution X-ray diffraction and no other structures have been solved yet. The knowledge on 3D crystallization will progress, not in the least because everybody wants to crystallize his or her protein nowadays, but it will always remain a difficult task to grow good 3D crystals.

For several reasons, electron microscopy, the ‘minor technique’ for studying crystallized membrane proteins, will remain important. First, with electron microscopy one can easily check the initial steps in crystal growth: the aggregation state of the protein, and its interactions with detergent molecules. Second, recent progress in high resolution (cryo-) electron microscopy has opened the way to solve the structure of membrane proteins with a resolution well under 1 nm (Baldwin et al., 1988). Third, many proteins are easier to crystallize into 2D than 3D.

This review deals with the techniques for obtaining 2D crystals from detergent-solubilized intrinsic
membrane proteins. It gives an overview of the methods that have been applied, the objects that have been crystallized and finally gives details and suggestions which may be of help for getting more proteins crystallized in a form suitable for electron microscopy.

Since projections of proteins on electron micrographs suffer from a poor signal-to-noise ratio, averaging is necessary. Methods for analyzing the various projections of single hydrophilic proteins are able to produce averaged images with a resolution of about 1.5-2 nm (Frank et al., 1988). Such methods are important for the larger proteins, which have a mass of at least several 100 kDa. For membrane proteins the resolution that can be obtained is even worse, since single particles of hydrophobic proteins can only exist as detergent protein mixed micelles. The attached detergents and lipids hinder the precise translational and rotational alignments of projections, a necessary step before averaging can take place. As an exception, Boekema et al. (1989) were able to separate the two types of top view projections of a trimeric Photosystem I particle with a diameter of 19 nm. However, most membrane proteins are much smaller than the 600 kDa of that Photosystem I particle and the alignments cannot be of significant accuracy to reveal much inner detail. Obtaining membrane protein crystals, therefore, is of utmost importance. In several cases it has been shown that from 2D crystals the projected protein structure could be determined with a resolution of better than 1 nm, which is even beyond the possibilities of single particle averaging for soluble proteins.

Table 1. Two-Dimensional Crystals from Membrane Proteins

<table>
<thead>
<tr>
<th>Protein / Complex</th>
<th>Space Group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH dehydrogenase</td>
<td>p22,2</td>
<td>Leonard et al., 1997</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>p22,2</td>
<td>Leonard et al., 1997</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>p5</td>
<td>Fuller et al., 1982</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>p21</td>
<td>Hebert et al., 1982</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>p21</td>
<td>Taylor et al., 1983</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>p21</td>
<td>Lepault et al., 1988</td>
</tr>
<tr>
<td>Photosynthetic reaction centre</td>
<td>p21</td>
<td>Mannoni et al., 1987</td>
</tr>
<tr>
<td>Photosynthetic reaction centre</td>
<td>p21</td>
<td>Miller and Jacob, 1983</td>
</tr>
<tr>
<td>Acetyl choline receptor</td>
<td>p21</td>
<td>Branson and Unwin, 1985</td>
</tr>
<tr>
<td>Proteolipid from cerebral cortex</td>
<td>p3</td>
<td>Unwin and Henderson, 1975</td>
</tr>
<tr>
<td>Proteolipid from cerebral cortex</td>
<td>p3</td>
<td>Unwin and Henderson, 1975</td>
</tr>
<tr>
<td>Proteolipid from cerebral cortex</td>
<td>p3</td>
<td>Dorset et al., 1983</td>
</tr>
<tr>
<td>Light-harvesting complex II</td>
<td>p321</td>
<td>Kuhlbrandt et al., 1983</td>
</tr>
<tr>
<td>Light-harvesting complex II</td>
<td>p321</td>
<td>Kuhlbrandt et al., 1983</td>
</tr>
<tr>
<td>NADH dehydrogenase fragment</td>
<td>p42,2</td>
<td>Brink et al., 1986</td>
</tr>
<tr>
<td>Gap junction</td>
<td>p622, p6m</td>
<td>Zamplighi and Unwin, 1979</td>
</tr>
</tbody>
</table>

The crystals are arranged according to their symmetry. The crystals belong to 7 out of the possible 17 plane groups. The plane group symbols are written in Holser notation (Holser, 1958) and in ‘International tables notation’ (Hahn, 1983; in brackets) for groups where the symbols differ. Some objects have been crystallized in several forms and appear in more than one symmetry group.

A. Why Crystallization?

Since projections of proteins on electron micrographs suffer from a poor signal-to-noise ratio, averaging is necessary. Methods for analyzing the various projections of single hydrophilic proteins are able to produce averaged images with a resolution of about 1.5-2 nm (Frank et al., 1988). Such methods are important for the larger proteins, which have a mass of at least several 100 kDa. For membrane proteins the resolution that can be obtained is even worse, since single particles of hydrophobic proteins can only exist as detergent protein mixed micelles. The attached detergents and lipids hinder the precise translational and rotational alignments of projections, a necessary step before averaging can take place. As an exception, Boekema et al. (1989) were able to separate the two types of top view projections of a trimeric Photosystem I particle with a diameter of 19 nm. However, most membrane proteins are much smaller than the 600 kDa of that Photosystem I particle and the alignments cannot be of significant accuracy to reveal much inner detail. Obtaining membrane protein crystals, therefore, is of utmost importance. In several cases it has been shown that from 2D crystals the projected protein structure could be determined with a resolution of better than 1 nm, which is even beyond the possibilities of single particle averaging for soluble proteins.

B. Crystallized Objects

The number of membrane proteins that have been crystallized is still small and is far behind that of the naturally occurring crystalline objects, such as the bacterial cell wall proteins. Table 1 summarizes the membrane proteins that have been crystallized in well defined arrays and from which the packing has been analyzed. Cell wall proteins and similar objects have been omitted.
During the solubilization and purification process the liquid membrane is, of course, fully dissolved. As a consequence, the orientation of the membrane proteins after purification is random. It is therefore not surprising that many membrane proteins give rise to 2D crystals in which the molecules are oriented in alternating up and down positions. The most common space group for 2D crystals with such ‘flip and flop’ molecules is \( p2_{1}2_{1} \) (Table 1). In these crystals the molecules are pair-wise oriented up and down. The techniques dealing with symmetry determination and crystal (image) analysis have been reviewed several times and are outside the scope of this review.

For making 2D crystals there have been several approaches, some of them taking advantage of the behaviour of membrane proteins to incorporate into two-dimensional (lipid) layers. The various techniques have been classified into four general methods, which are described subsequently in the following sections.

**II. CRYSTALLIZATION IN VESICLE CRYSTALS**

**A. General Description**

In 1979 Weiss and coworkers succeeded in making vesicle crystals of cytochrome reductase (Wingfield et al., 1979; Leonard et al., 1981). After the isolation step with Triton X-100, Triton X-100 phospholipid mixed-micelles were added to the purified cytochrome reductase. By slow removal of the detergent, vesicles were obtained in which the protein molecules were in a crystalline packing. In a similar way crystals were also formed from a subcomplex of this enzyme (Hovmöller et al., 1981). Typical vesicle crystals were closed lipid vesicles which were highly packed with protein. Upon preparation by the negative staining technique the vesicles collapse, often resulting in double-layer crystals. Depending on the pH, such preparations also contained single layers. Collapsed vesicle crystals or lipid vesicles are always characterized by a brighter stain excluding outer zone, in contrast to monolayer sheets.

In fact, this method of obtaining crystals is a modified form of the general method of reconstitution: the incorporation of proteins into artificially made lipid vesicles. The difference is mainly in the lipid to protein ratio. Whereas mass ratios of 20:1 or higher are usually applied in reconstitution, ratios between 3:1 and 1:3 are optimal for cytochrome reductase crystals. Under these conditions the protein concentration apparently is too high for free rotational and translational rotation, resulting in a crystalline packing (Hovmöller et al., 1983). PhoE Porin, which was reconstituted with phosphatidylcholine at a ratio of 1:4, forms highly ordered two-dimensional crystals, from which the projection normal to the membrane could be determined to a resolution of 0.65 nm (Jap, 1989). Another example is NADH dehydrogenase (Leonard et al., 1987), from which crystals were prepared in a way very similar to that for cytochrome reductase.

A further example of an enzyme crystallized in vesicle crystals is cytochrome oxidase. In fact, this was the first membrane protein ever crystallized (Seki et al., 1970; Vanderkooi et al., 1972). The original method is different from the one used for the cytochrome reductase crystals. First, the cytochrome oxidase was extracted from mitochondrial membranes with either Triton X-114 and X-100 or deoxycholate, and then further purified by salt fractionation with KCl. The green pellet showed already some crystallinity. If no crystalline structures appeared, further incubation with detergent (Triton X-100 or deoxycholate) was necessary. This probably improved crystallization by removing more phospholipids from the membraneous preparation (Capaldi and Zhang, 1986). Furthermore, cytochrome oxidase crystals could also be made by fusion of purified protein with preformed lipid vesicles in a similar way to that used for cytochrome reductase (Fuller et al., 1982).

The last, but not the least example is bacteriorhodopsin. This protein already exists in a crystalline form in vivo in the purple membrane of *Halobacterium halobium*. Triton X-100 solubilized monomers spontaneously rearrange into crystalline membranes after removal of detergent by dialysis (Cherry et al., 1978).
B. Phospholipase Treatment

In cases where proteins are incorporated in lipid vesicles one can induce or further improve crystal formation by removal of (part of) the excessive lipid by treatment with phospholipase A₂ or phospholipase C. This method was proposed by Mannella (1984), with application to a porin from mitochondrial outer membranes. The phospholipase enzymes work with Ca²⁺ as a cofactor, mainly by binding to the lipid which is arranged in a bilayer. Phospholipase A₂ treatment of phospholipids (lecithin) gives lysolecithin molecules as the breakdown product; phospholipase C cleaves phospholipids releasing diacylglycerols. Lysolecithin has detergent properties and it may therefore be advantageous in some cases to remove it finally by dialysis. The method has also been applied to several other proteins, for instance Na⁺-K ATPase (Mohraz et al., 1985). The effect of phospholipase treatment can be easily tested on a small scale where protein has been incorporated into vesicles. The method is therefore highly recommended as a tool in all crystallization trials dealing with lipid vesicles.

C. Sheet Forming Properties of some Detergents

In the last decade, many new non-ionic detergents have been synthesized and introduced in membrane protein chemistry (see also Section III). These detergents are all pure homogeneous compounds, in contrast to some older detergents such as Triton X-100, which is a mixture of very long (about 4nm) molecules. Several membrane proteins have a lower stability in Triton X-100, which is a mixture of very long (about 4nm) molecules. Several membrane proteins have a lower stability in Triton X-100.

D. Further Details on Crystallization Conditions

The conditions of the vesicle crystal method have never been tested on a large scale, although some of the factors have been systematically determined for the cytochrome reductase and cytochrome reductase subcomplex crystals (Hovmöller et al., 1981, 1983). Slight variance in the protein and detergent concentration was not very critical. The lipid used could be pure phosphatidylethanolamine or mixtures with phosphatidylethanolamine, and the protein:lipid ratio could vary between 0.5 to 2.0. Temperature (25-37°C), pH (5.5-6.0 optimal) and the methods of detergent removal were found to be the most important factors affecting the quality of the crystals. With cytochrome reductase the dialysis step takes at least 48 hr, since Triton X-100 dialysis is very slow. Engel et al. (1988) have also found that the exact conditions of the detergent removal are of importance. They constructed a flow-dialysis device that allowed precise control of temperature and flow-rate. By such dialysis it was possible to obtain well-ordered single-layered membranes from E. coli outer membrane porin.

E. Improving Crystallization Conditions

Reconstitution of proteins into preformed lipid vesicles is a widely applied technique in membrane biochemistry (see Jain and Zakim, 1987, for a review). It has, for instance, often been applied in studies on the enzymology of purified membrane proteins. In such cases, vesicles with one double lipid layer and a low protein content are preferentially used. There are, however, differences between such a general situation and our special case of reconstitution. For instance, in ordinary reconstitution it is often important that the proteins all...
have the same orientation, perpendicular to the membrane, and that the vesicles are impermeable to protons or ions. Reconstitution applied to making membrane crystals has other demands. The proteins may very well be alternating upside-down, as long as they arrange in a regular crystal, and proton gradients are irrelevant. Of more importance is the general structure of the vesicles. The usual vesicles produced have a diameter of 100–200 nm, which is too small to contain enough protein molecules and often the vesicle walls are of a multilayered type. Another big problem is that many isolated proteins do not easily fuse with the detergent–lipid vesicles.

Fortunately, many aspects of vesicle fusion processes have been described in the literature, and we should take advantage of this knowledge for our particular case of membrane crystals. There are a number of factors that greatly enhance fusion.

First, it matters which kind of lipid is used. Sonified phosphatidylcholine is commonly used for the protein crystals described previously. However, the general literature states that fusion is strongly inhibited by phosphatidylcholine (Düzgünes, 1983). Phosphatidylserine and phosphatidylethanolamine have a positive effect on fusion. Therefore, we suggest that pure phosphatidylcholine should be abandoned in any case where protein fusion is difficult to obtain, and that instead, mixtures with phosphatidylserine should be used.

Second, fusion conditions are further improved by addition of detergents to the lipid vesicles (‘mixed micelles’). Detergents such as Triton X-100 or octyl glucoside seem to have a similar effect (Alonso et al., 1982) and any of them could be taken advantage of.

Third, the fusion process is temperature dependent; membranes made of phosphatidylcholine or phosphatidylserine fuse at characteristic temperatures of 43 C and 38 C, respectively. The incorporation of lysolceithin, which was the product of phospholipase A treatment, reduces these temperatures by about 10°C (Breisblatt and Ohki, 1975). This suggests that the successful phospholipase method could have an additional effect in the crystallization: fusion could be stimulated if carried out at temperatures around 30°C–40°C.

Recently, vesicle fusion induced by the catalytic activity of phospholipase C at 37°C was demonstrated (Nieva et al., 1989). It was argued that the diacylglycerols, released by the enzymatic breakdown, enhance fusion by bilayer destabilization. However, raising the temperature can also be a disadvantage if the protein is unstable at elevated temperatures. Another factor that should be given attention when working at higher temperatures is the lipid phase transition. Cherry et al. (1978) found that above the phase transition temperature, the lattice of bacteriorhodopsin crystals disappears. This effect was found for bacteriorhodopsin reconstituted with dimyristoyl/phosphatidylcholine and dipalmitoyl/phosphatidylcholine which have transition temperatures of 23°C and 41°C, respectively. For bacteriorhodopsin, one should stay well under the phase transition temperature.

Fourth, the presence of 1 mM Ca2+ and Mg2+ ions also enhances fusion for phosphatidylserine, but not for phosphatidylcholine (Breisblatt and Ohni, 1976). The divalent ions have more effect at higher temperatures and generally the vesicle crystal method should have more success when applied at higher temperatures, as was done for cytochrome reductase. A number of other substances also enhance fusion, but their application is of less relevance for crystallization.

Recently, Mannella (1989) tested some of the factors generally influencing the fusion process on mitochondrial porin membrane crystals. Freeze–thawing treatment with metal ions or organic fusogens did not result in increased vesicle size. However, successful fusion was achieved with a procedure employing slow dehydration of the outer mitochondrial membranes at acidic pH.

### III. DETERGENT–PROTEIN CRYSTALLIZATION METHOD

The previous section describes the methods which take advantage of sheet-forming properties of lipids and some detergents. However, naturally occurring lipids are rather inhomogeneous and a heterogeneous protein–detergent sample may be a
disadvantage for getting well ordered, large crystals (Kühlbrandt, 1988). Therefore, crystallization of membrane proteins in the presence of one well defined type of detergent is perhaps the most promising general method. Both 2D and 3D crystals have already been obtained by this method. For about 15 objects 3D crystals are now available (reviewed by Kühlbrandt, 1988). Some of these crystals are suitable for determining the structure at high resolution by X-ray diffraction. In the extensive review of Kühlbrandt many details on the 3D crystallization methods can be found. Michel (1983) has formulated some useful concepts that have been helpful for making 3D crystals. A monolayer of right-sized detergent molecules should cover the hydrophobic parts of the protein making its total surface "hydrophilic". Such a small detergent-protein micelle could then be treated as a normal soluble protein and crystals could be made by application of classical crystallization methods using salts and other additives.

Many detergents have been tested for obtaining 3D crystals and several dozens of detergents have been found to be of potential interest for all the proteins studied so far, but for each new protein many have to be inspected in order to get crystals, since the success of a particular detergent is highly protein specific. Pikula et al. (1988) tested 49 detergents for getting 3D crystals for Ca-ATPase. Only four of them gave crystals; three Brij-type detergents and furthermore, octaethylene glycol dodecyl ether ("C12E8"). The 49 different detergents were already preselected on the basis of results with other proteins.

For 2D crystallization, the suitability of detergents has never been tested on such a scale, but the constraints for getting well-ordered crystals are perhaps generally less stringent. Some of the detergents used for 3D crystals have also been successfully applied for 2D crystallization, such as dodecyl (lauryl) maltoside and N,N dodecyl dimethylamine N-oxide (LDAO). Dodecyl maltoside gives 3D Photosystem I crystals (Witt et al., 1988) and 2D Photosystem II crystals (Dekker et al., 1989b). LDAO gives 2D crystals as well as 3D crystals for the photosynthetic reaction centre from *Rhodopseudomonas viridis* (Miller and Jacob, 1983; Michel, 1982). Unfortunately, Photosystem II, although having similarities to the photosynthetic reaction centre in two of its subunits, is not very stable in LDAO (Timmins et al., 1988). Therefore, for 2D crystals it is also true that an optimal combination of protein and detergent has to be found experimentally.

However, there are differences in the way 2D and 3D crystals can be made. Whereas Triton X-100 is a good detergent for 2D crystals as described previously, 3D crystallization generally needs smaller detergent molecules. This is exemplified by the light-harvesting complex II. Monolayer crystals were obtained with Triton X-100, whereas thin 3D crystals were obtained with nonylglucoside (Kühlbrandt, 1988). Another object that has been 2D-crystallized with Triton X-100 is bacteriorhodopsin of purple membrane from *Halobacterium halobium* (Michel et al., 1980). From this crystal form the projected structure was determined at 6.5 Å resolution. Unfortunately, the 3D crystals from this object, obtained with octylglucoside (Michel and Oesterhelt, 1980) did not diffract well enough to determine the structure by X-ray diffraction. Besides the four successfully applied detergents discussed previously, Tween 80 gave crystals for rhodopsin from frog retinal rod outer segments (Corless et al., 1982).

With the detergent-protein crystallization method, crystals have been found for five different detergents. These were also the detergents in which the proteins were purified. Perhaps in some cases there were remnants of lipids present in the crystalline sheets, which could be advantageous for sheet-forming, especially in combination with Triton X-100. But since high purity and homogeneity are vital for crystal growth, removal of lipid molecules seems to be more promising. Moreover, 2D crystallization can also start from a mono-disperse solution. For example, the crystals of Photosystem II were obtained, starting from a mono-disperse particle solution with dodecyl maltoside as detergent, by optimizing detergent concentration, salt content and pH. It seems therefore likely that many more detergents will give good crystals, if tested with highly purified proteins on a larger scale. Dodecyl maltoside, octyl-, nonyl- and decyl glucoside are among the most interesting newer detergents that should be tried out in any case.
IV. CRYSTALLIZATION ON LIPID MONOLAYERS

A third general method to induce 2D crystals makes use of a lipid monolayer which is formed on top of an aqueous solution in a small container. After the lipid layer has been formed, a protein solution is injected under the surface. The protein molecules specifically interact with the monolayer and shortly after the injection the monolayer plus attached proteins are picked up with a carbon-coated copper grid and stained. Since the interaction between protein and lipid is often specific, the protein solution may even contain minor impurities, without disturbing crystallization. The method was introduced by Uzgiris and Kornberg (1983) and applied to an antigen–antibody–complement complex. Five other proteins have been crystallized as well (cholera toxin (Ludwig et al., 1986; Reed et al., 1987); ribonucleotide reductase (Ribi et al., 1987); RNA polymerase holoenzyme (Darst et al., 1988); tetanus toxin (Reidler and Robinson, 1988) and p68 protein (Newman et al., 1989)] and work on another three protein crystals is in progress.

The wide variety of objects indicates that this method is of general interest, particularly for proteins that associate with membranes. However, none of these proteins actually belongs to the group of intrinsic membrane proteins, and the question as to whether this method is also applicable for these proteins is still open.

V. OTHER CRYSTALLIZATION METHODS

Soluble proteins are usually crystallized from concentrated solutions by addition of salts, such as ammonium sulphate and sodium chloride and/or water-binding substances, such as polyethylene glycol (PEG). In a few cases 2D crystals of membrane proteins have been grown by similar procedures and addition of salts in the 1 M range, as is usually performed in classical protein crystallization, was successful. Two examples are NADH dehydrogenase (Boekema et al., 1982) and bovine rhodopsin (Dratz et al., 1985). In both cases ammonium sulphate, which was added in the 1 M range, induced crystallization.

Addition of specific cations, such as vanadate, at low concentration has been applied to two different ATPases. Skriver et al. (1981) found crystals for Na, K ATPase and Dux and Martonosi (1983) found crystals for Ca ATPase.

For the crystals mentioned above, where salt addition is the main effect in the crystallization, other factors such as the lipid to protein ratio and the detergent to protein ratio, remain very important. If, for instance, salt addition is combined with removal of detergent by dialysis this will often lead to random aggregation of the protein molecules.

The effect of other small salt molecules and additives on 2D crystals have never been tested on a larger scale. Some small ‘space filling’ additives have been successfully applied in 3D crystallization, such as heptanetriol (Kühlbrandt, 1988). For frog rhodopsin and for Photosystem II, lipitane and taurine have been applied, respectively (Dratz et al., 1985; Dekker et al., 1989b). Polyethylene glycol 6000 is a well-known additive which binds water and therefore concentrates the protein solution. Preliminary results with crystalline sheets of ATP synthase from spinach chloroplasts (Böttcher, Gräber, Boekema, unpublished observation) indicate that this substance, which is widely applied in 2D and 3D crystallization, is also of interest for membrane proteins.

Older methods applicable for soluble proteins make use of a substrate, such as the carbon support film (Keegstra and van Bruggen, 1980) or mica (Harris, 1982). For membrane proteins these methods do not seem to have a particular value and no results for membrane proteins have been reported until now.

VI. CONCLUSIONS AND CLOSING REMARKS

In this paper, four general ways of making two-dimensional membrane protein crystals have been described. One of them, the lipid monolayer technique, gives an elegant way to obtain two-dimensional crystals for hydrophilic or membrane-associated proteins, but possibly it is not very
promising for intrinsic membrane proteins. The last method, the addition of salts and additives can only be useful in combination with detergents and/or lipids. That leaves us basically with the two other crystallization methods: the vesicle crystal method and the detergent–protein crystallization method.

From the limited number of crystallized objects (Table I) it is not clear which method is, in principle, the better one, in terms of its potential success or the ultimate resolution that can be achieved. Therefore, we suggest that generally both methods should be tried out as a starting point for getting good crystals. In cases of extreme hydrophobic membrane proteins the vesicle crystal method could, however, have slight advantages over the detergent–protein crystallization method, since the latter depends more on interactions between the hydrophilic protein parts extending from the membrane. The choice will also depend on the way the proteins are isolated and their stability against a particular detergent.

Well characterized, FPLC-purified and lipid-free membrane proteins should in principle give crystals without extreme effort. Indeed, some crystals were initially found by routine checking of isolated protein batches. Therefore, with the steady interest in the structural aspects of membranes there is much hope that the number of crystallized proteins will rapidly increase.

REFERENCES


