Crystallization of membrane proteins

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Five new membrane protein structures have been determined since 1995 using X-ray crystallography: bacterial light-harvesting complex; bacterial and mitochondrial cytochrome *c* oxidases; mitochondrial bc_1 complex; and α-hemolysin. These successes are partly based on advances in the crystallization procedures for integral membrane proteins. Variation of the size of the detergent micelle and/or increasing the size of the polar surface of the membrane protein is the most important route to well-ordered membrane protein crystals. The use of bicontinuous lipidic cubic phases also appears to be promising.

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Introduction

Up until now, the 3D structures of about 8,000 biological macromolecules, mainly soluble proteins, have been determined using X-ray crystallography. During the past few years, the availability of recombinant DNA technology to produce and to tailor the protein of interest has substantially contributed to the exponential growth in the number of new protein structures published each year. In contrast, the number of known membrane protein structures is still below 20. This fact is remarkable considering that close to 40% of the 6,000 gene products encoded by the genome of baker's yeast are expected to be integral membrane proteins. These numbers not only underscore the importance of membrane proteins, but also emphasize the enormous biochemical and structural work that remains to be done in the field of membrane proteins.

Membrane proteins are difficult to handle; the difficulties reside in the amphipathic nature of their surface. They possess a hydrophobic surface where they are in contact with the alkyl chains of the lipids, and they possess a polar surface where they are in contact with the aqueous phases on both sides of the membrane or with the polar headgroups of the lipids. In order to solubilize and to purify membrane proteins one has to add a vast excess of detergents—amphiphilic molecules that form micelles above their critical micellar concentration. The detergent micelles take up the membrane proteins and cover the hydrophobic surface of the membrane protein with their alkyl chains in a belt-like manner. The polar headgroups of the detergents face the aqueous environment.

Any crystallization strategy has to take into account the amphipathic nature of the surface of membrane proteins. Essentially, there are two possibilities for arranging membrane proteins in the form of 3D crystals [1].

First, one can try to form 2D crystals in the plane of the membrane, and then stack these membranes in an ordered way. These crystals are called 'type I'.

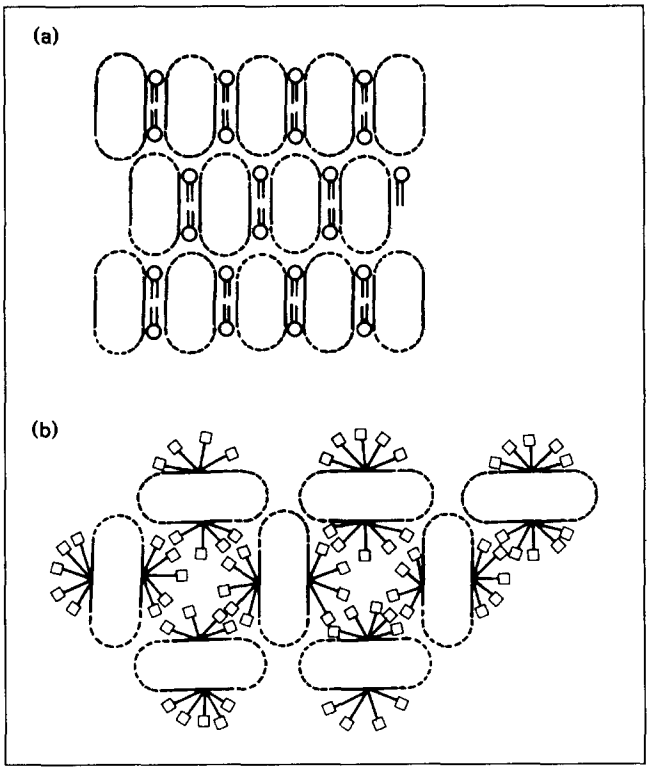
Second, one can try to crystallize membrane proteins within their detergent micelle. The crystal lattice will be established via polar contacts of the polar surface parts of the membrane protein extending out of the detergent micelles. In this case, the crystallization procedures are very similar to those for soluble proteins. For these 'type II' crystals, detergents with relatively small polar headgroups should be used in order not to cover too much of the membrane proteins polar surface.

Both types of membrane protein crystals are schematically represented in Figure 1. Mixed types of crystals appear to be possible; however, the overwhelming majority of membrane protein crystals belong to type II.

Since 1985, when the structure of the bacterial photosynthetic reaction center was presented as the first membrane protein, detailed structures from only seven families of integral membrane proteins have been published (Table 1; for reviews, see [2]). The past two years, however, have seen an enormous increase in the number of newly solved membrane protein structures, including two bacterial light-harvesting complexes [3,4], bacterial [5] and mitochondrial [6,7] cytochrome *c* oxidase, mitochondrial bc_1 complex [8], and α-hemolysin [9]. All integral membrane proteins crystallized so far are either pigmented or eubacterial outer membrane proteins. The latter proteins contain only β strands in their membrane-spanning section and are of extraordinary stability.

We discuss the recent advances in membrane protein crystallization. Clearly, obtaining well-ordered crystals is the bottle-neck of membrane protein structure determination. The problem of crystallizing membrane proteins cannot be reduced to the issue of which screening method or crystallization set up is to be used. Rather, thorough biochemical work and intensive protein characterization, in combination with comprehensive screening for the most suited detergent, may be the most efficient strategy to cope with the difficulties of membrane protein crystallization.

Figure 1



The two basic types of membrane protein crystals. **(a)** Type I: stacks of membranes contain 2D crystalline membrane proteins, which are then ordered in the third dimension. **(b)** Type II: a membrane protein is crystallized with detergents bound to its hydrophobic surface. The polar surface portion of the membrane protein is indicated by broken lines; lipids are indicated by spheres with two alkyl chains attached; detergents are indicated by squares with one alkyl chain attached. Reproduced with permission from [1].

The first step on the way to the structure of a membrane protein is to obtain a sufficient amount of pure and homogeneous protein. The second step is to find the one detergent needed to obtain well-ordered crystals for crystallization. In fact, this is the most critical step for crystallization; unfortunately, it is also the most error-prone step. Interestingly, finding the optimal crystallization conditions does not seem to be a bottle-neck. With respect to precipitating agents and pH, all membrane protein crystals obtained so far have been obtained under quite standard crystallization conditions. If the biochemist and the crystal-grower has done his/her job well, data collection and structure determination of a membrane protein are as straightforward as for nonmembrane proteins. Currently, flash cooling of membrane protein crystals is often used to enhance crystal stability in the X-ray beam [10] or for trapping reaction intermediates; however, establishing cryoconditions for membrane protein crystals seems to be much more difficult than for soluble proteins. This problem may be due to the presence of detergent micelles in membrane protein crystals.

Most important: the wet-lab biochemistry

For crystallization trials, up to 100 mg of pure protein must be isolated. Soluble proteins can often be obtained by overexpression of the gene or cDNA, combined with the use of affinity tags for detection and purification. Refolding from inclusion bodies sometimes works well. Engineering membrane proteins for crystallization is possible in principle [11] but less helpful, as a sufficient level of overexpression rarely can be achieved. In all published cases, membrane protein crystals have been grown from proteins isolated from natural sources. In nature, mainly photosynthetic membrane proteins and those from bioenergetics are abundant, which explains why these membrane proteins are the best characterized structurally. The majority of membrane proteins in the cell are present at only very low levels. Up until now, there seems to be no general way to obtain large quantities of functional membrane proteins using recombinant DNA techniques [12].

Recently, however, a strategy for the overproduction of membrane proteins, which are usually lethal to their host cells, has been published [13••]. This strategy involves usage of selected *Escherichia coli* strains and the bacteriophage T7 RNA polymerase system for the the overproduction of a number of membrane proteins. In these strains, membrane proteins are formed in large amounts as inclusion bodies. Protein yields in the range of 100 mg per liter of bacterial cell culture have been reported. Unfortunately, the refolding of membrane proteins from inclusion bodies is mostly an unsolved problem and is one of the main challenges for the future.

Table 1

Membrane protein familes for which crystal structures exist.

Membrane protein family	Resolution (Å)	Pigmented	β sheet
Photosynthetic reaction centers	2.3	Yes	No
Porins	1.8	No	Yes
Light harvesting complexes	2.4	Yes	No
Cytochrome *c* oxidases	2.7	Yes	No
α-hemolysin	1.9	No	Yes
Cytochrome bc_1 complex	3.0	Yes	No
Prostaglandin H_2 synthase	3.5	Yes	No

Detergents: expensive soaps

Since the early years of membrane protein crystallization, choosing the right detergent has been the key to success. Well-ordered crystals of the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis* could only be grown using N,N-dimethyl dodecylamine-n-oxide as detergent. Even use of the decyl homolog did not lead to crystals. Recent experiences confirm this observation. The cytochrome *c* oxidases provide illustrative examples. Crystallization attempts with the cytochrome *c* oxidase from bovine beef heart mitochondria continued in Yoshikawa's laboratory for about twenty years, and crystals have been obtained in a number of different detergents [14]; however, only the use of n-decyl-β-D-maltoside (C_{10}-maltoside) — a mild, well-known detergent — has yielded well-ordered crystals.

Cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* is another typical example. For the purification and crystallization of the four-subunit complex, only detergents of the maltoside-type can be used. All other detergents remove subunits III and IV leaving an active complex consisting of subunits I and II. Only n-dodecyl-β-D-maltoside (C_{12}-maltoside) leads to the formation of well-ordered crystals of the four-subunit oxidase as an complex with an Fv fragment [15]. Recently, the catalytically active two-subunit complex could be crystallized, again with the help of an antibody Fv fragment (see also below) in different detergents. Originally, crystals were grown using the C_{12}-maltoside, but these diffracted to only about 8 Å. Crystals grown with hexaethylene glycol monododecyl ether ($C_{12}E_6$) showed the same poor diffraction quality. With the C_{10}-maltoside, no crystals could be obtained at all. Recently, the C_{11}-maltoside became also commercially available. Crystals grown in this detergent diffract to better than 2.6 Å resolution (C Ostermeier, A Harrenga, U Ermler, H Michel, unpublished data). Similar crystals can be grown with cyclohexyl-hexyl-β-D-maltoside (CYMAL-6), but not with cyclohexyl-pentyl-β-D-maltoside (CYMAL-5). Cyclohexyl-heptyl-β-D-maltoside (CYMAL-7) is not yet commercially available.

These results show that even small chemical differences in the detergent can cause essential differences in the crystallization behaviour of these detergent–membrane protein complexes. The conclusion has to be drawn that more efforts should be put into screening various detergents for crystallization than into the variation of other parameters. A major problem may be the high costs of many detergents. The optimal way to cope with this hindrance is to purify the protein using a rather inexpensive detergent such as Triton X-100 or N,N-dimethyldodecylamine-N-oxide and then to exchange the detergent prior to the crystallization attempts. One should keep in mind that it may be difficult to control the completeness of the detergent exchange. In our opinion, the simplest and most efficient method for a complete exchange is ion exchange chromatography, or another method in which the membrane protein is bound to column materials and can be washed with a large amount of buffer containing the new detergent without being eluted from the column. Gel filtration or exchange by ultrafiltration is not recommended if one requires a complete exchange.

It would be helpful if a continuous set of alkyl chain lengths were commercially available for many detergent headgroups, for example, the C_xE_y-detergents are available only with an even number of C atoms. One should also keep in mind that mixtures of detergents often may be useful. Finally, a need still exists for new classes of detergents.

An alternative to the classic detergents may be the so-called 'amphipols' — polymers that can potentially keep membrane proteins in aqueous solution [16•]. These possess a strongly hydrophilic backbone that is decorated with hydrophobic sidechains, resulting in an amphiphilic structure. So far, amphipols have not yet been used for crystallization, but they might be useful in the future.

Crystallization: finding the needle (detergent?) in the haystack

For the time being, most promise lies with trying to obtain a type II crystal. This approach has the advantage that the membrane protein surrounded by its belt-like detergent micelle can be treated as an ordinary soluble protein, and standard crystallization procedures can be used (for general reviews, see [1,17–20]). Most membrane protein crystals have been obtained using standard precipitants like polyethyleneglycols or salts (ammonium sulfate, potassium phosphate). The vapour-diffusion method with sitting drops is most frequently applied to achieve supersaturation of membrane proteins.

As outlined above, the choice of the detergent is the most important factor apart from the stability and homogeneity of the protein. This is understandable because the detergent micelle has to fit optimally into the crystal lattice of the protein. Attractive, polar interactions between neighboring detergent micelles appear to be helpful and to contribute to the stability of the crystal lattice. Such contacts cannot occur when the detergents have a rather short alkyl chain, thus explaining why crystals are sometimes obtained only with longer homolog of the same detergent type. That attractive interactions between detergent micelles play a role is also indicated by the fact that crystallization often occurs close to conditions in which where phase separation into a detergent-rich and a detergent-depleted phase occurs. This phase separation is caused by attractive interactions between detergent micelles [21]. In the case of the bacterial cytochrome *c* oxidase crystal, formation normally starts at the physical boundary between the detergent-rich and the detergent-depleted phase.

Detergent micelles can be made smaller by adding small amphiphilic molecules such as heptane-1,2,3-triol [1,22,23]. This approach has been successful in the case of bacterial photosynthetic reaction centers and light-harvesting complexes [4], for which rather harsh detergents with small polar headgroups can be used. It is unsuccessful when rather mild detergents, such as the alkyl maltosides, are required.

The trick with the complex

Instead of trying to get a smaller detergent micelle, one can try to increase the surface area of the hydrophilic portion of the membrane protein. Binding a soluble protein to the membrane protein under investigation is one possibility for extending the polar regions. This strategy has been used successfully twice. The four- and two-subunit bacterial cytochrome *c* oxidases have been crystallized as a cocomplex with an Fv fragment of a monoclonal antibody ([5]; C Ostermeier, A Harrenga, U Ermler, H Michel, unpublished data). The crystallization conditions, the space group and the crystal packing of both complexes are completely different. In both crystal structures, the Fv fragment plays an essential role in forming the well-ordered crystal lattice. Another advantage of using engineered Fv fragments for cocrystallization is the possibility of using an affinity tag engineered to the antibody fragment for the rapid isolation of the whole membrane protein–antibody complex [24]. Thus, an affinity tag for purification of the membrane protein can be used even if genetic engineering of the membrane protein itself is not possible. In the case of the two-subunit oxidase, isolation has been simplified by this strategy. Starting with crude membranes, crystallization trials can be set up within six hours after starting purification (C Ostermeier, A Harrenga, U Ermler, H Michel, unpublished data). Producing the Fv fragments may be a labour-intensive and often cumbersome procedure; however, for many important membrane proteins well-characterized hybridoma cell lines are already available.

Use of bicontinuous lipidic cubic phases

When mixed with aqueous solvents, some lipids form a bicontinuous cubic phase, in which the lipids are arranged in a curved, continuous 3D bilayer. Landau and Rosenbusch [25••] have succeeded to incorporate monomeric bacteriorhodopsin prepared from purple membranes in such a bilayer, and to use this as a matrix for crystallization. The idea is that the protein can diffuse in the bilayer, but it is also able to form 3D contacts. Landau and Rosenbusch have been able to demonstrate that bacteriorhodopsin forms small, but well-ordered 3D crystals. The X-ray data obtained from the most well-ordered crystal form indicate that the same 2D crystal lattice is formed that is observed in the native purple membrane. These membranes appear to be stacked and well ordered in the third dimension; therefore, the crystals belong to type I. It is to be hoped that this method can also be used for membrane proteins that do not have a strong tendency to form 2D crystalline arrays spontaneously. In particular, this method appears to be the only chance for membrane proteins that are unstable in detergent micelles or in the absence of added lipids.

Conclusions

The picture that emerges is that the membrane proteins tend to form the crystal lattice; the crystal lattice that forms is strongly influenced by the polar headgroup of the detergent. Sometimes, for example in the case of the photosynthetic reaction center from the purple bacterium *R. viridis*, the headgroups are involved by forming critical protein/headgroup/protein contacts (CRD Lancaster, H Michel, unpublished data). Often, the length of the alkyl chain of the detergent has to be optimized in order to get a well-ordered crystal. A possible reason for this is that polar interactions between neighboring detergent micelles are needed to stabilize the protein crystal lattice.

The recent advances in structural membrane protein research raise some hope that crystallography of membrane proteins will be no longer a wallflower in the field of structural biology but will become a powerful tool for understanding essential functions of membrane proteins, such as cell–cell communication via hormones or neurotransmitters, transport across membranes or energy conversion. The prerequisite for membrane protein crystallography — membrane protein crystallization — is still far away from being straightforward or routine. Two of the most important problems to be solved in the near future are the overproduction of functional membrane proteins in their native membrane environments, and the refolding of recombinant membrane proteins from inclusion bodies. Patience and many long-term grants are necessary before we can state that membrane protein crystallography is no longer in its infancy.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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