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Introduction: Crystallization of Membrane Proteins—In Need of a New Focus?

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Crystallographic results are essential for understanding the mechanism of transmembrane signaling, and the rapid pace with which new results in research on receptor signaling are being obtained requires a concerted effort to unravel the three-dimensional structures of receptors playing key roles in those events. Crystallography stands to gain from the progress in the molecular biology of signaling as much as it has contributed to its progress.

Most transmembrane proteins are available in small quantities but are rather unstable. Furthermore, there are three essential factors to be considered in the analysis of successful crystallization. The first factor is the choice of detergents for both the solubilization and crystallization steps. Despite the slow progress in crystallization of transmembrane proteins, there is a considerable amount of data to indicate the most useful set of detergents. A set of these detergents, a screen, has been presented by Hartmut Michel[2] at the Membrane Proteins Crystallization Workshop in San Diego, and those detergents, along with a few additional ones, are presented in Table I. The second factor is the presence of heptane-1,2,3-triol which has been shown to compete with the detergent on the protein surface. The third factor is the presence of lipids, as shown by Kühlbrandt and his colleagues in the crystallization of plant light-harvesting complex (Nußberger *et al.*[5]).

There is a basic strategy for exchanging detergents and additives which involves two steps. The first is the solubilization/concentration step and the second is the detergent exchange step. The detergent exchange step is carried out on a small Q-sepharose column which is first equilibrated with a wash buffer containing the detergents listed in Table I. There are several factors that have to be considered in the selection of detergents for the screening experiments. Neutron crystallography has shown that in the crystal lattice of the bacterial photosynthetic reaction center of *Rhodopseudomonas viridis*, polar interactions are observed not only between proteins and micelles, but also between micelles and micelles and proteins and proteins. These interactions of detergents in the crystal lattice imply that the size of the detergent will determine the quality of the diffraction pattern. It has also been observed that both short alkyl chain detergents and detergents with small polar nonionic head groups denature membrane proteins more severely. The third important fac-

Table I. Transmembrane Protein Crystallization Detergents[a]

Number	Concentration (%)	Detergent
1	0.1	*N,N*-Dimethyldodecylamine-*n*-oxide[1]
2	0.2	*N,N*-Dimethyldecylamine-*n*-oxide[1]
3	0.5	*N,N*-Dodecyldimethylamine *n*-oxide[2]
4	0.5	*N*-Lauryl-*N,N*-dimethylamine-*n*-oxide[3]
5	0.3	Octyl-β-D-thioglucopyranoside[1]
6	0.9	Octyl-β-D-glucopyranoside[1]
7	3.0	*n*-Octyl-β-D-glucopyranoside[4]
8	0.2	Nonyl-β-D-glucopyranoside[1]
9	0.6	*n*-Nonyl-β,D-glucopyranoside[5]
10	0.05	lauryl-β-D-maltoside[1]
11	0.3	*n*-Octyltetraoxyethylene[1]
12	0.3	*n*-Octylpentaoxyethylene[1]
13	1.0	Octylpolyoxyethylene[6]
14	3.0	Octylpolyoxyethylene[7]
15	0.6	*n*-Octyl-rac-2,3-dihydroxypropysulfoxide[1]
16	0.4	*n*-Octyl-2-hydroxyethylsulfoxide[1]

[a] Concentration is in % w/v, except detergent Nos. 4, 7, and 14, where it is in % v/v.

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tor in the crystallization of membrane proteins is the presence of lipids. Two-dimensional crystallization of membrane proteins as illustrated by Kühlebrandt *et al.* has been accomplished by slow removal of detergents and addition of lipids. Two variables affect the crystallization, the nature of the lipid and the lipid:protein ratio.

All of the factors concerning crystallization are discussed by R. Michael Garavito of the University of Chicago, where the methodological emphasis is put on the purification and preparation of membrane protein samples. This approach stresses crystallization of most transmembrane proteins as an application of straightforward, standard methodologies used for standard soluble proteins, but incorporating the use of detergent in both the solubilization step and the crystallization step. Garavito's laboratory, which recently published the atomic structure of prostaglandin H-synthase,[9] focuses on the choice of detergents for maintaining stability and preventing the aggregation of membrane proteins before and during the crystallization trials.

When using molecular biology techniques, it is important to subdivide crystallization problems into distinct experiments by crystallizing those proteins that are partially embedded in the membrane, those that transiently associate, or those that form stable domains of large transmembrane assemblies. The atomic structure of prostaglandin H-synthase is an example of the first class, as this enzyme integrates only in one leaflet of the lipid bilayer with an extended hydrophobic domain. The second class of proteins is those known to interact with the membrane via myristylated N-terminal domain. Detergent binding to unmyristylated protein kinase A shows the structural implications for the role of the myristate. Comparison between the myristylated and the unmyristylated protein kinase A indicates that detergent locates itself in the same pocket as myristic acid does, and this binding results in the stable structural motif recognized by other proteins. This motif results from structural ordering of N-termini of helix A and most likely counts for the resolution extension of crystals (from 2.7 to 1.95 Å). The third class of experiments is the separate expression of ligand binding and cytoplasmic domains. This approach has been very successful, as documented by the insulin receptor tyrosine kinase structure.[10] The approach to the crystallization of transmembrane proteins where the receptor protein is subdivided into separate outside and inside domains which are crystallized using a standard methodology for crystallization of soluble proteins can provide an enormous amount of information about separate domains. However, since those domains have to be conceptually reassembled to provide models for the mechanism of action of the entire receptor, potentially important information concerning the molecular basis for signal transmission may be lost. The advantage of this approach is evident through the increasing number of high-resolution structures of receptor domains that have been solved.

Gil Prive of the University of California, Los Angeles has demonstrated the specific use of molecular biology in the crystallization of transmembrane proteins. In his approach, lac permease is used as a model system to increase the chances of obtaining crystals through modification of membrane proteins. Purification is simplified by the addition of six consecutive histidines at the C-terminus of the protein. This allows for rapid purification by nickel-chelate chromatography and the inclusion of the entire protein domain into the inner cytoplasmic loops of the protein to obtain a protein with a larger hydrophilic surface area. An increase in the polar surface transforms the fusion protein into one that behaves like most soluble proteins in the purification steps. In those constructs, the introduction of cytochrome B562 of *E. coli* into hydrophilic domain 7 of the lac permease results in a fusion protein with the visible absorbance spectrum of the cytochrome. The "red" permease is very easily monitored through the steps of expression, purification, concentration, and crystallization.

Due to the slow progress in successful protein crystallization there is a need for new approaches from both crystallographers and molecular biologists working together. A molecular biologist's help in obtaining protein samples is essential for a crystallographer's success in obtaining a structure; and a crystallographic structure provides an excellent platform for further research into mutagenesis and is essential to understanding the biochemistry of a system. The key strategy, therefore, should be to focus on different expression systems. It is still not clear whether or not membrane proteins can be expressed in the same quantities as the soluble proteins. However, due to the recent work of Xu *et al.*[8] (briefly reviewed in this series), the heterologous expression of mitochondrial transporters, specifically the tricarboxylate transport protein from rat liver and citrate transport protein from the yeast *Saccharomyces cerevisiae,* has been successfully carried out in *E. coli.* The enormous yield of 90 mg of the protein from 1 liter of the *E. coli* culture and 25 mg from another indicates that progress is imminent. Large amounts of these transmembrane pro-

teins, which are highly purified functionally competent transporters, would allow for more crystallization studies.

An enormous effort is required to generate high-resolution protein crystals, which warrants the need for different strategies for funding the crystallography of transmembrane proteins. Currently many studies are being pursued without such collaboration which is the single most important factor affecting the pace of research; funding is sorely needed for joint research focused on cloning, expressing, purifying, and crystallizing transmembrane proteins, all under one collaborative grant.

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