

New tools in membrane protein determination

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Abstract

The determination of membrane structures presents the structural biologist with many challenges; however, the last two years have seen major advances in our ability to resolve these structures at atomic resolution. My goal here is to summarize some of the most recent advances that have enhanced our prospects for understanding membrane proteins at the level of atomic structure.

Introduction and context

While the number of proteins for which the structures are known seems to increase exponentially each year (there are now almost 72,000 structures in the Research Collaboratory for Structural Bioinformatics [RCSB] Protein Data Bank), only a handful of intrinsic membrane proteins are among this group, despite the fact that they represent nearly 40% of all proteins. The main reason for this is a practical one: by necessity, the portion of the protein that passes through the membrane is hydrophobic, so most methods used to extract them are tuned to maintain the protein stably in a detergent-lipid-protein micellar structure. In situ, the associated lipid bilayer has limited the techniques that can be applied to determine structure to electron microscopy, or electron diffraction from rarely occurring two-dimensional crystals in the membrane. X-ray crystallography remains the only general method that can yield reliable insights into the atomic structure of membrane proteins of any size, at a resolution where interatomic atom positions can be determined. This requires solubilizing membrane proteins in detergent micelles, which then can be crystallized. Alternatively, use of lipidic phases can lead to crystallization in three dimensions within stacked planar lipid bilayers. As a result, elucidating the structures of membrane proteins presents particularly tough challenges. This is reflected in the recent commitment of funds from the National Institute of General Medical Sciences to support the Protein Structure Initiative's PSI:Biology program aimed

at membrane protein structure determination at no less than nine centers. This is partly driven by the therapeutic possibilities since many membrane proteins are implicated in disease, and a better understanding of their structures will allow more efficient focus on development of effective drugs, seen in the context of the molecular structure of their target.

Despite the great technical challenges, a combination of tenacity, great patience, and ingenuity has produced a raft of technical advances that have greatly improved our ability to determine some of these structures at a resolution useful for assistance in therapeutic development. Here, I outline some of the recent achievements in advancing this most demanding of disciplines.

Most membrane proteins are not expressed at high enough levels in cells of their natural environment, so determining their structures relies on inducing overexpression in cells, or by cell-free synthesis. Once expressed and inserted into the plasma membrane of cells that can be grown in quantity, membrane proteins must be solubilized from the membrane fraction, purified, and conditions optimized to maintain the protein either stably in solution over several weeks, or in lipidic environments for X-ray crystallography, electron microscopy, or nuclear magnetic resonance (NMR). A further level of difficulty is associated with eukaryotic, as opposed to prokaryotic, membrane proteins that can often be expressed in the membranes of

prokaryotic cells. Eukaryotic membrane proteins are normally assembled in the endoplasmic reticulum, an organelle not found in prokaryotes, and delivered to the cell surface via trafficking mechanisms and further post-translational processing in the Golgi apparatus. Thus eukaryotic membrane proteins are almost always expressed in eukaryotic cells.

Expression of membrane proteins

There have been several advances in improving expression systems in recent years.

One of the more dramatic achievements came with the structure of the eukaryotic chloride CLC transporter [1]. The elucidation of this structure was remarkable not only because the CLC transporter is a eukaryotic membrane protein and was successfully expressed in heterologous eukaryotic cells, but for the elegant replacement of each of 30 sites by hydrophobic methionine in 20 different mutant forms of the protein. These mutants were used because at limited resolution, which all too often is all that can be achieved, individual atoms cannot be placed unambiguously. The protein was then expressed in selenomethionine, which replaces the methionine sulfur atoms in the protein with larger selenium atoms to provide firm experimental assignment of the positions of mileposts along the protein chain. Thus this process effectively tagged the protein with visible markers at each of 30 labeled sites. This was done to unambiguously resolve potentially controversial assignments. This publication also dramatically coupled visual images of the structure and explanation by Rod MacKinnon with music, as Rimsky-Korsakov's "Flight of the Bumble Bee" was used as a beautiful accompaniment to the supplementary movie that illustrates the stochastic nature of chloride transport. It is a great example of how intricate mechanisms can truly become accessible to the broad community.

Several key factors have catalyzed a ready access to the membrane proteome. Cloning and expression of membrane proteins requires that they be correctly inserted into lipid bilayers or into the bacterial plasma membrane. However, while bacteria are excellent for producing many eukaryotic proteins, they tend to not be so good at folding them. For this reason, it is better, if possible, in many cases to use a eukaryotic expression system. Operating in compartmentalized cells, eukaryotic membrane proteins are, however, subject to much more involved trafficking through the endoplasmic reticulum and the Golgi apparatus, so protein insertion involves additional steps. A major advance has been the increasing ability to express such membrane proteins in eukaryotic cells, a key to understanding human membrane biology at the level of

molecular structure. One of the most attractive eukaryotic expression systems is in yeast *Saccharomyces cerevisiae* [2,3], in part because there is a wealth of genetics available and it is easily grown to high volumes within a week, whereas expression in HEK (Human Embryonic Kidney) cells can take 1 month to establish transient expression, and around 3 months to generate stable cell lines.

There have also been great improvements in tools to assess the proteins expressed. Rapid ways of screening expression levels by fusing a fluorescent protein to the membrane protein being tested, prior to analysis by fluorescence-detection size-exclusion chromatography to assess and eliminate misfolded and aggregated proteins, and even prior to solubilizing the membrane fraction or purification, have been instrumental in rapidly settling on satisfactory expression methods [4,5]. Multiple homologous genes can be tested and screened for expression in the membranes of eukaryotic cells including insect cells, HEK cells, and COS cells. This powerful screening method for proteins that express better in certain species compared with others has resulted in several exciting breakthrough structures of membrane proteins, by first rapidly identifying the species that best expresses for crystallization [6,7].

Gene redesign has become a powerful tool in controlling the expression level of membrane proteins, making structure determination possible. In a recent study [8], two redesigns of a *Plasmodium falciparum* gene gave us low-level expression in the plasma membrane fraction of *Escherichia coli*, whereas the wild-type gene was not expressed. This is a common problem in expressing eukaryotic proteins in bacteria, but it can be circumvented by "codon optimization", in which codons that are not copied well by the bacterium are replaced by those that are, while producing the same amino acid sequence. We generated so-called "codon-optimized" genes for 21 different proteins, which led to 7 of the best-expressed proteins progressing to crystallization trials. However, the rules for optimizing are still quite variable, and only about 50% of the constructs showed better expression levels than the wild-type gene, indicating that while it is a powerful tool, sometimes it requires several constructs for the same protein to find one that expresses at a higher level. Of the 21, 3 crystallized and 1 yielded a structure within a year of using the construct. Codon optimization for each of these proteins was essential for adequate expression, mostly in *E. coli*, and for moving towards crystallization trials.

Is the process of codon optimization reproducible? The sequence of bases is different every time the gene is designed. In attempts to calibrate effects of gene optimization and to extract algorithms that might be predictive of increased expression, two independent genes were

systematically codon optimized for expression in *E. coli*. The different gene variants within each set had as low as 65% DNA identity with the original gene while still encoding the same wild-type amino acid sequence. Protein expression levels varied from below the level of detection to 30% of cell mass. "Machine learning" algorithms were used to identify 12 variables of the gene and the amino acid sequence that could be used to build highly predictable and robust rule-based gene sequences that produce proteins for crystallization. Each of the important variables in *E. coli* was identified to be a specific codon.

Stabilizing the proteins expressed

The hydrophobic nature of their membrane-spanning regions makes stabilization of expressed membrane proteins more complex than soluble proteins. The importance of lipids in interacting directly with membrane proteins has become increasingly recognized, and hence lipid mixtures are now increasingly being incorporated into crystallization cocktails. One recipe uses buffers that contain 0.1mg/ml lipid in a 3:1:1 ratio of POPC:POPE:POPG (1-Palmitoyl-2-Oleoyl-sn-glycero-3-PhosphoCholine: 1-Palmitoyl-2-Oleoyl-sn-glycero-3-PhosphoEthanolamine: 1-Palmitoyl-2-Oleoyl-sn-glycero-3-[Phosphorac-(1-Glycerol)]), obtained from Avanti Polar Lipids, Inc., and 5mM of n-dodecyl β -maltoside (DDM) as detergent [9]. This recipe is fairly standard though small variations are used around these general conditions.

One example of a lipid playing such a direct structural role emerged in the structure of an engineered beta-2 adrenergic receptor, where a cholesterol molecule formed the interface between the protein molecules in the physiological state of the membrane protein [10].

Another such example is in the case of voltage-sensing domains of potassium channels, where lipid molecules are critical for correct channel conformation, and crystal structures determined without lipids show unphysiological distortions. NMR has shown that the voltage-sensing domains interact with short-chain and long-chain phospholipids in the context of a phospholipid micelle. The atomic description of the micelle environment and the difference in affinity for membrane lipids along the protein surface likely could not have been obtained without the use of NMR and micelles to stabilize the protein [11]. This NMR approach is broadly applicable and opens the door to understanding the structure, dynamics, and lipid interactions of other integral membrane proteins.

New detergents have also emerged as new tools for stabilizing and crystallizing membrane proteins. Facial amphiphiles, such as cholate-based amphiphiles in which

hydrophilic maltose units project from one side of the rigid and hydrophobic steroidal skeleton, are especially attractive [12]. Particularly interesting are tandem facial amphiphiles, termed TFAs, in which two deoxycholate moieties are coupled together as a covalent head-to-head dimer [13]. These form small, discrete micelles in water (with a molecular weight of approximately 13 kDa) whereas DDM forms much larger micelles (\sim 90 kDa). The radii of the TFAs were significantly smaller (\sim 19-30Å), relative to DDM micelles (\sim 34Å). Since the detergent micelle that surrounds the hydrophobic portions of membrane protein is essentially liquid-like, the crystal lattice contacts involve the protein regions that extend beyond the micelle. Hence the smaller micelle allows proteins to pack more closely together in the crystal, and increases the amount of protein outside the smaller micelle, allowing more access to the protein for lattice formation. Three TFAs showed excellent ability to maintain intrinsic membrane proteins or protein assemblies in native-like forms in aqueous solution.

Purification techniques

Purification of membrane proteins is critical to success in crystallization. Affinity purification using nickel affinity tags, or antibodies to protein antigenic sites, have been developed to capture poorly expressed levels of eukaryotic membrane proteins. Size-exclusion chromatography is then used to select well-folded protein and the correct oligomeric state of protein complexes. Such columns are run in detergent buffers to maintain solubility.

Detergents used to stabilize membrane proteins can interfere with crystallization, so tools to assess how detergent-free a sample is are another big advance. Triple and tetra detectors that incorporate right-angle light scattering, viscosity, and the refractive index can analyze the separation of the free detergent or detergent/lipid micelles from the protein-lipid-detergent micelle and can also, in principle, determine the molecular weight of the protein-detergent-lipid complex [3,14,15].

Optimizing proteins for structure determination

X-ray crystallography is the commonest technique used to determine protein structures at a resolution high enough to provide coordinates for its atoms, and for this proteins must be crystallized. This is not particularly easy for cytosolic proteins. For membrane proteins, it is much harder.

Crystallization in lipidic phases has only recently been developed but has already become an essential tool in the arsenal of membrane protein crystallization [16,17]. The method originated from Ehud Landau and Jürg Rosenbusch in 1996 [18] and was soon followed by

application to bacteriorhodopsin [19]. This protein had been crystallized and was the target for crystallization attempts for many years but without adequate resolution for an atomic structure determination. In the early 1980s, Larry Miercke, working with Edward Dratz and myself, had grown large-sized (~ 1 mm) crystals of bacteriorhodopsin in the dark under dim red lights to prevent photobleaching using detergents; Hartmut Michel similarly had crystallized this readily available, colored membrane protein. However, the so-called lipidic mesophase methods, a term describing lipid molecules that have properties between those of a conventional liquid and a solid crystal, proved the key to solving the structure, which eventually reached very high resolution (1.55\AA) [20], with the subsequent mapping of the structural changes in the photoreaction. This method was also applied to two other haloarchaeal rhodopsins, sensory rhodopsin and halorhodopsin, also colored membrane proteins, with success. But until recently, it remained a boutique method readily applicable to colored proteins that could be seen inside the clear viscous phase.

The biophysics of how the method works to “exclude” membrane proteins into a lamellar phase where they may crystallize is still an exciting question. But several developments established the method as broadly applicable to all membrane proteins. These include the high-throughput robotic screening of mesophase materials for embedded crystals using X-rays that can pick out crystals of even noncolored proteins, a key step making the method routinely applicable [21]. Another powerful approach seeks to assess the ability that membrane proteins have while in the lipidic bilayer-based mesophase and identify conditions that favor them diffusing in two dimensions as freely as possible in order to find other protein partners with which to build the two-dimensional array, which will become the nucleus for stacking up layers to form a three-dimensional crystal lattice. A major advance is the application of fluorescence to assay the diffusion rates, seeking conditions that maximize the diffusability. Dyes such as hydrophobic 5,5'-disulfato-1'-ethyl-3,3,3',3'-tetramethylindocarbocyanine (Cy3) are attached to the protein. In this method, labeled protein in solution (40% w/w) is mixed with molten monoolein (1-oleoyl-*rac*-glycerol, Sigma) (60% w/w) using a syringe lipid mixer to form lipidic cubic phase (LCP). The fluorescent dye-labeled proteins are used to assay effects of swelling agents such as butanediol on the lipid phase, and effects of cholesterol, for example, in slowing diffusion.

Crystallization in lipid-based bicelles and nanodiscs is a new development to watch. These methods seek to trap

each membrane protein molecule within a disc of lipid bilayer of finite size. The size of a bicelle is determined by the mass ratio of shorter chain lipids that form the rim around the disc; the nanodisc dimension is determined by a surrounding protein. Thus the method has the advantage of bilayer-based methods of preserving integrity of the membrane protein, and the advantage of the ability to diffuse in three dimensions in the process of forming a three-dimensional lattice. In the case of bacteriorhodopsin, clear density for a CHAPSO molecule inserted between protein subunits is seen within the layers indicating that an important interaction between lipid and protein has been preserved within the bicelle by the cholesterol-like detergent. The ability to grow crystals at room temperature, rather than under defined temperatures used to stabilize these heterogeneous states, significantly expands the applicability of bicelle crystallization [22]. Bicelle lipid/amphiphile mixtures tend to form small bilayer discs at low temperature, and form a perforated lamellar phase at higher temperature. The transition temperature depends on the lipid/amphiphile composition. In the perforated lamellar phase, the mixtures form gels, but at lower temperatures the mixtures are liquid and can be easily manipulated. Consequently, proteins in bicelles can be handled at low temperatures just like proteins in detergent, and crystal trials can be performed in the same manner as detergent crystallization. Thus, the bicelle approach has the advantage of convenience in handling membrane proteins within a bilayer, but in solution as opposed to being embedded in a large deformable lipid-bilayer membrane. This allows them to diffuse freely so they can find each other and build a three-dimensional lattice while maintaining the protein in a bilayer-like environment (as it would be in a natural membrane).

Nanodiscs, like bicelles, are yet another template for solubilizing membrane proteins with some advantages over liposomes or detergent micelles in terms of size and stability [23]. The nanodisc is a noncovalent assembly of phospholipids, typically POPC, dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), and a genetically engineered “membrane scaffold protein” based on the sequence of human serum apolipoprotein AI. They are formed by removing detergent from a mixture of the membrane protein, lipid, and detergents. The phospholipid bilayer is composed of two opposed leaflets, or layers in which all lipid chains are pointed in the same direction. The nanodisc is constrained by two molecules of membrane scaffold protein that wrap around the edges of the discoidal structure in a belt-like arrangement, such that one membrane scaffold protein coats each of the hydrophobic alkyl chains of each leaflet. To date, these bilayer-based, diffusible structures have

been used to best advantage with NMR methods, or for assessing the ligand binding or native state of membrane proteins.

Protein engineering has long been used to make proteins more amenable to crystallization. Truncations of the hydrophilic, often flexible termini of membrane proteins have often been used as an aid to crystallization since they tend to remove a sometimes flexible region that may impede formation of an ordered three-dimensional lattice. This can be done by truncating the gene expressed, or by mild treatment with proteolysis, as was used in the determination of the human aquaporin 4 structure [24]. Another approach is insertion of mutations that alter the protein sequence in a manner that stabilizes one particular conformation versus others, and screening of several to find those that might rigidify otherwise flexible regions. With the beta-adrenergic receptor, the insertion of the mutation E122W was found to make the protein stable as detected by the raised temperature of unfolding, measured as described above. Chimeric insertions were also used to insert a small, fairly rigid soluble protein into a supposedly flexible loop of the membrane protein under the theory that more ordered and rigid components make a more ordered crystal lattice, and hence yield better resolution in the structure. The soluble protein chosen was a lysozyme, chosen because it had its N- and C- termini close to each other in the soluble protein favoring a short circuit of a short excised loop. This strategy proved successful for this and other G-protein coupled receptors [10].

A powerful new strategy is to try to stabilize the membrane protein along with a screen of different engineered constructs, ligands, or lipids to assess their ability to increase the thermal stability of membrane proteins. This can be carried out in a high-throughput format using fluorescent dyes. In a recent application [25], exposure and reactivity of native cysteines embedded in the protein interior were used as a sensor to determine the temperature of overall unfolding of the protein as the temperature is raised. In this case, a fluorescent label is used to detect thermal "melting" of the membrane protein by covalent attachment to cysteines that become available upon melting. The thermal information gained by investigation of the protein stability landscape can be used to guide construct design, to select stabilizing ligands or lipids, to assess detergents, and to plan crystallization strategies based on increasing the thermal stability [25]. This LCP- T_m protocol offers a clear quantitative method of selecting conditions that are most stabilized by the variation in conditions and therefore the most likely to crystallize since they are more likely to be in a more stable state.

Future prospects

The last few years have seen dramatic progress in the techniques and prospects for determining structures of membrane proteins and how they function in response to binding a natural ligand or a drug, and specifically those selected for their impact on a particular biological or biotherapeutic application. At last, scientists can begin to approach the mechanisms of transmembrane processes involved in neurochemistry, metabolic signaling via G-protein coupled receptors, receptors that signal pain whose inhibition might assuage their debilitating effects in acute conditions, and neuroreceptors and transporters in mental illness. Understanding the transmembrane processes involved in mental illness perhaps most poignantly illustrates the potential applications for the future. Today's drugs act on membrane protein receptors and transporters, yet we often don't know which receptors, and we know little of what effect their binding evokes, or how many other targets are also affected. Such drugs are discovered by trial and error in the only animal model that relays a sense of mental disturbance: humans. We can now just begin to decode the action of today's drugs on each specific receptor or neurotransmitter transporter. These structures, and the purified membrane proteins that are produced even before the structures are resolved, will lead us to develop a rational approach to the treatment of schizophrenia, depression, and bipolar disorder. The introduction of a raft of effective new tools for the membrane structural biologist reflects the ingenuity of the current generation and lays the groundwork for application to numerous diseases.

Abbreviations

DDM, n-dodecyl β -maltoside; HEK, Human Embryonic Kidney; LCP, lipidic cubic phase; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; TFA, tandem facial amphiphile.

Competing interests

The author declares that he has no competing interests.

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