Riding the wave: structural and energetic principles of helical membrane proteins

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Genome sequencing efforts have revealed that perhaps as many as 20–40% of open reading frames in complex organisms may encode proteins containing at least one helical transmembrane segment. Contrasting with this approaching tidal wave of helical membrane proteins is the fact that our understanding of the sequence–structure–function relationships for membrane proteins lags far behind that of soluble proteins. This looming reality emphasizes the tremendous biochemical and structural work that remains to be done on helical membrane proteins in order to elucidate the structural and energetic principles that specify and stabilize their folds, which define their functions. These facts are not lost on the pharmaceutical industry, where successful therapeutics and major discovery efforts are targeting membrane proteins.

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Abbreviations
GPCR G-protein-coupled receptor
PLB phospholamban

Introduction

The paradox posed by the sheer number of potential helical membrane proteins [1] and the lack of high-resolution structural and thermodynamic information for them emphasizes the extensive biophysical and structural work that remains to be done in the field of helical membrane proteins. The potential payoff may be great as this class of proteins has historically contained excellent targets for therapeutics. Advances in our ability to understand and manipulate membrane proteins may lead to the discovery or design of pharmaceutical agents that can modulate their functions. The protein superfamily of seven-transmembrane segment, G-protein-coupled receptor (GPCR) proteins, provides an illustrative example: all major pharmaceutical firms are currently engaged in GPCR research, and over half of the drugs on the market today are thought to target GPCR signaling pathways [2–4]. This review will highlight recent experimental methodologies that were successful in advancing our understanding of helical membrane proteins.

Catch a wave

Obtaining functionally active purified protein

Much of the effort expended by the membrane protein structural biologist focuses on obtaining high enough quantities of functionally active protein for subsequent study. In contrast to genetic assays, relatively large amounts of purified protein are required for structural and biophysical characterization. Although transmembrane proteins can, in principle, be expressed in a functional form in all expression systems, the degree of success varies because of differences in protein and host-cell characteristics [5,6]. Insect cell expression systems have proven to be a valuable tool for the high-level production of a multitude of recombinant proteins. Incorrect folding and processing, however, often hamper production of membrane proteins in infected insect cells, which results in the accumulation of non-functional protein. In cases of massive protein production, often only low levels of functional protein can be detected. This can possibly be avoided by coexpression of molecular chaperones with the protein of interest, as Tate *et al.* [7] have found that the proportion of active serotonin transporter can be increased three-fold when coexpressed with calnexin.

When expression of inactive forms is unavoidable, efficient refolding strategies need to be employed in order to recover functionally active protein. In fact, the production of proteins in the form of inclusion bodies (i.e. protein aggregates) may be advantageous, as long as they can subsequently be folded *in vitro*. Inclusion bodies are significantly enriched with the protein of interest, which may make them easier to purify, and can be economically generated in *Escherichia coli*. To provide logic to the folding task, Gouaux and co-workers [8,9•] have advanced a factorial protein-folding scheme. Grounded in statistics, the system represents an analytically meaningful process of evaluating protein-folding conditions from a minimal number of experiments, which makes the search for favorable protein folding conditions a focused and doable endeavor. The approach is particularly powerful because it can estimate the main effects of each factor, as well as identify multifactor interactions. Although the usefulness of this system was demonstrated using soluble proteins, such a strategy should also be applicable to the folding of membrane proteins.

Beyond purification

Following purification, one of the primary difficulties with working with membrane proteins is their poor stability in currently available detergent systems. This impediment has been addressed by two complementary strategies. Because the presence of free micelles is a source of difficulty in structural and biophysical studies, Popot and co-workers [10] have developed a class of polymers, termed ‘amphipols’ that are designed to keep membrane proteins in solution in the absence of free surfactant. Amphipols are comprised of a hydrophilic backbone chain that is randomly derivatized with hydrophobic acyl chains, thus yielding an amphiphilic polymer. Following reconstitution and removal of excess detergent and amphipol,

protein–amphipol complexes are present as small monodisperse particles, which can maintain several large protein complexes in their native state for days longer than traditional detergent solutions can.

Bowie and co-workers [11•] have taken the complementary tack of engineering the membrane protein in order to optimize its stability in commonly used detergents. Their studies have demonstrated that simply changing single sidechains in diacylglycerol kinase can greatly enhance the resistance of a membrane protein to irreversible inactivation. They further found that a combination of mutations at specific sites results in a double mutant that is more stable than either single mutant alone. Although the structural basis of such stabilization remains to be elucidated, the enhanced stability of the protein means a facilitation of biophysical and functional studies. Moreover, such an engineering approach might be widely applicable to other membrane protein systems, as it has been observed that several membrane proteins isolated from the thermophilic organisms have higher stability in detergent than their mesophilic counterparts [12,13].

Genetically probing structure and energetics

Genetic assays can also inform on structural and energetic principles of membrane proteins. Heterologous expression of GPCRs in yeast has proven to be especially useful when such expression systems have been used to explore ligand binding, G protein and effector coupling, and structural aspects of the receptors. Several groups have recently reviewed these technologies and their uses [6,14].

The transmembrane α-helix represents a fundamental building block of the helical transmembrane protein [15], and examination of transmembrane helix–helix interactions is one experimental pathway for probing specificity–stability principles of helical membrane proteins [16,17•]. Russ and Engelman [18•] have improved upon previously published protocols [19] for measuring self-association between transmembrane helices by using a genetic assay, which they term 'TOXCAT'. The TOXCAT system couples transmembrane helix association with the expression of chloramphenicol acetyltransferase, which can be easily and quantitatively measured. The assay is designed such that the level of expression of chloramphenicol acetyltransferase will reflect the strength of the transmembrane helix association. TOXCAT distinguishes a known dimerizing transmembrane helical domain from a non-associating mutant, and modulation of expression in the TOXCAT system results in a concentration-dependent response in *E. coli*. Perhaps most exciting, TOXCAT has been used to select associating transmembrane segments from randomized sequences, demonstrating the potential to identify previously unidentified interacting domains. Comparison of these novel segments with sequences derived from databases will lead to testable hypotheses on the formation of helical membrane protein complexes.

Minor *et al.* [20•] employed a yeast genetic screen to derive a model of an inwardly rectifying potassium channel. Functional channels were identified from libraries containing mutagenized transmembrane domains. Consideration of the positional tolerance to mutation coupled with phylogenetic and biochemical data permitted a preliminary model to be created in which the 'protein–protein', 'protein–lipid' and 'protein–water' faces of the M1 and M2 transmembrane domains could be identified. The model was further refined by sequence minimization and by second site suppressor analysis experiments. This proposed organization of the M1 and M2 transmembrane domains suggests a geometry of subunit–subunit interactions distinctly different from the structure of a bacterial channel with the same topology whose structure is known [21]. Interpretation of the rescue data is simplified by consideration of the structural constraints imposed by the phospholipid bilayer, and this genetic approach thus provides a method for obtaining structural information in the absence of high-resolution data.

Biophysical dissection

Biophysical analysis of membrane proteins using traditional techniques is complicated by the need to solubilize them in detergents. Electrospray ionization-mass spectrometry of intact protein generates a profile of the native covalent state of the gene product and its heterogeneity, but its application to membrane proteins necessitates removal of detergents and lipids required for solubilization [22]. Recent experiments with lactose permease have demonstrated that large hydrophobic intrinsic membrane proteins are amenable to mass spectrometric analysis if they are first transferred into chloroform/methanol/1% aqueous formic acid solution by gel filtration [23•]. The resultant acidified aqueous organic solvent mixture is highly compatible with electrospray ionization, and experiments with the permease showed an excellent signal-to-noise ratio. Combination of electrospray ionization-mass spectrometry with suitable chromatography should allow rapid analysis of complex protein mixtures, such as entire membrane fragments, with high accuracy within less than one hour.

Spectroscopic techniques [24–28] continue to be useful for determination of polypeptide segment structure (e.g. helical or not), depth of residues within a membrane, distance measurements between labeled sites, and association propensities of labeled segments. In particular, Thomas and co-workers [24,25] have used both fluorescence and electron spin resonance spectroscopy to show that the helical transmembrane protein phospholamban (PLB) exists as a mixture of oligomeric and monomeric forms in lipid vesicles. PLB is known to regulate the Ca-ATPase in cardiac sarcoplasmic reticulum. Further experiments evaluating the oligomeric state of PLB show that the presence of the Ca-ATPase results in a PLB depolymerization [26]. Additional experiments with mutants suggest that the regulatory effects of PLB upon the Ca-ATPase depend on

its propensity to dissociate into monomers [27]. It follows that directed engineering of PLB self-association may lead to designed strategies for regulation of the Ca-ATPase activity. Hubbell *et al.* [28] have highlighted the continued evolution and application of the spin label technology in a recent review.

For detailed consideration of the thermodynamics of transmembrane helix–helix interactions, analytical ultracentrifugation protocols have been developed whereby the free energy change of an interaction can be determined [29]. Under conditions where the detergent is invisible to the gravitational field, one can use sedimentation equilibrium to measure the association state of the protein moiety alone of a protein–detergent complex, from which the free energy of interaction can thus be calculated for a set of defined conditions. Recent experiments suggest that specificity in helix–helix interactions may define a hierarchy of stabilities for sequence variants that is independent of the hydrophobic environment (KG Fleming, DM Engelman, unpublished data). Moreover, the structural consequences for changes in energy can be explained by invoking simple principles for protein–protein recognition. It is worth noting that the advent of modern, user-friendly analytical ultracentrifuges is generating a renaissance in this field [30]. In particular, with respect to membrane proteins, the ultracentrifuge can be especially useful for identification of environmental conditions that produce monodisperse membrane-protein–detergent complexes, which might subsequently be amenable to structural analysis.

Obtaining high-resolution structures

The number of crystal structures of membrane proteins has nearly doubled in the past two years, and the solution of members of new classes of membrane proteins allows further glimpses into these proteins residing in membranes [21,31–33]. An in-depth discussion of these new structures is beyond the scope of this review. Nevertheless, the major constraint in attaining high-resolution structures of membrane proteins by X-ray crystallography continues to be the growth of well-ordered three-dimensional crystals. Strategies to obtain crystals and solve structures have been reviewed [34] and continue to evolve. These focus on modulations to both the membrane protein environments as well as to the protein itself.

Cubic lipid phases

The addition of bicontinuous cubic lipid phases to the protein crystallization matrix represents growth in the area of productive crystallization environments for membrane proteins. Their use may be advantageous because they provide a means to crystallize membrane proteins in a bilayer-like environment. Their implementation in the crystallization of bacteriorhodopsin has yielded crystals that diffract X-rays to <2 Å resolution [32,35,36,37•,38]. Although their general applicability to other membrane proteins remains to be demonstrated, cubic lipid phases offer the possibility of an additional approach to obtaining crystals of membrane proteins [36]. In the meantime, this technology has allowed the long-awaited initiation of elucidation of the cycle of structural changes that occurs upon bacteriorhodopsin activation, thus probing the interplay between bacteriorhodopsin structure and function [39••].

Engineering membrane proteins for crystallization

Rational redesign of the membrane protein itself is also being pursued in order to increase the likelihood of crystallization. Kaback and co-workers (see [40]) have used this strategy to engineer a fusion protein comprised of lactose permease and cytochrome b_{562}. The cytochrome was placed between loops of lactose permease resulting in a fusion protein in which the internal degrees of freedom should be reduced. Expression of lactose permease as a fusion protein increases the amount of polar surface area, which renders the fusion protein easier to handle, and which may also favor the formation of well ordered crystals. This particular design has the additional advantage in that, due to the cytochrome moiety, the 'red permease' is easier to monitor throughout its handling from expression until crystallization. The redesign efforts resulted in two-dimensional crystals of 'red' lactose permease [41], which represent the first step toward solving the lactose permease structure.

Conclusions: surfing the wave – predictive value

The usefulness of an understanding of helical membrane protein structural and energetic principles lies in its predictive value. The requisite solvent environment of a membrane protein imposes serious technical obstacles both to biophysical analysis and to obtaining high-resolution structures using conventional techniques. Consideration of the structural constraints imposed by the phospholipid environment may, however, actually simplify the problem because it limits the number of structures a transmembrane protein can adopt. Thus, a scaffold for membrane protein engineering may already be in place. In particular, the fact that helix–helix interactions can be energetically stable and highly specific suggests that such associations might be targets for directed interactions or for pharmaceutical agents that may either be discovered or designed to modulate membrane protein function. If advances in techniques for studying membrane proteins can keep pace with the wave, the next millennium will be filled with designer membrane proteins.

Acknowledgements

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