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Jose Manuel Perez Aguilar
University of Pennsylvania, perezagu@sas.upenn.edu

Jeffery G. Saven
University of Pennsylvania, saven@sas.upenn.edu

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Abstract
Membrane proteins are involved in a wide variety of cellular processes, and are typically part of the first interaction a cell has with extracellular molecules. As a result, these proteins comprise a majority of known drug targets. Membrane proteins are among the most difficult proteins to obtain and characterize, and a structure-based understanding of their properties can be difficult to elucidate. Notwithstanding, the design of membrane proteins can provide stringent tests of our understanding of these crucial biological systems, as well as introduce novel or targeted functionalities. Computational design methods have been particularly helpful in addressing these issues and this review discusses recent studies that tailor membrane proteins to display specific structures or functions, and how redesigned membrane proteins are being used to facilitate structural and functional studies.

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Computational Design of Membrane Proteins

Jose Manuel Perez-Aguilar and **Jeffery G. Saven**
Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104

Summary

Membrane proteins are involved in a wide variety of cellular processes, and are typically part of the first interaction a cell has with extracellular molecules. As a result, these proteins comprise a majority of known drug targets. Membrane proteins are among the most difficult proteins to obtain and characterize, and a structure-based understanding of their properties can be difficult to elucidate. Notwithstanding, the design of membrane proteins can provide stringent tests of our understanding of these crucial biological systems, as well as introduce novel or targeted functionalities. Computational design methods have been particularly helpful in addressing these issues and this review discusses recent studies that tailor membrane proteins to display specific structures or functions, and how redesigned membrane proteins are being used to facilitate structural and functional studies.

Introduction

Residing within lipid bilayers, integral membrane proteins are ubiquitous in cells and it is estimated that roughly 15–30% of the proteins in currently known genomes are integral membrane proteins (Almen et al., 2009; Wallin and von Heijne, 1998). These proteins participate in a wide diversity of cellular processes, including selective molecular transport across the bilayer, uptake of nutrients, discharge of toxins and waste products, respiration, cell motility, and cell signaling. Given their relevance to cellular physiology, membrane proteins--particularly receptor proteins and ion channels--are of great significance for a wide variety of therapeutics, with approximately 60% of currently available drugs having a membrane protein as a target (Overington et al., 2006). In recent years, advances in their study and characterization have furthered our understanding of the general molecular properties and biophysical principles germane to membrane proteins, but additional work needs to be done to acquire a detailed, predictive knowledge of the molecular basis of their structures, stabilities, and activities (White, 2009).

In this brief review, we highlight advances toward the design and redesign of integral membrane proteins with special emphasis on recent applications of computational methods.

Contact information: Jeffery G. Saven, Department of Chemistry, University of Pennsylvania, 231 South 34th Street, Philadelphia, PA 19104 USA, Telephone: 215-573-6062, Fax: 215-573-6416, saven@sas.upenn.edu.

The development of approaches to engineer membrane proteins has exploited advances involving both experimental and computational techniques. After briefly introducing some of the major challenges in working with membrane proteins and recent advances to address these, examples of designed membrane proteins are presented. Such designs can be used to explore membrane protein structure, introduce new functionality, modulate membrane integrity, control the activity of receptors and integrins, and arrive at water-soluble variants of membrane proteins. We focus on cases where designed proteins have been characterized experimentally.

Challenges in working with membrane proteins

Membrane proteins are notoriously difficult to study in their natural forms and are often difficult to obtain in large quantity. Typically present in low amounts in native tissues these proteins are difficult to isolate in quantities sufficient for detailed characterization. The situation is rendered even more challenging due to the fact that they are also difficult to over-express. Given that structural studies of proteins (e.g., using diffraction-quality crystals or NMR methods) typically require large amounts (> 10 mg) of protein, it is clear that naturally low abundances of membrane proteins in their native sources poses a significant impediment to their detailed examination. Consequently, most membrane proteins are over-expressed in heterologous bacterial and eukaryotic expression systems, a process with its own obstacles. Recombinant production and/or purification of membrane proteins present major hurdles in their study and structure determination. In this respect, eukaryotic recombinant proteins are particularly problematic, and mammalian membrane proteins are some of the most challenging. As a result, most membrane protein structures determined thus far are from bacteria and archaea (Alguel et al., 2010; Bill et al., 2011).

Even when sufficiently large amounts of protein can be obtained and purified, determining the structures of membrane proteins can be arduous. These proteins typically have low stability in the detergents used for dispersion in aqueous media and are susceptible to aggregation. Obtaining conditions under which such proteins can be crystallized or studied using NMR methods can be delicate and time-intensive, often involving extensive trial and error. Particulary elusive is obtaining quality crystals suitable for high-resolution x-ray diffraction studies (Alguel et al., 2010; Bill et al., 2011). Despite these difficulties, a significant number of membrane protein structures have been determined over the past 25 years. The number of nonredundant structures is currently around 300 (see (White, 2009) and the associated online database), which is only about 2% of known protein structures (Arinaminpathy et al., 2009). Of note, many of these membrane protein structures have been realized as protein mutants and chimeras (Chien et al., 2010; Jaakola et al., 2008; Rasmussen et al., 2011; Rosenbaum et al., 2007; Shimamura et al., 2011).

Significant progress in preparing membrane protein samples for structural and functional studies has been made in recent years. Novel approaches have been developed for improving the stability of membrane proteins and have facilitated determination of their structures. Rapid screening assays have been used to optimize the conditions that confer stability using GFP fusion proteins (Drew et al., 2008; Newstead et al., 2007). Complexes involving membrane proteins and monoclonal antibody fragments can increase the effective surface

area and introduce additional sites for crystal contact formation (Dutzler et al., 2003; Hunte et al., 2000; Iwata et al., 1995; Rasmussen et al., 2007; Zhou et al., 2001). Engineered fusion proteins have also been used in structure determination; for example a breakthrough in the structural biology of G protein-coupled receptors (GPCRs) came when a chimera was created that included a robust, crystal-forming protein (T4 lysozyme) and a GPCR, the human β_2 adrenergic receptor (β_2-AR; (Cherezov et al., 2007; Rosenbaum et al., 2007)) resulting in a high resolution β_2-AR structure. Short-chain detergents can form small micelles that leave large hydrophilic regions exposed. These detergents can be used in combination with thermostabilization strategies, where alanine scanning allows identification of stabilizing mutations so as to make the protein/micelle complex stable enough for crystallization (Lebon et al., 2011a; Lebon et al., 2011b; Magnani et al., 2008; Serrano-Vega et al., 2008; Shibata et al., 2009; Warne et al., 2008). The development of improved detergents has yielded stable protein-detergent micelles suitable for crystallization (Popot et al., 2003; Yu et al., 2000). Though not specific to membrane proteins, improvements in robotic technologies enhance both the exploration of a large number of crystallization conditions and the collection of the X-ray diffraction data. In addition, microfocus X-ray diffraction has the promise to reduce the necessary size of crystals required for solving structures as well as the ability to examine different regions of the same crystal, which may have different diffraction qualities (Bowler et al., 2010).

Design of Membrane Proteins

Structural and functional studies of nature's membrane proteins are frontier areas of structural biology, and a variety of beautiful structures have recently been elucidated. These include GPCRs (Chien et al., 2010; Jaakola et al., 2008; Palczewski et al., 2000; Rasmussen et al., 2011; Rosenbaum et al., 2007; Shimamura et al., 2011; Warne et al., 2008; Wu et al., 2010) and pentameric ligand-gated ion channels (Bocquet et al., 2009; Hibbs and Gouaux, 2011; Hilf and Dutzler, 2008, 2009; Miyazawa et al., 2003). The natural systems are often highly complex, however, and it is desirable to create systems where the details of function and structure are more readily at the control of the researcher. Designing and redesigning membrane proteins is one such route to better understand and engineer the structure and function of membrane proteins. Such design endeavors also, provide a way to test hypotheses concerning structure, function, and protein-protein interactions within membranes. Successful design can lead to proteins having new or tunable properties while residing in the membrane and facilitate studies of their functions and structures. Indeed, many of the recent structures of eukaryotic integral membrane proteins are mutants and/or chimeric constructs, where domains have been deleted or exogenous domains have been added, so as to render proteins that are more readily over-expressed, more soluble, or more likely to crystallize (Chien et al., 2010; Jaakola et al., 2008; Rasmussen et al., 2011; Rosenbaum et al., 2007; Shimamura et al., 2011; Wu et al., 2010).

Early successful efforts in membrane protein design focused on hydrophobic patterning of exterior residues in a manner consistent with a lipid bilayer. Synthetic α-helical amphiphilic peptides were designed to mimic properties of protein ion channels. Comprising only leucine and serine residues, a self-assembling 21-residue model peptide was designed to span the membrane and form an ion channel (Lear et al., 1988). The designed oligomeric

protein had properties similar to those of the acetylcholine receptor with regard to its channel conductance, cation selectivity and open state lifetime (Lear et al., 1988). An alternative peptide with one heptad position changed from serine to leucine produced a proton-selective channel, while a shortened version with only 14 residues was too short to span the phospolipid bilayer and failed to form discrete stable channels (Lear et al., 1988). Another early success employed one, two and four hydrophobic transmembrane segments. Using a highly simplified amino acid composition (mainly leucines and alanines), sequences of the α-helical segments were tailored for efficient insertion into the inner membrane when expressed in *E. coli*. The appropriate positioning of positively charged lysine residues was found to control the overall orientation of the protein in the lipid bilayer (Whitley et al., 1994).

Redesigned water-soluble proteins often provide a starting point for understanding the association of proteins in membrane environments. An early model system for studying the association of coiled-coils in aqueous media was the dimeric leucine zipper from a eukaryotic transcriptional regulator protein, GCN4 (Harbury et al., 1993). This system contains buried asparagine residues that mediate dimerization. The identities of surface exposed residues of the dimer were changed from polar to apolar to create membrane-soluble versions (Choma et al., 2000; Zhou et al., 2000). In the membrane-soluble analogs, the same buried asparagines mediate the formation of dimer and trimers; mutation of the buried asparagines to valines eliminated oligomerization. The oligomeric equilibrium was modulated by modification of the relative detergent concentration, and trimer formation was predominant at the highest peptide/detergent ratios (Choma et al., 2000). Using similar analogs, the roles of different residues at the “a” positions (where “a” is the first position in the heptad repeat of the coiled-coil structures) along the dimer interface were investigated with regard to their impact on dimer stability (Zhang et al., 2009). The results reveal that the size and hydrophobic character of the side chain regulates helix association. The helix association propensity of amino acids can be rank ordered: Gly > Ala > Val > Ile. This ranking is reversed relative to that observed for dimerization in water-soluble structures (Acharya et al., 2006; Wagschal et al., 1999). These findings suggest that van der Waals and electrostatic interactions dominate the stability and orientational preferences of the intramembrane dimers.

The approaches described in this section used largely empirical knowledge of structure, sequence and natural proteins to create novel membrane proteins. The work has revealed the degree to which knowledge acquired from studies of globular proteins can be extended to membrane proteins. Such studies also reveal features and trends that are unique to membrane associated proteins. Protein design provides a route to probe and characterize the biophysical principles governing protein-protein and protein-lipid interactions in membranes and opens the potential for more detailed, challenging, and sophisticated designs.

Design of Functional Membrane Proteins

Leveraging the insight gained from studies of transmembrane helical peptides and proteins, functionality can be introduced in to designed systems. Membrane associated proteins have been designed to provide a “switch” that can be used to modulate the integrity of a lipid

bilayer. In particular, amphiphilic α-helical peptides are known to be antimicrobial and to rupture cell membranes. In this regard, mastoparan X, a natural α-helical cell-lytic peptide has been redesigned to bind divalent cations. Upon binding of Zn(II) or Ni(II), the amphiphilic structure of the designed peptide is stabilized, which triggers the lysis of cells and vesicles (Signarvic and Degrado, 2009). The strategy demostrates the feasibility of designing proteins that can be selectively triggered to disrupt membranes.

Fusion of protein domains can yield chimeras that are useful for structural studies and can also be used to realize designed membrane proteins with targeted functionalities. One such effort yielded a pentameric ligand-gated ion channel, where each subunit comprised a prokaryotic extracellular domain and a eukaryotic transmembrane domain (Duret et al., 2011). The extracellular segment of the chimera was the proton-gated ion channel from *Gloeobacter violaceus* (GLIC), while the transmembrane segment was the anion-selective human α1 glycine receptor (Figure 1). Putative mismatches at the interface between the prokaryotic and eukaryotic domains of the chimera were minimized. The site of fusion was carefully selected and specific interfacial motifs were switched from the extracellular identity (GLIC) to the transmembrane identity (α1 glycine receptor). The chimera functions as a proton-gated ion channel, as evidenced from electrophysiological data obtained in *Xenopus* oocytes. Moreover, using patch-clamp experiments in baby hamster kidney (BHK) cells it was shown that the chimera displays anion selectivity identical to that of the glycine receptor. The activity of the chimera does not require posttranslational modifications typical of eukaryotic extracellular domains, and therefore the protein is good candidate for bacterial expression systems. This work provides a starting point for studies of the coupling between ligand gating and ion channel activity, as well as drug development; the findings suggest that GLIC and α1 glycine receptors may possess highly similar structures.

Another example of creating functional membrane proteins was based on a natural transmembrane dimer motif. Using the structure of the transmembrane region of glycophorin A, a bis-histidine binding site was designed to bind the cofactor Fe-protoporphyrin IX (Cordova et al., 2007). Five out of 32 transmembrane residues were modified, and the resulting structure was characterized in dodecylphosphocholine (DPC) micelles (Figure 2). The protein binds the cofactor with submicromolar affinity and retains the dimeric oligomerization state. Moreover, the catalytic activity of the complex was characterized by the oxidation of the organic substrate TMB (2,2′,5,5′ -tetramethyl-benzidine). TMB undergoes two successive oxidations in the presence of peroxide to produce TMB-ox, and formation of the latter indicated that the complex presents modest peroxidase activity. A single mutation (G25F) was introduced to assess aromatic-porphyrin interactions. The mutant binds heme with a lower dissociation constant (by a factor of 1/10), displays a change in the midpoint potential, and presents a decrease in peroxidase activity. The changes were ascribed to the stabilization of the Fe(III) form in the mutant. The findings illustrate the use of designed proteins to control the properties of the porphyrin cofactor within a membrane localized environment.

Computational Design of Membrane Proteins

The design efforts described thus far draw heavily upon structures and sequences derived from natural membrane proteins and/or qualitative and coarse-grained representations of protein sequence and structure (e.g., hydrophobic patterning). The interactions within a structured protein can have many levels of complexity, and often more molecular detail is required to specify structure and activity. Atomistic approaches to design are potentially more powerful and versatile, particularly when large numbers of candidate sequences are possible. Nature uses variation of sequence and selection to arrive at proteins with precisely tuned structures and functionalities. In the laboratory, well-folded proteins can potentially be identified through the careful choice of sequences, but this choice can be nontrivial. Proteins contain tens to thousands of amino acid residues, and even for a single sequence, many conformations of the backbone are possible. Even if we consider just a single backbone tertiary structure, exponentially large numbers of side chain conformations are possible. In addition, the folded states of proteins are stabilized largely by noncovalent forces: van der Waals, hydrophobic, electrostatic, and hydrogen-bonding interactions. Given the subtlety of these interactions, reliable estimates of stability with respect to unfolding can be difficult to calculate using molecular modeling methods. Furthermore, the large numbers of potential sequences can lead to combinatorial complexity in protein design: using just the 20 naturally occurring amino acids, a small protein of only 100 amino acids has more than 10^{130} possible sequences.

To address many of these difficulties, computational methods have been developed for the design of proteins. Most methods take as input a target structure, which can be a natural one or one created de novo via computational modeling. Energy-based objective functions are used to quantify interactions within a given structure and assess the compatibility between potential sequences and targeted structures and functions. The methods can identify individual sequences or the properties of sequences in an ensemble likely to possess targeted structural and functional properties. Optimization-based methods for identifying such low-energy sequences employ algorithms such as pruning methods (dead-end elimination) (Desmet et al., 1992) (Dahiyat and Mayo, 1997), Monte Carlo simulated annealing (Hellinga and Richards, 1994; Shakhnovich and Gutin, 1993) (Kuhlman and Baker, 2000), and genetic algorithms (Butterfoss and Kuhlman, 2006; Desjarlais and Handel, 1995; Kang and Saven, 2007; Samish et al., 2011; Saven, 2011). Such methods identify low-energy sequences. Alternatively, probabilistic methods characterize an ensemble of sequences and use methods derived from statistical thermodynamics to estimate the site-specific probabilities of the amino acids at variable sites within the protein (Calhoun et al., 2003; Kono and Saven, 2001; Park et al., 2005; Samish et al., 2011; Yang and Saven, 2005). Computational protein design may be used to design novel nonbiological protein-based molecular systems, to better understand protein stability and folding, and to facilitate the study of natural proteins.

The computational design of water-soluble proteins has seen significant progress (Nanda and Koder, 2010; Samish et al., 2011; Saven, 2011) and has been extended to the design of membrane proteins recently. Many of the biophysical principles involved in protein/protein and protein/membrane interactions within the anisotropic environment of the lipid bilayer are at least partially understood. Leveraging this understanding, particularly with the

assistance of computational design methods, stands to open new routes to the design and investigation of membrane proteins. Excellent related reviews of membrane protein design have also recently appeared (Ghirlanda, 2009; Senes, 2011).

In applying computational protein design, the features and structures that are specific to membrane proteins must be identified and quantified. Though they often have well-packed hydrophobic interiors, membrane proteins also have features that are distinct from water-soluble proteins. Efforts have been made to discern and characterize transmembrane helix-helix interaction motifs (Walters and Degrado, 2006). Energy functions have been developed based upon the observed frequencies with which the amino acids reside in particular regions of the lipid bilayer, and these depth-dependent propensities have been parameterized so as to reproduce the observed positioning of amino acids in helical transmembrane proteins (Senes et al., 2007). Relevant to membrane protein design is work involving the modeling and computational analysis of membrane protein structures, particularly with an eye toward structure prediction (Barth et al., 2007). Adjustments to energy functions developed for soluble proteins have been performed using a set of 18 high-resolution crystal structures of membrane proteins (Barth et al., 2007). Features frequently observed in membrane proteins were included explicitly: a membrane depth-dependent term for the amino acids and a bifurcated hydrogen bond term in which a carbonyl oxygen accepts more than one hydrogen bond. Furthermore, a weak Cα-H hydrogen bond with a carbonyl group was also considered. Such Cα-H interactions have been observed in transmembrane helices and suggested to stabilize helix-helix interactions (Bowie, 2011; Senes et al., 2001). The results obtained using proteins of less than 150 residues displayed an accuracy of structure prediction quantified by an RMSD < 2.5 Å when the model is compared to the known crystal structure (Barth et al., 2007). The method was extended to larger proteins (ranging between 190 and 300 residues) with the addition of experimental constraints on the structure (Barth et al., 2009). The constraints were extracted mainly from helix-helix packing arrangements, from a library of 79 high-resolution membrane proteins, cofactor vicinity geometries, and mutagenesis information.

Computational de novo protein design has been used to explore the residues involved in dimerization of GPCRs. Computational methods were used to identify 3–5 mutations at the putative dimer interface of rhodopsin. The predicted effects of mutations on the oligomerization state were consistent with experimentally derived mutation and alanine scanning data. The results suggest computational design and provide powerful tools for exploring the role of oligomerization in these important receptor proteins (Taylor et al., 2008).

Water-soluble proteins have been designed to selectively bind nonbiological porphyrin-based cofactors (Bender et al., 2007; Cochran et al., 2005; Fry et al., 2010; McAllister et al., 2008), and one of these complexes has been redesigned to yield a redox-active membrane protein (Korendovych et al., 2010). This membrane protein (PRIME) was designed to form an antiparallel D_2 symmetric homo-tetramer. The 24-residue helix bundle contains two bis-histidine binding sites that accommodate two nonbiological iron diphenylporphyrin cofactors ($Fe^{III}DPP$), which are poised to form a multicentered pathway for transmembrane electron transfer (Figure 3). Using threonine residues, an interhelical second-shell hydrogen

bond was designed to stabilize and orient the metal-coordinating histidine residues (Cochran et al., 2005; Korendovych et al., 2010). The stability of the complex derives from complementary van der Waals interactions throughout the interior, designed bis-his cofactor coordination, and His-Thr hydrogen bonding between helices (Cochran et al., 2005; Korendovych et al., 2010). The energies for the designed membrane protein were scored using the CHARMM potential with an implicit membrane solvation model (Lazaridis, 2003). The suitability of the sequence was assessed from calculations using a statistical potential (EZ) based upon the observed positioning of amino acids within the lipid bilayer (Senes et al., 2007). The designed membrane protein PRIME displays specificity for the Fe^{III}DPP cofactor and has the expected stoichiometry. The CD spectra are consistent with the targeted helical structure and orientation of the cofactors. The measured reduction potentials ($E_{1/2}$) are consistent with the target structure, and the difference in $E_{1/2}$ for reduction of the first and second Fe^{III}DPP is similar to that measured for natural four-helix/ bis-Fe-porphyrin proteins. This study establishes that guiding principles used for the design of soluble proteins, e.g., complementary van der Waals interactions and metal ion coordination, can be augmented to include features specific to membrane proteins to arrive at designed transmembrane complexes containing nonbiological redox-active cofactors.

Computational design has been used to examine the role of cooperative interactions between residues in a serine-zipper transmembrane helix motif. A probabilistic method was used to partially design sequences for a pair of associating helices. The designed protein forms a parallel helix dimer, but mutation of the central serine residues to alanines yields dimers of comparable stability, suggesting that complementary van der Waals interactions rather than hydrogen bonding plays a dominant role in stabilizing the dimer (North et al., 2006).

Leveraging the expertise gained from studying transmembrane proteins, it is possible to design transmembrane proteins that modulate the activities of the natural ones. Helical peptides have been designed that associate with the transmembrane domains of integrins and modulate their functions using a computational method (CHAMP: Computed Helical Anti Membrane Protein) (Yin et al., 2007). The native activity of the human integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ is modulated by association of the helical transmembrane domains of the α and β subunits. Together with interaction of their cytoplasmic domains, the interaction of the transmembrane helices stabilizes the inactive state, whereas stimulation by an agonist such as adenosine 5′-diphosphate (ADP) shifts the integrin to an active state. The interaction of the transmembrane domains of the integrin $\alpha_{IIb}\beta_3$ with a peptide from the transmembrane domain of α_{IIb} (α_{IIb}–TM) induces platelet aggregation and yields dissociation of the α and β heterodimer (Yin et al., 2006). This information was used to guide the identification of peptides that selectively recognize the transmembrane domains of α_{IIb} and α_v (Yin et al., 2007). The sequence of the target transmembrane domains of α_{IIb} and α_v were compared to existing structural motifs. A suitable backbone geometry was identified from a library of structurally defined helix pairs that were already local minima with respect to interhelical backbone-backbone interactions. This transmembrane structure and sequence of α_{IIb} on one (integrin) helix provided a “mold” for design of a second helix, the CHAMP peptide. Complementary van der Waals interactions were obtained by designing the sequence using a Monte Carlo algorithm. The calculations used energies derived from van der Waals interactions and a statistical potential for transmembrane orientation (EZ potential (Senes et

al., 2007)). The residues residing in the lipid interior were restrained to the amino acids most frequently found in transmembrane helices (G, A, V, I, L, S, T, and F). The resulting anti-α_{IIb} and anti-α_v peptides (Figure 4) recognize their targets with high specificity and formed heterodimers in micelles. Moreover, the anti-α_{IIb} and anti-α_v peptides strongly activated $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in mammalian cells, resulting in platelet aggregation ($\alpha_{IIb}\beta_3$) or adhesion ($\alpha_v\beta_3$). Despite the similarity of these integrins and of the templates used, cross-reactivity of the designed peptides was not observed. Lastly, the roles of the GxxxG-like motif (Russ and Engelman, 2000), complementary van der Waals interactions, and the Cα-H hydrogen bonding with a carbonyl group (Senes et al., 2001) were suggested as major driving forces in the selective association of the transmembrane helices. The approach has been further validated in studies of the direct interaction of the designed anti-α_{IIb} CHAMP peptide with isolated full-length integrin $\alpha_{IIb}\beta_3$ in detergent micelles. The designed peptides assume αhelical conformations that span the membrane and do not disrupt the bilayer integrity (Caputo et al., 2008).

The structure determination and functional characterization of membrane proteins remain challenging. Much of the difficulty arises from the poor expression level and poor solubility of the typical membrane protein. As a result, a variety of cell free, prokaryotic and eukaryotic over-expression systems are commonly employed to generate proteins in sufficiently large amounts, and then surfactants or co-proteins are employed to disperse, and when possible, crystallize the protein. Such efforts to realize large quantities of a membrane protein in a form suitable for structural studies are time-intensive and often involve extensive trial and error.

An alternative approach is to redesign a membrane protein as a water-soluble variant, while retaining the overall structure and many of the functionally related properties, e.g., ligand binding. Soluble proteins are usually much easier to obtain in large quantity, purify and analyze structurally. Transmembrane proteins have large numbers of exterior hydrophobic residues that complement the hydrophobic character of the bilayer interior. For many membrane proteins, the interior of the protein structure is similar to that of a typical water-soluble protein, in that it has large numbers of complementary hydrophobic residues. The solubilization approach focuses on redesigning the hydrophobic exterior transmembrane positions of the protein. Using computational design, water-soluble variants of transmembrane proteins have been designed, providing a proof of principle for the creation of variants that facilitate structural and functional studies (Bronson et al., 2006; Slovic et al., 2004). An initial target was the bacterial potassium ion channel KcsA, specifically the tetrameric integral membrane region (Zhou et al., 2001). The computationally designed water-soluble variant contains 29 designed exterior mutations in each of the four 104-residue subunits of the transmembrane domain. The protein was redesigned using a probabilistic design method so as to have exterior residues in the transmembrane domain that are consistent with each other and with those expected on the surface of a water-soluble protein (Slovic et al., 2004). The site-dependent probabilities of the amino acids at variable exterior positions were determined using a molecular potential (Amber) and an environmental energy (solvation propensity) for the amino acids that was tuned to a value consistent with that observed among soluble proteins of similar size. The designed proteins

express in large yield in *E. coli*, and one designed variant forms predominantly the target tetramer in solution. This protein (WSK-3) binds an ion channel toxin with the stoichiometry and affinity of the wild type protein. The solution structure of WSK-3 has been determined using NMR methods (Ma et al., 2008). The tertiary and quaternary structures are in excellent agreement with those from the X-ray crystallographic structure of the membrane-soluble wild-type protein (Figure 5) (Ma et al., 2008; Zhou et al., 2001). As expected, the water-soluble form exhibits larger structural fluctuations than the membrane bound form (Bronson et al., 2006; Ma et al., 2008). The chemical shifts of the residues bordering the selectivity filter of the protein are highly sensitive to potassium ion concentration, consistent with their native functional significance. The study suggests that identification of water-soluble variants of membrane proteins for biophysical, functional, and structural studies may be achieved via the computational redesign of sequence.

A similar solubilization approach was applied to obtain water-soluble variants of the transmembrane domain of the α1 subunit from a nicotinic acetylcholine receptor (nAChR), a eukaryotic pentameric ligand-gated ion channel (Cui et al., 2012). Using the transmembrane domain of the α1 subunit from the cryo-EM structure from *Torpedo* nAChR as template (Miyazawa et al., 2003), 23 exposed hydrophobic residues were computationally redesigned. To express the designed protein as a single chain, a polyglycine linker was used to connect the TM4 helix with a fragment containing the other three helices. The designed sequence (WSA) was expressed in *E. coli*, and its NMR structure was determined. The structure of WSA displays the expected four-helix bundle topology, but a detailed structural comparison indicates that the WSA structure resembles more the structure seen in GLIC, a prokaryotic homolog, than that in the original template structure. This was somewhat surprising, since the sequence of WSA is more similar to the wild-type transmembrane domain of nAChR (~83%) than to the transmembrane domain of GLIC (~11%). In addition, anesthetic binding studies in WSA showed excellent agreement with the binding site identified in the recent co-crystal structures of GLIC with similar general anesthetics (Nury et al., 2011). This result indicates that the designed water-soluble analogs retain the putative binding sites and thus, they could serve as good surrogates of membrane proteins for drug screening. Also, to disperse WSA at pH values suitable for NMR studies, 2% of LPPG detergent was utilized. Based on inter-molecular NMR cross-peaks, WSA-detergent interactions were identified and compared with the positions of lipid molecules present in the GLIC structure (Bocquet et al., 2009; Hilf and Dutzler, 2009). The location of the interaction site of WSA-LPPG is in excellent agreement with the location of the lipid in GLIC. These findings further support the notion that the designed water-soluble variants are able to retain important features of the structure, function and intermolecular interactions of their parent wild type membrane proteins.

Application of computational approaches in membrane protein design has established flexible, formal and physically grounded protocols to address the subtle interactions present in membrane proteins while exploring new functionalities such as the regulation of integrin activity. The examples described in this section exemplify the power and tunability of computational approaches. Additionally, the strategy involving membrane protein solubilization by redesign is supported by the two successful studies; these findings point to

the potential of using this approach to obtain proteins in large quantity in forms suitable for biophysical, ligand-binding, and structural studies.

Conclusions and outlook

Membrane protein design provides new routes to explore the structures and functions of these important but recalcitrant proteins, particularly when such design efforts are assisted by computational methods. Design can provide routes to novel proteins that can introduce new functions to membranes and modulate their integrity. Designed proteins can be conduits for transmembrane flow of ions, electrons, small molecules and (in the case of signaling) information. Furthermore, designed proteins can also be used to modulate the functional properties of membrane associated proteins, as in the case of transmembrane helical peptides targeted to the transmembrane domains of specific integrins. Some of the long-standing problem areas in structural biology are the difficulties associated with structural and functional studies of membrane proteins from a molecular perspective. The redesign of natural membrane proteins to facilitate their study—including removing them from the membrane altogether in the form of water-soluble proteins—provides a promising route to obtain protein in large quantities and explore functions and structures. Such studies have obvious relevance to drug development. Computationally guided protein design stands to make studies of membrane proteins more informative and effective. The design of proteins having the complexity, efficiency and specificity of nature's proteins is likely to remain challenging, but combining computational design and experimental studies will advance our abilities to engineer novel proteins, control biological activity, and better understand the natural systems. Incorporating nonnatural functions and components, e.g., nonbiological amino acids and cofactors, will yield protein-based systems that have properties not available in nature. Perhaps most importantly, attempts to design novel membrane proteins will provide stringent assessments of our understanding of the important structural, energetic, and functional features of these vital molecular systems.

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Highlights

- Computational design methods have advanced our capability to design membrane proteins.
- Designed membrane proteins bring new functionality to membranes and regulate lipid bilayer integrity.
- Designed proteins can regulate the activity of natural membrane proteins such as integrins.
- Designed water-soluble variants facilitate structural and functional studies of integral membrane proteins.

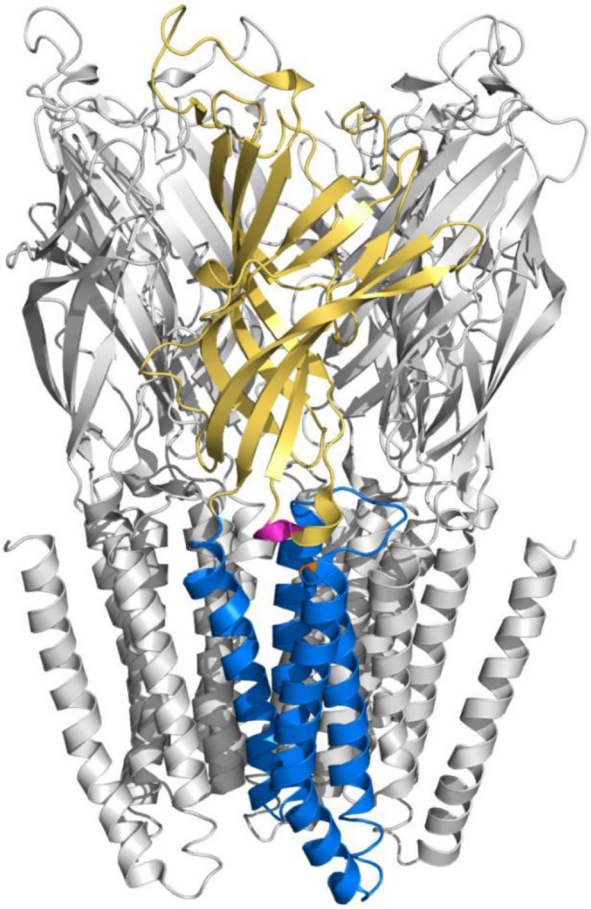

Figure 1. Pentameric Ligand-Gated Ion Channel Chimera

Rendering of the chimera membrane protein structure based on the structure of GLIC (pdb accession code: 3EHZ). The extracellular domain (yellow) is from the prokaryotic proton-gated ion channel GLIC and the transmembrane domain (blue) is from eukaryotic anionic-selective α1 glycine receptor (Duret et al., 2011). Small modifications at the interface of the two domains are colored in magenta and orange. For clarity, the other subunits are colored gray.

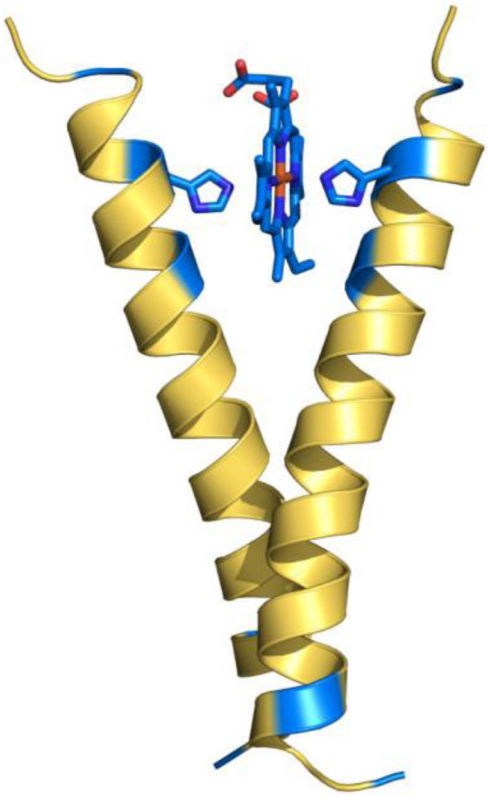

Figure 2. Structure of the Redesigned Glycophorin A in Complex with the Cofactor Protoporphyrin IX

The designed bis-histidine binding site is depicted together with the protoporphyrin IX ligand (Cordova et al., 2007). The modified positions in the structure of glycophorin A are colored in blue.

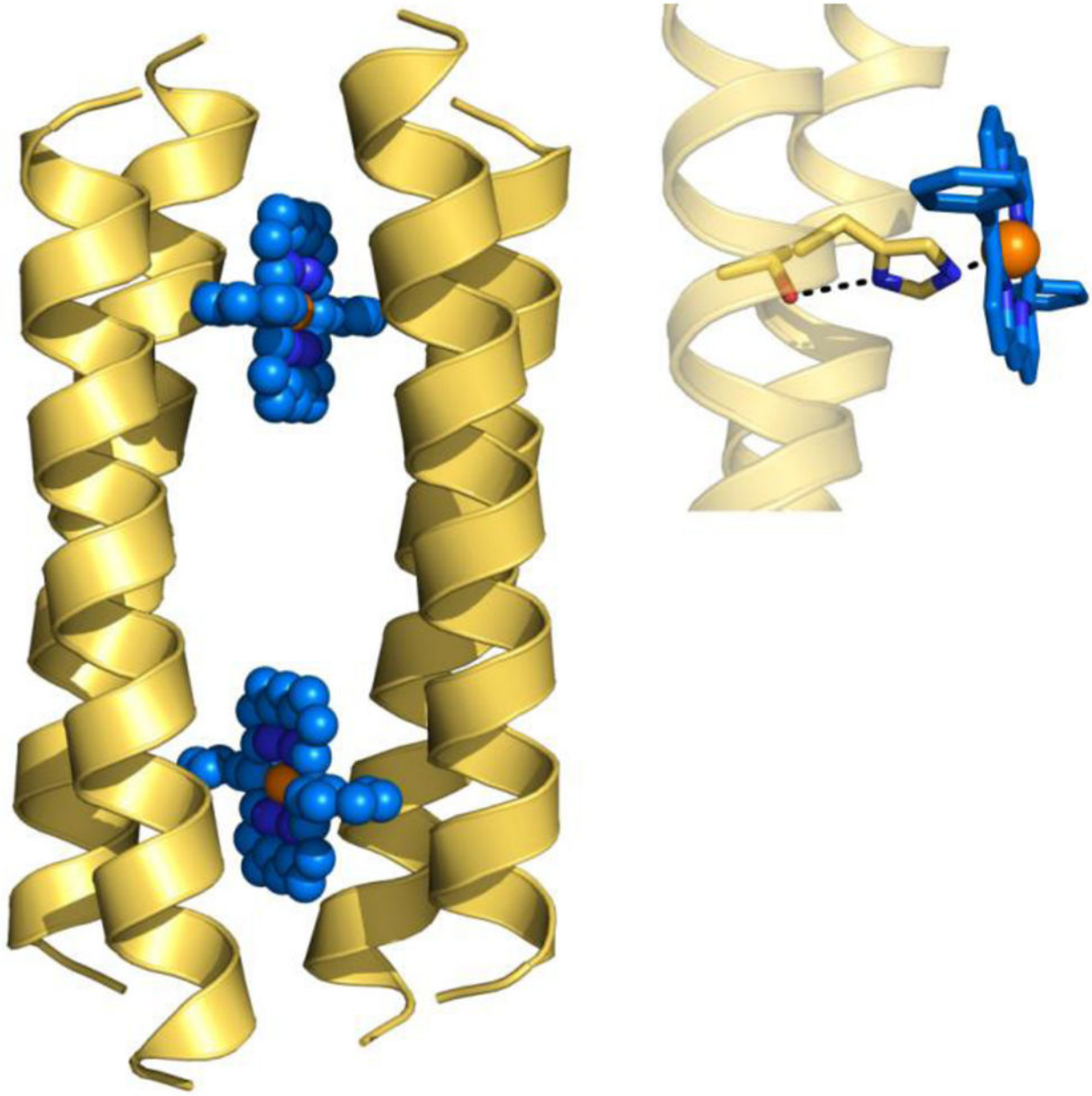

Figure 3. Topology of the De Novo Designed Membrane Protein PRIME

The de novo designed membrane protein PRIME is depicted with two nonbiological iron diphenylporphyrin (Fe^{III}DPP) cofactors (in blue) (Korendovych et al., 2010). The cofactor binding site is displayed in more detail showing the axial interaction of the histidine residue and the iron metal. The second-shell hydrogen bond with threonine residue is also indicated.

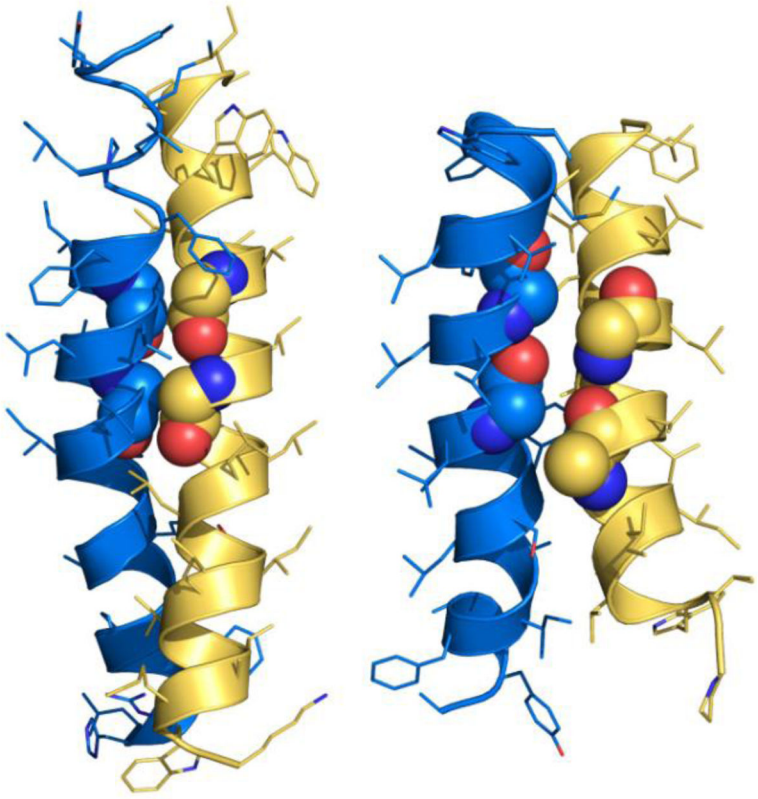

Figure 4. CHAMP Transmembrane Peptides

Structural models of the CHAMP transmembrane peptides (blue) designed to bind (yellow) α_{IIb} (right panel) and (yellow) α_v (left panel) integrins with high specificity–both transmembrane motifs naturally bind integrin β_3 (Yin et al., 2007). The GxxxG motif is highlighted with space-filling representations in both cases.

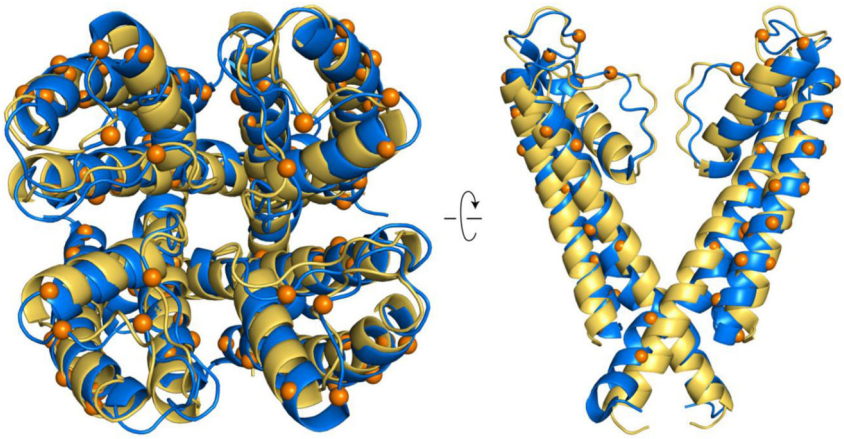

Figure 5. Transmembrane Portion of the Bacterial Potassium Ion Channel KcsA and its Water-soluble Variant

Comparison of the structure of the bacterial potassium ion channel KcsA (yellow) (pdb accession code: 1K4C) (Zhou et al., 2001) and its water-soluble variant (blue) (pdb accession code: 2K1E) (Ma et al., 2008). The water-soluble variant was expressed in *E. Coli* and contains 29 computationally designed exterior mutations in each of the four 104-residue subunits. Depicted as orange spheres in the water-soluble structure (blue), are the Cα atoms for the exterior positions that were computationally designed (Slovic et al., 2004). In the right image all four subunits are depicted while in the left image (side view) only two subunits are rendered.

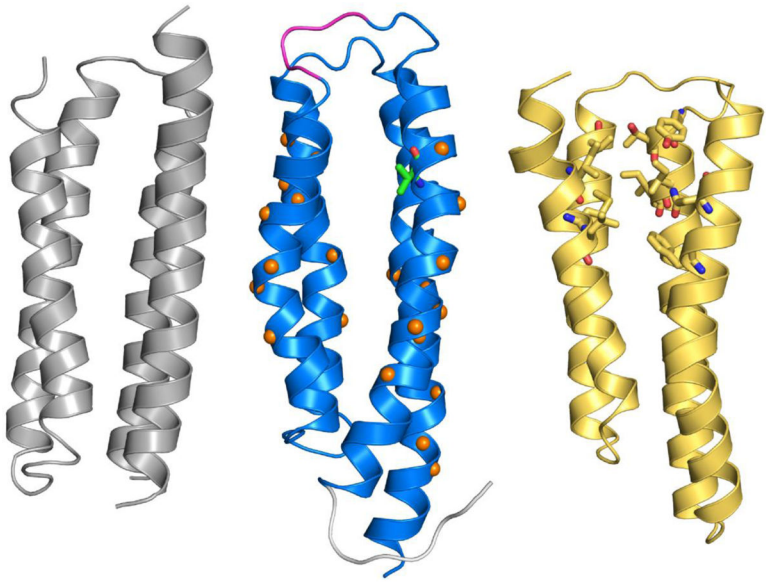

Figure 6. Cryo-EM Structure of a Transmembrane Domain from the Nicotinic Acetylcholine Receptor, NMR Structure of its Water-Soluble Analog (WSA) and X-ray structure of the Prokaryotic Homolog GLIC

Comparison of the 4-Å-resolution cryo-EM structure of the transmembrane domain of the α1 subunit from the nicotinic acetylcholine receptor (gray) (pdb accession code: 1OED) (Miyazawa et al., 2003), the NMR structure of a water-soluble analog from the same segment (blue) (pdb accession code: 2LKG) (Cui et al., 2012), and transmembrane domain of the prokaryotic homolog GLIC (yellow) (pdb accession code: 3EAM) (Bocquet et al., 2009). The water-soluble variant was expressed in *E. Coli* and contains 23 computationally designed exterior mutations (Cα atoms of these positions are depicted as orange spheres). To link the TM4 helix with the rest of the bundle, a polyglycine linker was inserted (magenta). Based on photoaffinity labeling studies, V31 (colored in green) was identified as potential binding site in WSA for general anesthetics (azi-propofol and azi-isoflorane). For comparison, the residues forming the anesthetic binding site in the co-crystal structure of GLIC (Nury et al., 2011) are shown in sticks representations.