Computational design of membrane proteins
Alessandro Senes

This article reviews the recent successes of computational protein design techniques applied to integral membrane proteins. This emerging area is still handicapped by significant difficulties in the experimental characterization of the stability and structure of the designed proteins. Nevertheless, by focusing on oligomeric complexes of single-span transmembrane (TM) peptides with detectable activity, the computational design of membrane proteins has already produced very exciting results. The ‘take-home message’ is that optimization of van der Waals packing and hydrogen bonding (both ‘canonical’ and weak C–H⋯O bonds) can produce functional structures of remarkable stability and specificity in the membrane.

Address
University of Wisconsin-Madison, Department of Biochemistry, 433 Babcock Dr., Madison, WI 53706, United States

Corresponding author: Senes, Alessandro (senes@wisc.edu)

Introduction
Thanks to the development of fast sampling algorithms and the formulation of effective energy functions, computational protein design has made impressive advancements — such as enabling the design of artificial proteins with enzymatic activity [1] — and promises in the long term to create macromolecules with any desired activity and specificity for applications in research, biotechnology, and medicine. Since its early days, however, design has been primarily a tool for investigating the factors that govern the folding, the interactions, and the activity of proteins. In this capacity, design has been an important contributor to our understanding of integral membrane protein folding, a field that has yet to reach the level of maturity of its soluble counterpart. The advanced computational methods that have been developed for soluble proteins have begun to be applied to membrane proteins, but before the same level of success can be achieved, the technology needs to be adjusted to take into account an environment that is very different from bulk aqueous solution and in many ways more complex.

Unlike methods such as molecular dynamics, in which the motions of all atoms — including the solvent — are evolved by simulating the fundamental interatomic forces, the computational methods used in protein design incorporate a number of simplifications and assumptions that are necessary to make the problem tractable. For example, the solvent can only be represented with implicit models, and the mobility of the main chain and the side chains is generally restricted or sampled at discrete intervals. Because of these simplifications, specialized energy functions become necessary [2]. The energy functions used in protein design often contain empirical terms of statistical derivation and physical terms, such as van der Waals and electrostatics whose functional forms may also be specifically formulated for the problem. Moreover, in order to estimate the free energies of folding across any possible sequence, the functions need to incorporate an implicit model of the unfolded state, whose energy cannot be directly calculated. As discussed in the next section, the folding of proteins in the membrane follows dramatically different rules, and thus proper energy functions need to be rederived specifically for this environment. This effort is still in its early stages, slowed primarily by the difficulties in obtaining structural information and stability data for membrane proteins, but the recent past has seen very promising achievements. This brief article reviews these developments, focusing on the technical aspect and the barriers that need to be overcome to advance the field of computational design of integral membrane proteins. For a comprehensive and general review on membrane protein folding and design, we refer to the excellent article by Ghirlanda [3].

Membrane protein folding: insertion
Figures 1 and 2 illustrate the basic concepts of folding in the membrane. The primary driving force for folding in solution — the sequestration of nonpolar surface away from water — is spent to insert the hydrophobic transmembrane (TM) segments into the lipid bilayer (stage 1 in Figure 1). Once inserted in this environment, the TM segments have a strong propensity to assume an α-helical conformation. The helices thus act as stably folded domains that associate to form the tertiary assembly of the protein (stage 2). The schematic division of membrane protein folding in two thermodynamically distinct stages — insertion and helix–helix association [4] — is not completely general, because not all TM helices are required to be independently stable in a protein, as long
Membrane protein folding can be subdivided into two thermodynamically distinct stages: (1) insertion of the hydrophobic transmembrane segments (highlighted in gray) into the membrane, where they assume a stable helical conformation; and (2) association of the helices to form the final folded state of the protein [4]. While the model is not completely general, it is likely to apply when all transmembrane domains of a protein are sufficiently hydrophobic. Because the release of the hydrophobic domains into the aqueous solvent would be highly unfavorable, the unfolded state of a membrane protein can be thought of as a membrane-inserted, helical state in which the domains orient in the bilayer so as to match their hydrophobicity to the environment. This concept is extremely useful for guiding membrane protein design, because it energetically decouples membrane insertion from the final bundle formation.

The stability of the insertion of TM domains in the bilayer is a long studied and well understood phenomenon [5,6], as testified by the fact that one of the earliest successes in membrane protein design was a sequence designed to insert in the membrane, but not necessarily assemble into a folded bundle [7]. The propensity of amino acid sequences to insert in the membrane can be derived from different sources, such as the partitioning of the individual amino acids between water and nonpolar phases [8–10], the recognition of model TM segments sequences in translocon-mediated biological insertion [11], and the statistical distribution of the amino acids across the TM segments of known crystal structures [12,13]. The results of these three very complementary types of analysis are in remarkable agreement, implying that the evolution of membrane proteins has selected TM sequences that are well matched to the hydrophobicity of their environment. These data have been the basis for the creation of empirical potential functions that can be used in protein design to rapidly calculate the depth and orientation preferences of a TM helix in a lipid bilayer [12–14].

### Membrane protein folding: helix–helix association

Figure 2 schematically summarizes the factors that are likely to contribute to the thermodynamic balance of membrane protein folding, after the helices have been inserted in the lipid bilayer. Folding will produce a loss of conformational entropy, although this factor is likely to be reduced with respect to the cost paid by soluble proteins, to the extent that the helices are preformed and preoriented. The energetic balance should include a loss of protein–lipid interactions and a gain of protein–protein interactions, as well as lipid–lipid interactions. The balance may also include a cost of reorienting the helices from their most favored depth and angle of insertion to that assumed in the folded state. Although the precise nature and balance of the forces that contribute to helix–helix association is still poorly understood, experimental evidence suggests that van der Waals packing and hydrogen bonding (including those hydrogen bonds formed by weaker C–H donors) are important for the stability of tertiary and quaternary structure in the membrane.
stabilization effect of hydrogen bonding derives from the notion that the hydrogen-bonding potential of the polar side chains embedded in the center of a bilayer cannot be satisfied by lipids once the protein unfolds. An elegant double mutant cycle study of bacteriorhodopsin suggests that this contribution is more modest than theoretically expected, but it is still significant [22]. A similar argument can be made for backbone-to-backbone $\text{Co} - \text{H} \cdots \text{O} = \text{C}$ hydrogen bonds, which are individually weaker but appear in extensive networks at helix–helix interfaces [23]. The formation of these networks is enabled by $\text{G} \cdots \text{G}$ and other motifs of small residues clustered on one face of the helix that permit the backbone to come into extensive contact [26–28]. For a comprehensive discussion on the role of hydrogen bonds in membrane proteins see the recent review by Bowie [29].

The role of van der Waals packing as a primary driving force for folding in the membrane is suggested by mutational analysis [16,17] and structure-based arguments [15]. Most recently, an experimental and computational analysis of MS1 variants – a membrane-soluble redesign [30] of the GCN4 coiled-coil — brought further support to the theory that van der Waals interactions can promote folding [18]. Zhang et al. analyzed a series of MS1 variants containing Gly, Ala, Val, or Ile at interfacial positions of MS1, and demonstrated that in this system stability ranks inversely with the size of the amino acid side chains (Gly > Ala > Val > Ile). Computational analysis suggested that the closer interhelical distances enabled by the smaller side chains promote more extensive packing. Because van der Waals interactions also occur in the unfolded state between the helices and the lipid molecules that solvate them, the logical implication is that protein–protein interactions tend to produce more effective packing than protein–lipid interactions. Although there is currently no direct proof for this theory, the data provide, at the very least, a strong indication that good packing is a necessity. An argument in its favor is the absence of a known strong driving force, such as the hydrophobic effect for soluble proteins, which would leave membrane proteins with little ‘spendable energy’ to compensate for imperfections and voids in their packing [16].

While the precise contribution of hydrogen bonding, van der Waals packing, and other factors is still under investigation, it is clear that they play an important role in the folding of membrane proteins. Further confirmation is provided by the success of recent computational design studies that have been based around these features, which is the topic of the following sections.

**Co$\alpha$–H$\cdots$O=C based design of anti-transmembrane peptides**

A successful example of design performed around a stabilizing feature is of the CHAMP peptides (Computed Helical Anti-Membrane Protein), developed by DeGrado and colleagues as dominant-negative inhibitors of TM association against two closely related human integrins (α$\text{IIb}$$\beta$3 and α$\text{v}$$\beta$3) [31]. The TM domains of the integrins are characterized by GxxxG (or GxxG-like) motifs, and are thus ideal targets for designs based on networks of $\text{Co} - \text{H} \cdots \text{O} = \text{C}$ hydrogen bonds. As stated, the authors ‘circumvented the need for accuracy [of the energy function] by using a library of structurally defined helix pairs that are already in local minima with respect to interhelical backbone–backbone interactions’ [31]. This was accomplished by selecting from the structural database TM helical pairs with extensive networks of $\text{Co} - \text{H} \cdots \text{O} = \text{C}$ hydrogen bonds, such as the one illustrated in Figure 3. One of the helices in the pair was threaded with the target’s sequence, while the opposing helix was the template for the design of the CHAMP. The side chains were repacked to maximize the van der Waals interactions, using a simple potential composed by a softened Lennard–Jones function and a statistical potential for TM orientation (EZ potential [12]), while restricting the composition of the CHAMPs in the inner core of the TM region to the most frequent amino acid types found in TM helices (G, A, V, I, L, S, T, and F, which account for about 80% of TM composition [26]). The resulting anti-α$\text{IIb}$ and anti-α$\text{v}$ CHAMPs had good affinity to their respective targets and were biologically active. They were also specific and not cross-reacting, despite the fact that the sequences of α$\text{IIb}$ and α$\text{v}$ are highly homologous and that both designs were based on backbones belonging to the same structural family of GlycophorinA-like motifs (sub 7 Å interhelical distance and a right-handed crossing angle near $−35°$). This demonstrates that Co$\alpha$–H$\cdots$O=C networks not only can provide stability but also support a high degree of specificity, because their formation depends on intimate backbone-to-backbone contacts which can be easily disallowed by incompatible packing [23].

**Design of an electron-transfer membrane protein based on polar interactions**

Very recently, we reported a functional membrane protein designed de novo with the objective of facilitating electron transfer across a lipid bilayer (called PRIME) [32]. The design is a 24 amino acid long $D_2$-symmetrical antiparallel homo-tetramer that sandwiches two nonnatural iron diphenylporphyrins (Fe$^\text{III}$DPP) (Figure 4a). The backbone was based on a section of a longer water-soluble design that contained four porphyrin moieties [33]. The central part of this precursor contained two porphyrin molecules in close proximity and had a topology that was well suited for an electron-transfer membrane protein, but the sequence had to be optimized for stability in a hydrophobic environment. We based our design on the hypothesis that the assembly could be supported by three main interactions: first, the coordination bond between the His and the iron atoms; second,
the interhelical hydrogen bonds between the subunits (His 8 to Thr 18); and third, van der Waals forces from complementary packing. The same three elements were also deemed critical for determining the specificity of the design. The design was performed with a two-step procedure. In the first stage, we optimized the geometry of the three critical elements (His 8 to Thr 18 and the porphyrin, Figure 4b) in the absence of all the other side chains. We sampled the conformation of His 8 and Thr 18 within one rotameric region (trans/g+ and g+ respectively) to find the ideal compromise between the geometry of coordination of the His residues (distance and angle with the porphyrin plane), the distance between the two porphyrin rings, and the second shell hydrogen bond between His 8 and Thr 18, which is important for locking the His ring in the desired position. During this phase the porphyrins were shifted along the central axis of the helical bundle to keep the iron midway between the Nε atoms of the coordinating His residue (the porphyrins were also rotated according to the superhelical screw to maintain D2 symmetry). The final step was the identification of a sequence that could stabilize the assembly. To optimize packing and maximize favorable van der Waals interactions we explored the conformational space of the side chains with a very large conformer library optimized for protein design, using an ‘unsoftened’ van der Waals potential and full atomic radii. The solutions were ranked by binding energy, calculated by subtracting the energy of the sequence in a monomeric helical state from energy of the tetramer. The energies were scored using the CHARMM 22 [34] force field with Lazaridis implicit membrane solvation model [35]. The favorable TM orientation of the resulting sequences was checked using the Ez potential [12]. The experimental characterization of PRIME determined that the above procedure produced a stable design with remarkable specificity,
characterized by a tight-binding isotherm and a geometry of metal coordination consistent with the design, according to EPR data. PRIME has a significantly reduced affinity for a closely related heme. Mutation of the critical Thr 18 to Ala substantially reduced binding affinity, demonstrating that the hydrogen-bonding network (Figure 4b) is critical for the assembly of the complex. Similarly, disruption of the favorable packing severely affects the assembly. Preliminary data show that PRIME can perform electron transfer in a phospholipid bilayer using a chemical assay (manuscript in preparation).

Structural prediction of membrane proteins
Protein structural prediction and protein design have different objectives but they share much of the underlying theoretical framework. Prediction and design have in common the ultimate aim of identifying the lowest energy state within their respective search spaces. Because of this commonality, similar methods and energy functions are applied to both problems, and it is therefore relevant here to discuss briefly the methodology introduced by a successful membrane structural prediction program, Rosetta-Membrane [36*]. RosettaMembrane represents an adaptation of the classical Rosetta potential, a weighted sum of terms of empirical and physical derivation, that was recalibrated specifically to recover the amino acid identities in a set of 18 high-resolution structures of membrane protein. The energy functions were altered for different environment. Solvation was represented using a depth-dependent membrane model [35]. The hydrogen bond potential was modified to have variable strength depending on the depth in the membrane and to support bidentate bonds. A Ca–H hydrogen bond was also introduced, calibrated to be roughly half the strength of a ‘canonical’ hydrogen bond. The results obtained with this procedure were remarkable, with an accuracy of prediction <2.5 Å for the smaller proteins, and the method can be extended to larger protein if experimental constraints are introduced in the calculation [37]. It is worth mentioning that the authors noted that ‘the success of [their] model in the prediction and design tests suggests that short-range VDW and hydrogen bond interactions are essential for the stability and structural specificity of TMH bundles.’ [36*]

Conclusion
So far the membrane protein design field has demonstrated its enormous potential by focusing on the areas in which the chances of success are most promising — the design of oligomeric complexes of single-span TM peptides with a detectable activity. The single-span TM helices — both natural and designed — have been a fertile ground for studying membrane helix association...
because a variety of methods exist to determine their interactions in detergents and — with more difficulties — also in lipids [38]. In this respect, a ‘steric trap’ method recently proposed by Bowie and colleagues represents a step forward for measuring single-TM interactions in bilayers [39]. The most pressing need, however, is the development of methods to readily and reliably measure the thermodynamic stability of multi-span proteins. This is something that to date is only achievable with a few systems [17,40–42], although a recently published method, based on pulse-proteolysis, is potentially, generally applicable as long as reversible unfolding conditions are identified for a membrane protein [43]. This progress is important because it is clear that the main barrier to the development of computational membrane protein design lays, paradoxically, in the limitations of the experimental methods. The relatively small number of high-resolution structures of membrane proteins available, the still developing understanding of the principles that govern their folding, and the great difficulties that are faced in determining experimentally the stability and the structure of a design are problems that will likely continue to handicap the development of this field in the near future. However, the success obtained by the rational application of basic principles is extremely encouraging, demonstrating that protein design represents a fundamental tool for understanding membrane protein folding and interaction, and for the creation of new functional systems.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

19. The authors analyzed a series of MS1 variants containing Gly, Ala, Val, or Ile at the putative ‘a’ position of this membrane-soluble GCN4 derivative. The experimental characterization demonstrated an order of stability (G > A > V > I) that is the inverse of the one observed for the same substitutions on a soluble coiled-coil. Computational analysis suggested that van der Waals packing and backbone electrostatics are the primary forces for determining the association and orientation preferences of this system.


Slusky and colleagues designed TM peptides targeted to bind the TM domains of two human integrins. The guiding principle for their design was the selection of naturally optimized backbones characterized by networks of interhelical Cα-H—O=C, followed by optimization of van der Waals packing. The method created so-called 'CHAMP' anti-TM peptides that displayed excellent affinity and specificity for their respective targets.


Design of an electron-transfer protein. PRIME is a four-helix bundle sandwiching two nonnatural porphyrin moieties. The design was based on the optimization of a number of polar interactions (iron coordination and second-shell hydrogen bonding) followed by side chain repacking to maximize the van der Waals interactions. Experimental characterization demonstrated that the tetramer assembles with high affinity and specificity.


Extension of the Rosetta method to the prediction of the structure of integral membrane proteins. Of interest here is the modification of the energy functions to account for folding in the membrane. In particular, the authors introduced a depth-dependent solvation potential and significantly altered the hydrogen-bonding function, extending it to also include weak Cα hydrogen bonds.


The authors present a new method for measuring thermodynamics of association of single-span TM proteins. The method is based on the dissociation of biotinylated TM peptides upon addition of a streptavidin variant due to a steric effect. A major advantage of this method is that association is monitored by varying the amount of streptavidin rather than by dissociation of the peptides via dilution, which is notoriously problematic to achieve for samples in lipid bilayers.


42. Barrera FN, Renart ML, Poveda JA, de Kruijff B, Killian JA, González-Ros JM: Protein self-assembly and lipid binding in the folding of the potassium channel KcsA. Biochemistry 2008, 47:2123-2133.


Park and colleague extend to membrane proteins a method previously developed for use in solution. The method allows to measure the fraction of unfolded protein in denaturing conditions by determining the amount of protein digested after a short pulse of proteolysis. The method is particularly useful for membrane proteins because their unfolding does not typically result in significant changes in spectroscopic quantities. The method was tested on the model system bacteriorhodopsin but could be generally applicable to any other membrane protein as long as reversible folding conditions are identified.