



Membrane proteins enter the fold

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Abstract

Membrane proteins have historically been recalcitrant to biophysical folding studies. However, recent adaptations of methods from the soluble protein folding field have found success in their applications to transmembrane proteins composed of both α -helical and β -barrel conformations. Avoiding aggregation is critical for the success of these experiments. Altogether these studies are leading to discoveries of folding trajectories, foundational stabilizing forces and better-defined endpoints that enable more accurate interpretation of thermodynamic data. Increased information on membrane protein folding in the cell shows that the emerging biophysical principles are largely recapitulated even in the complex biological environment.

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Introduction

Included among the National Academy of Engineering grand challenges for the 21st century are goals to advance health informatics, to engineer better medicines, to reverse engineer the brain, and to engineer the tools of scientific discovery [1]. Achieving these goals will rely on overcoming the contemporary biophysical problem of describing how a polypeptide sequence encodes the structure and function of a protein. Because membrane proteins play key roles in human health, cognitive functions [2], and are thought to bind over half of the therapeutics on the market today, advancing an understanding of how and why membrane proteins attain their native folds will be key to meeting the grand challenges. There are two important perspectives to be addressed: (1) a biophysical description of driving forces underlying how a sequence encodes a structure and (2)

a biological description of folding within a complex cellular environment. Here, we review the major advances from the biophysical vantage and comment on how these may be manifested in the cell (see [Figure 1](#)).

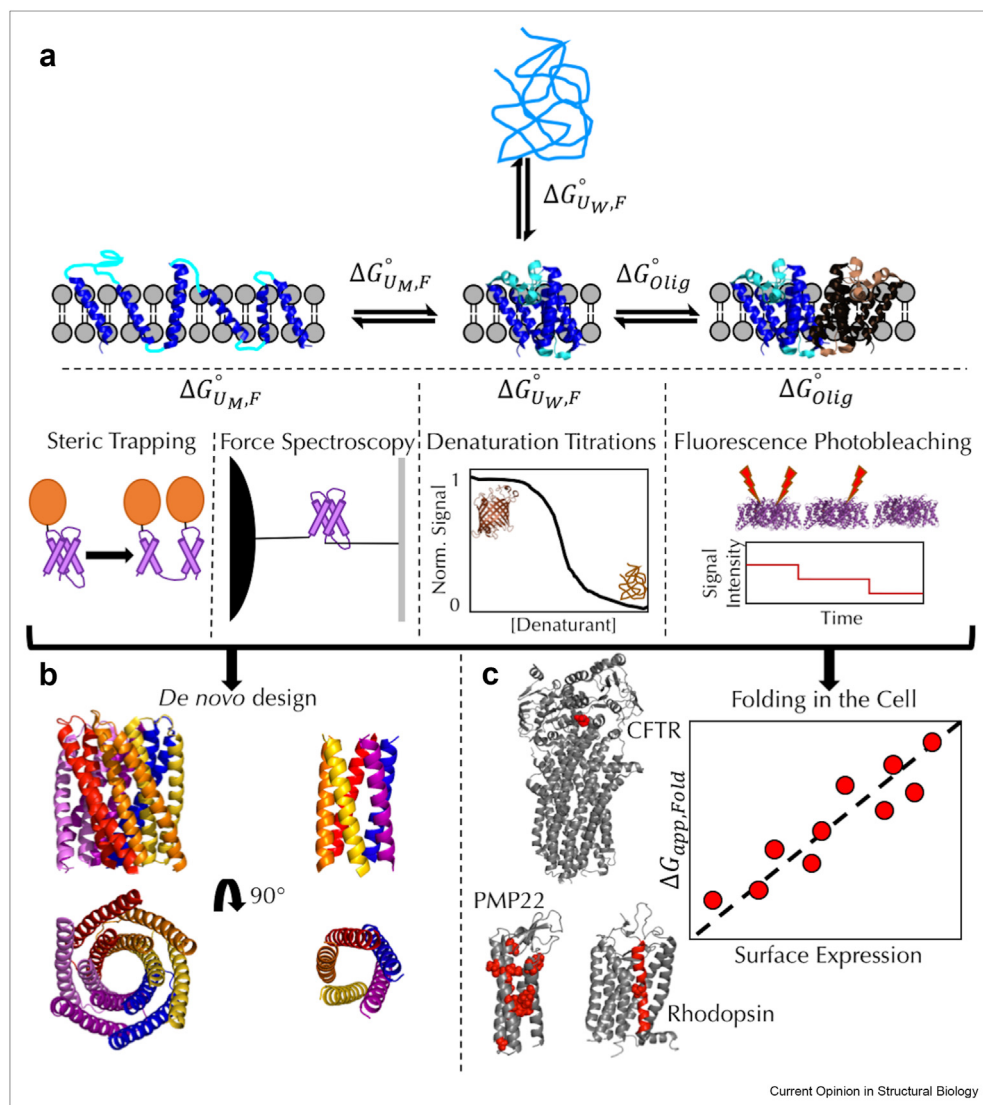
The value of water-to-bilayer end points

Water-solvated unfolded, U_W , and bilayer-embedded folded states, F , represent the two most extreme end-points of biophysical interest for membrane-protein folding reactions. A deceptively simple parameter — the free energy of folding ($\Delta G_{U_W, F}^0$) — captures the population bias at equilibrium, and the free energy change between these end states reveals the maximum energetic contributions of the various atomic interactions responsible for stabilizing a particular folded state over its aqueous-unfolded conformational ensemble. Although the water-soluble unfolded state is not typically observed in a cellular context, these end-points are nevertheless useful in theoretical considerations that seek to describe the underlying chemical reactions. Taking cues from the water-soluble protein-folding field, a number of groups used chemical denaturation titrations and extensive condition tweaking to measure path-independent equilibrium values for several transmembrane β -barrels [3–6]. These experiments reveal an extremely favorable folding stability for β -barrels, ranging from -18 to -32 kcal mol $^{-1}$, and the systems have proved useful in addressing the energetic contributions of side-chain partitioning and backbone hydrogen bond formation [4,7–10].

Hydrophobicity energies from water-to-bilayer folding

Statistically, membrane-embedded segments are highly enriched in apolar side chains that favorably interact with the nonpolar core of the bilayer [11]. One key question concerns how much energy is gained by the removal of a nonpolar moiety from water and its placement within the bilayer. The answer is captured by the driving force known as the hydrophobic effect. Historically, water-to-octanol partitioning of peptide segments has been employed to mimic this energetic contribution as manifested through the construct of a hydrophobicity scale [12]. More recently, folding studies using two different transmembrane β -barrels employing a host-guest system strategy and a phospholipid bilayer instead of octanol have enabled a novel hydrophobicity scale [4,5]. These new measurements demonstrate that the magnitudes of the membrane partitioning energies

Figure 1



Membrane protein folding and stability flow chart. (a) The relevant thermodynamic equilibria describing membrane protein stability and the experimental approaches used to measure each free energy are shown. $\Delta G_{U_{W,F}}^\circ$ describes the coupled folding and insertion of an unfolded, water-soluble membrane protein into the bilayer and is calculated from chemical denaturation titrations of β -barrels (PDB: 1QD5). This approach has been used to investigate side-chain transfer free energies [3–5,8,13,53] and folding transition states [3]. $\Delta G_{U_{M,F}}^\circ$ describes the association/folding of helices in a membrane unfolded state and has been measured using both steric trapping [20,23] and single-molecule force spectroscopy [29,30]. ΔG_{Olig}° describes the oligomerization of membrane proteins and is currently measured using single-molecule fluorescence photobleaching (PDB: 3Q17) [25,26]. (b) The growing knowledge of the thermodynamic parameters that define membrane protein folding and structure have led to the successful design of functional membrane proteins (PDBs: 6TMS (left) and 6MCT (right)) [27,35]. (c) *In vitro*-derived parameters of membrane protein stability (Panel A) have also been applied to membrane protein folding. Model systems for investigating folding *in vivo* include CFTR (PDB: 5UAK), PMP22 [54], and rhodopsin (PDB: 1L9H). The residues for each protein that have been discussed here are shown with a space-filling representation and are colored red. For rhodopsin, the entire TM7 helix has been investigated using deep mutational scanning [43]. For each system, the general trend is that stability is correlated with the surface expression of each membrane protein.

are nearly twice as high as previously concluded from the octanol scale.

Moving the membrane mimic from an organic solvent to an actual bilayer brought an understanding of hydrophobicity closer to the cellular condition; however, the

membrane itself is still not a uniform solvent. Rather, the bilayer interface is a chemically complex environment with a steeply changing water concentration. How does this aqueous gradient change the driving force energy of the hydrophobic effect along the bilayer normal? The favorable stability of the transmembrane

β -barrel scaffold enabled nonpolar partitioning energies to be assessed at different locations in the bilayer and thus, under widely varying water concentrations [13]. This work reveals a continuously changing nonpolar solvation parameter function that connects the value for the energy of insertion of nonpolar moieties at the interface to that at the center of the bilayer. By relating the energy of this important driving force to chemical parameters of the membrane, and not, for example, the position along a transmembrane α -helix, these results have the potential to be adopted for proteins in any bilayer.

Energetic features of native folds

Since the availability of the earliest membrane-protein structures, it has been observed that most membrane proteins are enriched in either the transmembrane α -helical or the β -sheet (barrel) secondary structure that is formed by regular patterns of backbone hydrogen bonds. Backbone hydrogen bond (bbHB) formation is favored in membrane-embedded regions because there is a larger energetic penalty for the water-to-bilayer partitioning of the nonhydrogen bonded backbone. Recent advances in NMR experimental methodologies have allowed for bbHB strengths to be measured both in α -helical and β -barrel transmembrane proteins using a hydrogen—deuterium exchange [14]. Cao et al. reported that bbHB strengths for the transmembrane α -helical amyloid precursor protein reach -6 kcal mol^{-1} , a value much more favorable than previous estimates using organic solvents and small peptides, or even soluble proteins [10,14]. Lessen et al. performed similar experiments using the transmembrane β -barrel OmpW and found strengths ranging from -3 to -4 kcal mol^{-1} on average [9]. In contrast to the partitioning free energy changes of nonpolar side chains discussed above, both NMR investigations found bbHB strengths to be relatively insensitive to the position of the membrane. Together, these studies indicate that bbHB energies appear to be affected by neither sequence nor secondary structure. In sum, the unchanging bbHB energy in membrane proteins across the bilayer implicates side-chain partitioning interactions as the main driving force for transmembrane protein insertion into the bilayer.

Side-chain entropy can be another energy source in protein folding. Compared to U_w , in which the polypeptide chain can assume a large and heterogeneous conformational ensemble, the folding of a transmembrane α -helix upon insertion limits the conformational space and perhaps the motions of side chains [15]. In contrast to this assumption, solution NMR relaxation studies suggest that membrane proteins are extraordinarily dynamic with fast internal motions on methyl-bearing side chains [16]. This finding was equally true for the α -helical sensory rhodopsin II as well as the

OmpW β -barrel and was independent of the hydrophobic, membrane-mimicking cosolvent. The energetic contribution of side-chain motion to folding will depend on the extent to which it is preferentially enhanced in F as compared to U_w . Crucially, this remains to be tested [16].

Membrane-embedded unfolded-to-folded endpoints dominate α -helical membrane protein measurements

To date, there are no water-to-bilayer stabilities measured for α -helical transmembrane proteins. This is presumably due to the enhanced aggregation propensities of transmembrane α -helical regions that are composed of continuous stretches of nonpolar amino acids. Stability measurements of α -helical membrane proteins have accordingly been tractable only in experimental setups in which unfolded states remain embedded in a membrane or in a membrane mimic, which we term U_M , regardless of its secondary structure. In these reactions, the energy derived from the hydrophobic effect is attenuated because the water concentration is not bulk, and a smaller energy difference between U_M and F is expected. If the α -helical secondary structure is stable in isolated segments in the unfolded ensemble, for example $U_{M, H}$, these experiments should report on transmembrane helix–helix interactions, for example $U_{M, H} \leftrightarrow F$.

The classic example of this reaction includes the dimerization of the single-transmembrane domain of glycophorin A, GpATM [17–19]. However, new methods that interrogate helix–helix interactions in more complex multispan proteins show that the lateral interactions are not going to be simple to understand. Local interactions show varied stabilities in the intramembrane rhomboid protease GlpG as assessed using a ‘steric trapping’ strategy [20–23]. In contrast, the CLC-ec1 Cl^-/H^+ antiporter has a high affinity in bilayers using a promising new single-molecule microscopy technique [24–26]. The method is model-independent and can be carried out in any bilayer of choice using single-molecule fluorescence bleaching steps to quantify the membrane protein oligomer size following equilibration in what is essentially an “infinite” bilayer. In 2:1 POPE:POPG, the authors found that CLC-ec1 forms a high-affinity dimer with a mole fraction equilibrium dissociation constant equal to 4.7×10^{-8} subunits lipid $^{-1}$. For context, this is only $\sim 1.3 \text{ kcal mol}^{-1}$ less favorable than the GpATM dimer in POPC [19], which was a surprising outcome because the CLC-ec1 dimerization interface is much larger by comparison. Because the CLC-ec1 lacks a so-called GxxxG dimerization motif, future mutational analysis on this protein will be needed to rationalize the distinct physical mechanisms these two proteins employ in subunit recognition. The distinction between these two structural modes for

dimerization also begs the question of whether the packing of nonpolar side chains is sufficient to drive protein–protein interactions in lipids, which is an area of high interest in the membrane protein design field [27].

Aspects of the folding trajectory as assessed by force spectroscopy

The folding reaction of ClC-ec1 has also been measured using single-molecule force spectroscopy, a second single-molecule technique that is gaining popularity in its ability to probe folding at infinite dilution [28,29]. In the ClC-ec1 experiments, a force ramp strategy interrogated the unfolding of the monomeric ClC-ec1 protein in a DMPC bilayer wrapped in CHAPSO [29]. This protomer possesses an inverted topological arrangement of structurally similar N- and C-domains connected by a linker. The authors found that the ClC-ec1 N- and C-domains unfolded in separate events suggesting the idea that the protein evolved from gene duplication of subunits that fused together. The work further revealed that aggregation is not the only factor subverting folding; even under these single-molecule conditions, misfolded states of the two ClC-ec1 domains refold slowly and inefficiently and are prone to forming a non-native structure.

Showing its versatility to a wide variety of proteins [28], single-molecule force spectroscopy was recently used to elucidate intrinsic folding pathways for GlpG and the human β -adrenergic receptor β 2AR [30]. Of significance is the observation that the β 2AR folding occurred N- to C-terminal, which is intriguing because it implies that transmembrane α -helices may have evolved to laterally interact as they are inserted into the bilayer using the translocon.

Designer membrane proteins

Design efforts challenge our current understanding of how a sequence encodes a structure. The driving questions in this area may be summarized by two pithy phrases, *What I cannot create, I do not understand* [31] and *Do I understand what I can create?* [32] These two are at odds because design efforts take advantage not only of advances in fundamental thermodynamic principles but also of the ever-increasing structural knowledge base to create novel proteins. Despite the balance of input arguments, engineering efforts have led to some exciting successes that foretell the power of this approach (Figure 1b). The landmark achievement of the Rocker coiled-coil $\text{Zn}^{2+}/\text{H}^{+}$ antiporter [33] was followed by Rosetta-driven design of α -helical transmembrane bundles of varying stoichiometries [34], a dodecameric-helix pore that conducts ions with a selectivity of K^{+} over Na^{+} [35], and the *de novo* design of β -barrel transmembrane proteins [36]. Promise has also been demonstrated for the rational control of cellular signaling by the design of single-pass transmembrane

domains that may alter receptor signaling through competition for helix–helix interactions in integrins [37]. Complementing these structural achievements is the continued development of energy functions that seek to more explicitly model interactions between the surfaces of transmembrane proteins and the lipidic membrane environment with additions that include differentiable models of multiple membrane compositions, nonpolar energy functions that increase the variety of side chains in design so that they more accurately reflect the biological diversity, and a lipophilicity-based force field for scoring [38,39].

Membrane protein folding in the cell

Recognizing that this entire literature cannot be summarized in a short review, we conclude with some comments on how the biophysical measurements discussed above impact our understanding of folding in the cell. The biophysical experiments are carried out under controlled conditions with purified components and carefully assessed endpoints. In contrast, it is widely appreciated that there is additional complexity within the living biological system. Foremost is the concept that evolution selects for fitness over stability, and it does so within the context of the cellular machinery. For example, there can be coupling between the biological processes of insertion and helix–helix association that can be difficult to disentangle [40]; putative transmembrane α -helices may be sorted by the translocon while simultaneously exploring conformational space in an unanticipated manner [41]; cotranslational forces are increasingly recognized in their ability to influence folding [42]; and the biogenesis process itself may place limitations on allowed mutations [43]. Thus, it is expected that mechanistic adaptations from the biophysically derived principles may arise because of constraints or benefits imparted by the proteostasis networks or cellular trafficking. Even in face of the complex cellular environment, works on the cystic fibrosis transmembrane conductance regulator (CFTR) and peripheral myelin protein 22 (PMP22) proteins involved in cystic fibrosis and Charcot-Marie-Tooth diseases, respectively, demonstrate the protein folding rules gleaned in the test tube are guiding principles largely applicable to the cellular context (Figure 1c).

There is a large body of literature supporting the conclusion that the most commonly occurring mutation in cystic fibrosis, ΔF508 , is at its heart a protein folding defect [44]. The mutant protein has a propensity to sample misfolded conformations and is degraded before reaching the plasma membrane. Early in these studies, it was appreciated that ΔF508 is temperature sensitive and could undergo conditional rescue at the permissive temperature [45]. Consistent with this observation, the severity of the disease correlates with the fraction of

folded CFTR protein that is trafficked to the plasma membrane [46]. This led to the discovery of folding correctors, including an FDA-approved drug (VX-809 [47,48]), and more recently to the demonstration that the peripheral quality control system can rescue the fold by suppression of the CFTR $\Delta F508$ mutant instability in cells [49].

Charcot-Marie-Tooth disease is a second example in which pathogenic severity is related to protein folding. In this case, the connection was directly established by showing that conformational stability and cellular trafficking of 12 variants of the PMP22 protein are linearly correlated [50]. Importantly, the work discovered that motor nerve conduction velocities in affected patients *in vivo* also tracked with thermodynamic stability of PMP22 assessed by classical protein-folding experiments *in vitro* [50]. The recent finding that overexpression of PMP22 leads to mistrafficking implies that overwhelming the proteostasis network is deleterious in the cell and is consistent with the very slow folding kinetics observed for PMP22 *in vitro* [51,52].

Conclusions and future directions

The work reviewed here highlights the creative applications and concomitant expansion of technical approaches that can be used to elucidate fundamental principles governing membrane protein folding. Continued increases in computational power and the advent of more widespread cryoEM structural solutions of recalcitrant membrane protein complexes will significantly add to the knowledge database from which design efforts can be drawn. Library expression of variants coupled with functional assays *in vivo* and deep mutational scanning methods are already showing promise in shaping the biologically allowed sequence space [43]. As the distinct steps of membrane protein folding are interrogated in the cellular context [41], scientists will gain greater insight into how the biophysical rules are played out within the living cell.

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Conflict of interest statement

Nothing declared.

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