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Folding of helical membrane proteins: the role of polar, GxxxG-like and proline motifs

Alessandro Senes[1], Donald E Engel[1,2] and William F DeGrado[1,3]*

Helical integral membrane proteins share several structural determinants that are widely conserved across their universe. The discovery of common motifs has furthered our understanding of the features that are important to stability in the membrane environment, while simultaneously providing clues about proteins that lack high-resolution structures. Motif analysis also helps to target mutagenesis studies, and other experimental and computational work. Three types of transmembrane motifs have recently seen interesting developments: the GxxxG motif and its like; polar and hydrogen bonding motifs; and proline motifs.

Addresses
[1] Department of Biochemistry & Biophysics, [2] Department of Physics and Astronomy, [3] Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059, USA
*e-mail: wdegrado@mail.med.upenn.edu

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Abbreviations

bR	bacteriorhodopsin
CFTR	cystic fibrosis conductance regulator
DGK	diacylglycerolkinase
EM	electron microscopy
GpA	glycophorin A
MCP	major coat protein
TCR	T-cell receptor
TM	transmembrane

Introduction

Membrane proteins are of critical importance to nearly every aspect of cell physiology, comprising one-quarter to one-third of all proteins [1]. To date, two distinct classes of membrane proteins have been structurally characterized. The all-β class of protein tends to form large transmembrane (TM) pores; many are toxins, whereas others facilitate the diffusion of small to large molecules across membranes [2]. The larger, α-helical class includes cell-surface receptors, ion channels, transporters and redox proteins of widely different structure and function. Many have a single TM helix that homo-oligomerizes or associates with other TM helices to form helical bundles. These TM assemblies are of critical importance in a variety of biological situations and also have particular advantages for the study of protein folding in membranes. They will be the primary focus of this review.

Popot and Engelman [3,4] have proposed a simple two-stage model for the folding of α-helical membrane proteins. In the first step, helices are formed and inserted into the membrane. In the second key step (or series of steps [5]), the helices come together and associate into the final, folded structure. The process of inserting TM helices is becoming increasingly better understood [6,7], but our comprehension of the subsequent folding step is just developing. Here, we review recent findings concerning the recognition of motifs involved in helix–helix association within membranes, the contributions of these motifs to the thermodynamics of membrane folding, and motif involvement in membrane protein assembly and function. These studies have led to a deeper understanding of a variety of biological processes, while also providing a toolkit for probing them further.

What are the features that stabilize the folded conformations of membrane proteins? An emerging answer to this question is that the primary determinants are relatively weak van der Waals packing and occasional hydrogen bonding or electrostatic interaction. An early study by Rees and Eisenberg [8] showed the interiors of membrane-soluble proteins to be similar in packing and polarity to those of water-soluble proteins [8]. More recent surveys of the growing database of high-resolution structures of membrane proteins have provided additional detail [9–15]. The solvent-inaccessible cores of both types of proteins predominantly consist of well-packed apolar residues. Although buried polar sidechains are hardly rare in the cores of either membrane proteins or water-soluble proteins, they do occur there less frequently than apolar residues. Typically, membrane proteins have at least one interhelical hydrogen bond per helix [10]. Membrane proteins show a slightly greater number of right-handed crossing angles compared to water-soluble proteins and membrane proteins have a greater tendency to bury small sidechains (alanine, glycine and serine) at helix–helix interfaces [9,16]. Of particular importance in membrane proteins are Cα to amide carbonyl hydrogen bonds [17,18], which will be discussed in the section on glycine patches.

Although these database studies are not controversial, their interpretation is less than straightforward. At one end of the spectrum would be the view that the folding of

helical membrane proteins can be driven by van der Waals interactions alone [19]. The other view is that, because hydrogen bonding is so much stronger in the membrane, such bonds could be the primary factor in TM helix association, with the packing interfaces playing primarily a permissive role [17,20]. Of course, these two views represent limiting cases, which may, in fact, be found in some proteins. The issue is fundamentally a quantitative one and can be addressed through detailed thermodynamic studies of systematically mutated membrane proteins, as will be discussed in the final section.

GxxxG and GxxxG-like motifs in helix–helix association

GxxxG in glycophorin A

The GxxxG motif (two glycine residues separated by any three residues) and 'GxxxG-like' motifs (in which one or both glycine residues are substituted by other small residues, such as alanine or serine) are often found to be important for mediating the interaction of TM helices. The discovery and characterization of the GxxxG motif were, in a large part, a result of the work of the Engelman laboratory at Yale. Early studies on the glycophorin A (GpA) TM dimer, including mutagenesis [21], computational modeling [22–24] and thermodynamic characterization [25–29], showed the central role of GxxxG in the L^{75}IxxGVxxGVxxT^{87} interfacial motif (Figure 1a). In the NMR structure of GpA [30], the groove of the GxxxG motif and the ridge of the neighboring valine residues form a large and almost flat central contact surface of the dimer. The right-handed crossing angle about this pivot point appears to be constrained by lateral contact of the terminal Leu75, Ile76 and Thr87 residues.

GxxxG as a framework for transmembrane helix association

The GxxxG motif has a strong propensity for TM helix interaction [31]. Sequence analysis shows that GxxxG-like motifs occur more frequently in TM helices than their random expectation [32,33] and are conserved among families [1]. Glycine and, to a lesser extent, alanine and serine residues allow very close contact between TM helices. This proximity permits extensive interhelical van der Waals interactions [16,34]. The strength of the interaction may be increased when the motif is associated with nearby β-branched residues. This is the case in GpA, which undergoes minimal loss of entropy in the sidechain χ_1 distribution when folding [34,35]. Intimate backbone contact between the helices also favors the formation of networks of weak Cα-H···O hydrogen bonds, which may add stability and/or specificity to the interaction [17]. As recently reviewed [20], the GxxxG motif may confer both stability and structural plasticity, as in the Ca^{2+}-ATPase, where the motif is at the pivot point of the structural rearrangement observed upon Ca^{2+} dissociation [36]. In the following section, we will cover recent reports showing the involvement of GxxxG-like motifs in a variety of protein families, including signal transduction proteins, channels, transporters, toxins and enzymes. Next, we will review the current debate on whether networks of weak Cα-H···O hydrogen bonds provide stability in TM helix association.

Biological examples of GxxxG-like motifs

ErbB receptor

The members of the ErbB family of growth factor receptor tyrosine kinases (ErbB1 or EGF; ErbB2 or HER2 or Neu; ErbB3 or HER3; ErbB4 or HER4) play a fundamental role in proliferation and differentiation, and are implicated in many types of cancers. These receptors have a single TM domain with two conserved GxxxG-like motifs at their N- and C-terminal ends. Some of the instances of the motif play a role in the homodimerization of the receptors [37]. Very recently, Shai and co-workers [38] have demonstrated that one of the 'orphan' interaction motifs, the N-terminal motif of ErbB1, is important for heterodimerization with ErbB2, displacing the homodimeric form of the second. They used an assay based on Langosch's ToxR assay [39] and determined heterodimeric interactions by competition, detecting the dose-dependent dominant negative effect of synthetic peptides added to the bacterial culture. Therefore, ErbB1 has two instances of the GxxxG-like motif, one for homodimerization and the other for heterodimerization.

APH-1

APH-1 is a multispan integral membrane protein that participates in the γ-secretase complex and is essential for *notch/glp-1* signal transduction in development. The fourth TM domain of APH-1 contains a conserved GxxxG tandem repeat (GxxxGxxxG). Lee *et al.* [40] have determined that these three glycines are important for the stable association of APH-1 in the γ-secretase complex.

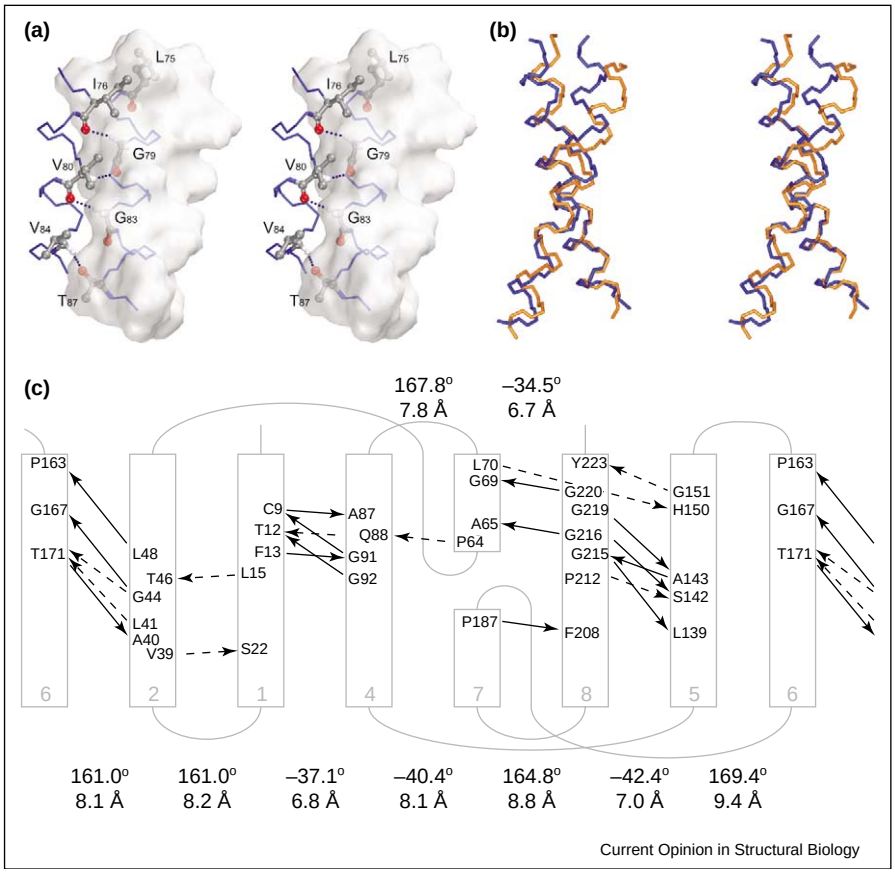
F_0F_1-ATP synthase

F_0F_1-ATP synthase is the enzyme that produces ATP in the mitochondrial membrane. Arselin *et al.* [41] reported that a GxxxG motif in the single TM helix of subunit *e* is critical for oligomerization of F_0F_1-ATP synthase. Mutagenesis followed by native gel analysis showed that glycine to leucine mutants have a greatly impaired ability to form oligomeric complexes in native gels. The mutants also had altered mitochondria morphology, as detected by EM.

G-protein-coupled receptors

Blumer and colleagues [42] have determined *in vivo* using fluorescence resonance energy transfer (FRET) and fluorescence microscopy that a GxxxG motif in TM helix 1 of the G protein α-factor receptor is essential for the oligomerization of the receptor. Sequence alignment of G-protein-coupled receptor (GPCR) families reveals that many of them have conserved GxxxG-like motifs in TM

Figure 1



GpA-like structures and formation of networks of Cα-H···O hydrogen bonds. **(a)** Stereo view of the structure of the GpA TM dimer [30]. One monomer is shown with a semi-transparent surface representation. Only the sidechains of the interaction motif (LIxxGVxxGVxxT) are shown. The central GxxxG motif, and the ridge of V80 and V84 form a wide contact area. Terminal sidechains L75, L76 and T87 extend to the side of the interaction surface, forming contacts that appear to stabilize the specific geometry of the dimer. The dots show the network of Cα-H···O hydrogen bonds. **(b)** Stereo view of a GpA-like structure in the PsaL subunit of *S. elegans* photosystem I (PDB code1jb0) [65]. GxxxG-containing helices *d* and *g* form an apparent network of Cα-H···O hydrogen bonds [64]. The helix pair has a parallel right-handed crossing angle of –40.8° and a short interhelical distance of 6.4 Å. Its backbone (blue) is superposed on the GpA dimer (red) with a rmsd of 1.41 Å. **(c)** Scheme showing the network of apparent Cα-H···O hydrogen bonds in the structure of aquaporin Z [57]. The crossing angles and the interhelical minimal axial distance are indicated. The interactions between helices 1 and 4, and 5 and 8 are GpA like.

helix 1 and other TM helices. However, other authors found that the motifs in TM helices 2 and 6 of the α_{1b}-adrenergic receptor [43], and TM helix 6 of the β_2-adrenergic receptor [44] do not appear to be involved in homo-oligomerization.

Helicobacter pylori vacuolating toxin

VacA, a toxin from *H. pylori* implicated in gastric disease, has a triad of GxxxG motifs: PxxxGGxxxGxxxGxxxG. This water-soluble toxin inserts itself into the bilayer to form an anion-selective channel. McClain *et al.* [45] have mutated these glycines and a critical proline to alanine. Using the TOXCAT *in vivo* assay in the *Escherichia coli* membrane [46], they showed that Gly14 and Gly18 are critical for association and vacuolating activity. The hexameric channel has been recently modeled computationally by Bowie and colleagues [47] using a Monte Carlo procedure. In the proposed model, the glycine residues pack against the valine and alanine ridge on the opposite side of the helix, with a right-handed conformation and a crossing angle of -25°.

Integrins

The integrin family is composed of receptors that mediate bidirectional communication between cells, and between the cell and the extracellular matrix. Integrins are type I integral membrane proteins with a conserved GxxxG-like motif in their TM domains, which are likely to be functionally involved in the oligomerization events that are critical for signaling. Recently, Schneider and Engelman [48] studied the propensity of the TM helices of several α and β integrin subunits for homo- and hetero-

oligomerization using the GALLEX *in vivo* system [49]. Although αβ hetero-oligomer formation was observed, in many cases there was a stronger propensity to form αα and ββ homo-oligomers.

An extensive leucine, alanine, valine and isoleucine scanning mutagenesis study of the entire TM domain of αIIb identified the homo-association interfacial motif as V^{971}GxxGGxxxL^{980} [50]. Periodicity analysis indicated that the motif is compatible with a crossing angle of −40°, similar to that of GpA, but with the interface estimated to be rotated by 50° about the main axis of the helix. A computational model consistent with the mutagenesis data was also produced using a Monte Carlo exhaustive search of conformational space. Interestingly, mutation of L980 to smaller alanine or valine residues produced a marked increase in dimerization. A computational model of L980A was found to be closer in structure to GpA, suggesting that position 980 could be important for maintaining the relative rotation of the helices. Several computational models for other integrins have been proposed, many featuring glycophorin-like motifs [51,52].

'BH3-only' apoptotic proteins

MacKenzie and colleagues [53] have mutated the single TM domain of BNIP3, a regulatory protein of mitochondria-mediated apoptosis whose activity is dependent on its TM domain. BNIP3 homodimerizes in SDS-PAGE and yeast two-hybrid analysis. The authors have mutated some of the positions of a multiple repeat of small residues at *i* and *i*+4 (SxxxSxxxAxxxGxxxG). Using TOXCAT, they found that mutations at Ala176 and Gly180 disrupt dimerization in SDS-PAGE and in membranes. They also mutated a histidine residue at position 173 and found that, whereas a tryptophan mutation was mildly destabilizing, an alanine mutation was completely disruptive. The second glycine of the GxxxG motif was not mutated in this work.

Lactose permease

The structure of *E. coli* lactose permease at 3.5 Å resolution has been recently solved by Kaback, Iwata and coworkers [54]. The crystallized molecule is a Cys154Gly mutant. The mutant was chosen because it is better behaved than the wild-type sequence: it can bind substrate, it is thermostable and it does not aggregate [55]. The mutation converts a GxxxC motif into a GxxxG motif at the interface of a right-handed parallel helix–helix interaction with short interhelical distance that is well superimposable to the GpA dimer. The introduced glycine residue, however, does not occur at the tightest point of the interaction (as in GpA), but one turn down (roughly corresponding to Tyr87). The mutation impairs transport and appears to lock the protein in one conformation. Stability-enhancing mutations are not rare in TM proteins [56] and thus glycine mutations may be particularly useful for engineering ultrastable membrane proteins for crystallization [57].

'Glycophorinization' of the major coat protein

The single TM domains of the major coat protein (MCP) from the M13 bacteriophage and GpA share a similar GxxxG-containing interaction motif; however, the MCP has a much weaker tendency to oligomerize than GpA. In a recent collaboration by the Deber, Bowie and Engelman groups [58], the MPC interface (VVxxGAxxGIxxF) was converted in steps to the complete GpA motif (LIxxGVxxGVxxT). TOXCAT experiments showed a gradual increase in CAT signal as single, double and triple mutations were introduced; the complete conversion (five mutations) recovered more than 60% of GpA wild-type activity. The two critical residues appear to be on the N-terminal (V30L) and C-terminal (F42T) side of the motif, and were sufficient to recover almost 70% of the final CAT activity. The minimal consensus motif for stability was therefore LxxxGxxxGxxxT, consistent with the findings of previous authors [31,59,60] and suggesting that the GxxxG motif requires stabilization at either side of the crossing point (Figure 1a). An F42V mutation was introduced at the terminal position to probe the specific role of the threonine hydroxyl group and, interestingly, the mutation suppressed dimerization. In GpA, Thr87 appears to form interhelical hydrogen bonds [61] when in bilayers and these bonds would be lost in the valine mutant. However, it is unlikely that this hydrogen bonding is a major contributor to stability, because threonine could form an intrahelical hydrogen bond in the unfolded (monomeric helical) state. A possible additional source of destabilization would be the loss of a hydrogen bond between the hydroxyl oxygen of Thr87 and the Cα-H of residue 84 on the opposite chain.

Technical note

Some authors have occasionally chosen substitutes for mutagenesis that do not appear to be ideal. Alanine scanning is very conservative when applied to glycine and serine residues. Proline mutations can disrupt interactions even when they are introduced at non-interfacial positions, as they perturb the helical backbone [62]. Finally, as we will discuss later, the strong potential for interaction of polar residues also makes the interpretation of this type of mutation problematic. Small-to-large (i.e. glycine and alanine to leucine or phenylalanine) and large-to-small (i.e. leucine to alanine) mutations are likely to be more effective for a first gross mapping of a specific interface, whereas more conservative isosteric mutations (e.g. glycine to alanine, threonine to valine) could be added for detailed exploration.

Does the Cα-H···O hydrogen bond stabilize helix–helix interactions?

Carbon atoms can act as very weak hydrogen bond donors in organic molecules, including proteins and other

biopolymers [63]. The strength of the interaction is increased for activated carbon atoms, that is, those that are bound to electronegative groups, such as sterically accessible Cα groups and the Cδ of proline. The energy of the bond has been estimated to be approximately half as strong as an O-H···O hydrogen bond. Smith and co-workers first proposed the involvement of glycine residues in Cα-H···O interactions in TM domains [16]. A structural survey of TM helix interactions by Engelman *et al*. [17] showed that networks of several backbone to backbone Cα-H···O=C interactions can occur between TM helices separated by a short interhelical distance. The close approach is favored by glycine and by other small residues; however, the Cα of any type of interfacial residue can act as a donor in the networks. In addition to the backbone carbonyl oxygen, the hydroxyl oxygen of threonine and serine can also act as acceptors. The evidence is based mostly on mid-resolution crystallographic data, but the overall picture is likely to hold. Right-handed parallel interactions, similar to those of the GpA dimer in their crossing angle (approximately −40°) and close interhelical distances (6–7 Å), were predominant in the survey. However, GxxxG and GxxxG-like motifs were also present in left-handed and antiparallel interactions. Recently determined structures of helical TM proteins have confirmed and expanded this view. Loll *et al*. [64] have analyzed the structure of *Synechococcus elegans* photosystem I at 2.5 Å resolution [65]. They report a total of 75 apparent Cα-H···O hydrogen bonds in 34 TM helices, mostly intrasubunit. Most interesting in this context is the interhelical contact with the greatest number of Cα-H···O bonds, which occurs between helices *d* and *g* of subunit PsaL. Both of these helices are characterized by GxxxG motifs. This interaction has a crossing angle of −40.8° and an interhelical distance of only 6.4 Å, and superimposes well onto the GpA dimer, with an rmsd of 1.41 Å (Figure 1b). Helix *d* has a tandem $G^{47}xxxG^{51}xxxG^{55}$, whereas helix *g* contains $G^{123}xxxG^{127}xxxS^{131}$. Similar GpA-like structures are present in the glycerol facilitator, in the calcium ATPase [17] and in soluble proteins [66,67].

The presence of apparent Cα-H···O bonds has also been confirmed in the recent structure of aquaporin Z. The authors reported 15 occurrences in 8 helices [57]. We found 13 and 23 bonds, using stringent and inclusive criteria, respectively (Figure 1c). As observed in the homologous glycerol facilitator, the more extensive networks are present in the two GpA-like structures (between helices 1 and 4, and 5 and 8) and in the antiparallel interaction between helices 2 and 6. A very interesting feature is the adjacent GGxxGG motifs at *i* and *i*+1 on helix 8. The two GxxxG motifs pack against two different helices, forming Cα-H···O bonds in both cases.

Although there may be convincing structural evidence that Cα-H···O hydrogen bonds often participate in TM helix–helix interactions, their actual contribution to structural stability is still under debate. Very recently, two groups have presented the first experimental measures of the bonds' energetic contribution. Arbely and Arkin [68] have selectively deuterated Gly79 of GpA, which appears to act as a Cα donor. Using FT (Fourier transform) IR, they measured the CD_2 asymmetric stretching mode in both the monomeric and the dimeric states. From the observed difference in the stretching frequency between the two states, they estimated an energy of 0.88 kcal/mol from the relationship $\Delta G = 0.31\cdot(\Delta v^{0.5})$ [69]. The conclusions of this study are in apparent disagreement with the findings of Bowie and co-workers [70], obtained by thermodynamic folding measurements of bacteriorhodopsin (bR) mutants. In these studies, Thr24, which receives a hydrogen bond from the Cα of Ala51, was mutated to an isosteric valine residue. They compared the stability of the wild type with that of three mutants (T24V, T24S and T24A), using SDS denaturation, and also crystallized the mutants to verify the structural impact of the mutations. The stability was only marginally affected and, in the case of T24A, it actually increased. Taken together, these two initial studies suggest that isolated Cα-H···O interactions (T24 in bR) may not be as stabilizing as those present in extensive networks (G79 in GpA). However, more data on both systems are required before a final conclusion can be drawn.

Interactions between polar sidechains in membranes

The inclusion of a single asparagine, aspartic acid, glutamic acid or glutamine in an otherwise uniformly hydrophobic helix provides a strong driving force for self-association in micelles and biological membranes [71,72]. In previous studies, an asparagine sidechain was placed at an 'a' position of a heptad repeat, with valine at the remainder of the 'a' positions and leucine at each 'd' position. The peptides formed dimers and trimers in micelles, and the interaction was eliminated when the asparagine was changed to a variety of other apolar residues. Among the polar sidechains, only glutamine or protonated aspartic acid or glutamic acid supported the association of the helices [73,74]. Similarly, asparagine and aspartic acid residues interact favorably when placed in an antiparallel helical hairpin translated *in vitro* in the presence of rough microsomes [75].

Although a single Ser–Ser contact between TM helices may not be sufficient to drive association, cooperative interactions between multiple serine residues appear to provide adequate stability for assembly. Statistical pairwise contact potentials have shown that serine residues have a high tendency to form interhelical interactions [9,10]. Using TOXCAT, Engelman and co-workers showed that multiple serine residues are able to drive the association of parallel TM helices [76] and similar

serine-rich motifs have been employed in the design of ion channel peptides [77,78]. A parallel motif with the heptad repeat SxxLxxx forms the dimerization interface of the erythropoietin receptor [79]. On the other hand, Liang and co-workers recently described an antiparallel 'serine zipper' motif in which interacting serine sidechains are spaced at seven-residue increments in the crystal structures of TM proteins [10]. This serine zipper features an antiparallel arrangement of two helices, with opposing serines making hydrogen bonds to the backbone carbonyl of the serine on the opposite helix.

Effects of sequence context and membrane locations on polar interactions

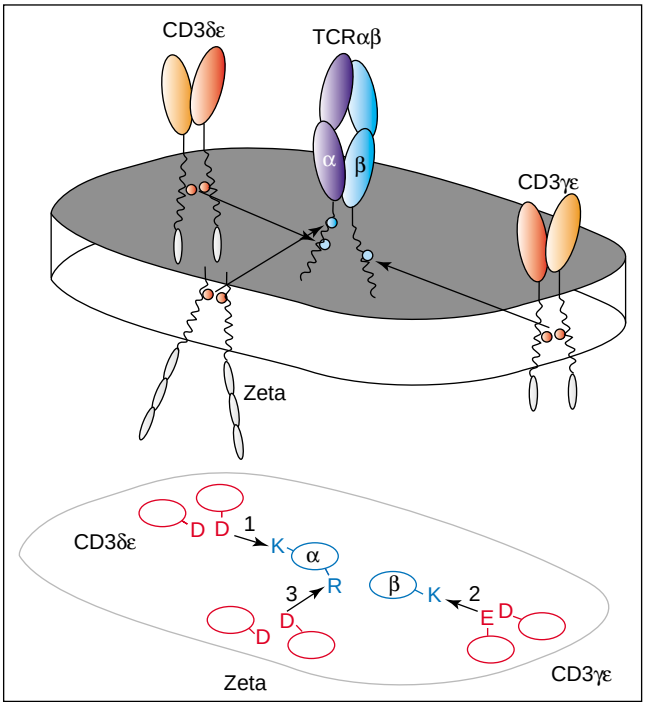
The effect of asparagine to valine mutations at the 'a' position of a model membrane-spanning helix (MS1) has been investigated as a function of location within the membrane. When the additional asparagine was located in the apolar region of the TM helix, it increased the stability of MS1 by approximately 2 kcal per mole of monomer [80]. When placed at the interface between the polar and apolar regions of the TM helix, the asparagine failed to contribute significantly to the free energy of association. Furthermore, in a water-soluble coiled coil, the same substitution of a single asparagine is destabilizing by 2–3 kcal/mole·helix [81]. These free energy changes correlate closely with the statistical tendency of asparagine and valine to occur in buried versus exposed positions in the corresponding regions of water-soluble and membrane proteins [80].

Langosch and co-workers [79] have placed an asparagine residue at each position of a poly-Leu sequence, and determined their effects on association in the inner membrane of *E. coli* and in SDS micelles. In micelles, as well as biological membranes, the asparagine sidechain mediated TM helix–helix association only when placed at or near the middle of the helix. To assess the association in biological membranes, the poly-Leu sequence was fused to the ToxR repressor, which controls the level of expression of reporter genes in response to dimerization of the membrane helix. The level of activation, when considered as a function of the position of the asparagine in the sequence, showed an α-helical repeat, suggesting there was a preferred orientation of the helix relative to the fused ToxR receptor. The importance of the phase of the TM helix relative to the fused ToxR domain has also been seen in other studies with natural TM helices [50,82]. Similar results were obtained by Dawson *et al.* [83] in a study of natural TM helices containing glutamine or glutamic acid. When these residues were near the N terminus of the TM helix, they led to weak association, whereas when they occurred near the middle of the helix, they led to a strong association that was eliminated upon mutation to alanine. Also, the authors examined the degree of activation of ToxR-dependent transcription in response to the introduction of asparagine or aspartic acid into the TM helix of M2. The degree of activation was position dependent, probably reflecting both differences in the phasing of the mutations within the helix and differences in the ability of the polar residue to reinforce a weak dimerization interface in the TM helix.

Polar contacts in the association of transmembrane helices, receptor activation and signal transduction

Appropriately placed polar residues are proving to be essential features in the association of a wide variety of TM helices and in the folding of membrane proteins. Perhaps the most dramatic example is the T-cell receptor (TCR), in which an intricate network of interactions between ionizable sidechains serves to stabilize a complex of individual helices from eight different protein chains [84,85]. The stoichiometry and specificity of association were recently determined using a novel method of immunoprecipitating the native complex. The TCR α-chain TM helix contains a lysine and an arginine sidechain, whereas the β-chain TM helix contains a single lysine residue. As shown in Figure 2, each of these basic sidechains recruits two additional chains from CD3 and the zeta subunit, all of which have a single acidic residue. Thus, the resulting complex consists of a network of intermingled three-helix bundles, each stabilized by

Figure 2



An intricate network of interactions between ionizable residues specifically stabilizes TM helix interactions in the TCR–CD3 complex. Positive sidechains are donated by the TCR heterodimer (blue circles) and interact with a pair of carboxylate groups (red circles) from the CD3γε, CD3δε and zeta dimers. Figure reproduced from [84] with permission.

two carboxylate-containing residues interacting with either an arginine or lysine residue. Most probably, the acidic residues form a carboxylate–carboxyl hydrogen bonded pair, which bears a single negative charge to balance the charge on the lysine or arginine residue with which it is interacting.

Studies with synthetic peptides have implicated polar residues in the association mechanisms of several TM proteins. The aspartate receptor has two TM helices that associate to form a TM four-helix bundle [86]. Synthetic peptides spanning the sequence of the first TM helix (helix-1) form dimers in detergent micelles, whereas helix-2 fails to associate [87]. These findings are fully consistent with disulfide cross-linking studies on the intact protein, which showed that helix-1 and helix-1′ are in physical contact, whereas helix-2 and helix-2′ do not physically interact in the TM four-helix bundle [86]. A polar QxxS motif is critical to the association of helix-1, as assessed by the ToxR dimerization method [39]. A mutational analysis showed that the glutamine was more important for dimerization than the serine and that the association could be enhanced by mutating the serine to either glutamine or glutamic acid [88]. In a related approach, peptides were synthesized corresponding to the three TM helices of the enzyme diacylglycerolkinase (DGK). A peptide corresponding to TM helix 2 was able to interact with intact DGK and inhibit its activity. This peptide also had a polar glutamic acid and replacement of this residue eliminated activity.

Polar residues can play a decisive role in the aberrant association and misfolding of TM domains. For example, the receptor tyrosine kinase encoded by the *neu* proto-oncogene is constitutively activated by a single valine to glutamic acid substitution in the predicted membrane-spanning sequence of the receptor [89,90]. Mutations in the TM domain of the cystic fibrosis conductance regulator (CFTR) also lead to the accumulation of misfolded protein [91,92] or aberrant association of helices in mutant forms of the protein [92]. Similarly, mutating Thr617 to asparagine in the granulocyte colony-stimulating factor receptor TM helix gave a growth-factor-independent phenotype in patients with acute myeloid leukemia [93], and mutating Ser498 of the thrombopoietin receptor to asparagine rendered this receptor constitutively active [94].

Asparagine-scanning mutagenesis

Transient associations of TM domains are important for the regulation of a variety of proteins. The introduction of an asparagine sidechain can improve the energetics of association of TM helices, by reinforcing a weak motif when placed in the proper position in the sequence. Thus, by scanning asparagine throughout a TM peptide, it is possible to modulate the propensity for oligomerization and to help reveal its role in a biological response. Langosch and co-workers [79] scanned asparagine sidechains throughout the sequence of a TM serine zipper important for dimerization of the erythropoietin receptor. When placed on the polar side of the helix, an asparagine sidechain can strongly stabilize the formation of dimers, as assessed by the ToxR assay. However, when stabilizing mutations were introduced into the full-length receptor, they failed to further increase the level of activation. These findings are consistent with previous studies, which had suggested that the wild-type receptor was fully dimeric *in vivo* and that signaling involved a transition between two different dimeric states, rather than a shift in the monomer/dimer equilibrium [95].

The TM helices of the α and β chains of integrins have been proposed to associate in the inactive state of this highly regulated family of proteins, but to move apart in the activated state [96–99]. Furthermore, when released from cytoskeletal restraints, the cytoplasmic and TM domains of these proteins have a strong tendency to self-associate [100], which may be an important step in the clustering of these integrins into focal adhesions [101]. To probe the effects of homo-oligomerization in integrin activation, Li *et al.* [101] scanned asparagine residues throughout the TM helix of the β subunit. Two mutations along one face of the helix, G708N and M701N, enabled integrin αIIbβ3 to constitutively cluster and bind soluble fibrinogen. This mutation also enhanced the tendency of the TM helix to form homotrimers. Thus, interactions that cause dissociation of the TM helix from the α subunit appear to activate the integrin.

A few words of caution on the interpretation of asparagine-scanning studies are in order. Firstly, the introduction of asparagine may enhance association only if the pre-existing interaction is relatively weak. For example, the introduction of asparagine into a highly evolved GxxxG motif might disrupt, rather than facilitate, dimerization. Secondly, when considering complex interaction networks, the introduction of asparagine might disrupt one helix–helix interaction while facilitating another. For example, in the case of mutating the TM helix of integrin β3, it is possible that some of the effects may arise from disrupting an interaction with αIIb, as well as from enhancing the homotypic association.

Proline as a determinant of structure, folding and interaction in transmembrane helices

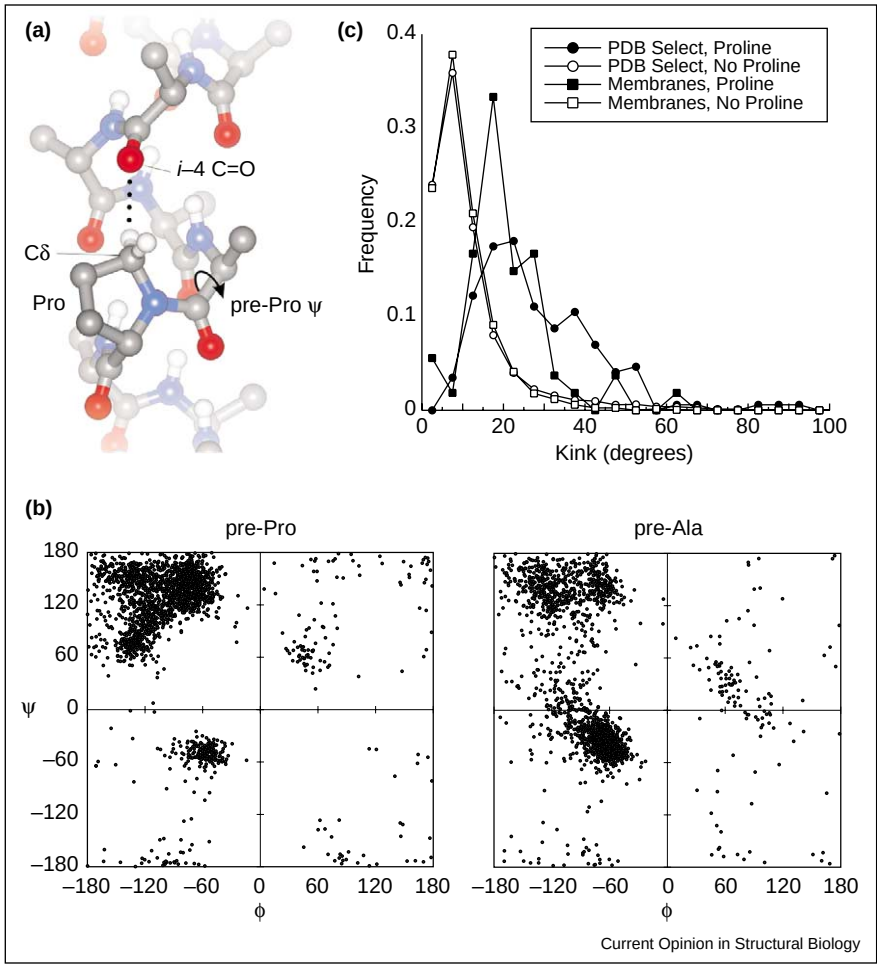
There is little doubt that proline residues are important for the function and structure of integral membrane proteins, as evidenced by the recent observation that mutations of proline have one of the highest phenotypic propensities in the analysis of TM sequences from the Human Gene Mutation Database [102]. Recent years have seen a refinement in our understanding of the role played by proline in defining the structural and thermodynamic properties of membrane proteins. In the

following sections, we will summarize recent findings concerning: the structural properties of proline in TM helices; how these properties influence the tertiary structure and hence function of membrane proteins; the role of proline in the avoidance of misfolding; and emerging proline-containing motifs involved in helix–helix association in membranes.

Structural aspects

The fusion of the backbone and sidechain atoms into a five-membered pyrolidine ring imparts special conformational characteristics to proline (Figure 3a). The ring induces the residue to adopt a ϕ angle of approximately -60°, thereby stabilizing the helical conformation. Thus, proline is strongly helix stabilizing, but only when placed in the first position (N1) of an α helix. At other positions, it is destabilizing for two reasons. First, it lacks an amide proton that could form a hydrogen bond to a carbonyl at position *i*–4 in the helix. Second, it influences the torsional angle preference of the residue preceding it in the sequence (the pre-Pro position) [103,104], stabilizing more extended conformations relative to the helical conformation (Figure 3b). Using the PDBselect database [105], we calculated that the residue preceding proline has a 5.4-fold lower tendency to adopt ϕ/ψ angles in the generously allowed α_R-helix region of the Ramachandran plot (as defined in [106]). Also, the population of the remaining regions of conformational space is altered, with

Figure 3



Effect of proline residues in TM helices. **(a)** Proline in a helical conformation. The pre-Pro residue ψ angle and the *i*–4 carbonyl are labeled. The $C\delta H_2$ group can form weak hydrogen bonds with the *i*–3 or *i*–4 carbonyl oxygen. **(b)** Ramachandran plots of pre-Pro versus pre-Ala residues in soluble proteins. The pre-Pro ψ angle is significantly restricted by steric interactions with proline Cδ, resulting in a 5.4-fold lower tendency to adopt ϕ/ψ angles in the generously allowed α_R-helix region. A novel 'pre-Pro' region (centered near -130°,$+70^{\circ}$), normally not significantly populated, becomes sterically accessible. **(c)** A comparison of kink angles in the mostly water-soluble protein PDB Select database ([105]; December 2003, 25% version) with kink angles in a current database of TM helices of all high-resolution TM proteins using an updated database described in [9]. A sliding 14-residue window was used on regions of α_R residues (α_R as defined by Balaram [106]). Axes were determined for each seven-residue half of the window and the angle between these axes is defined as the kink.

a novel 'pre-Pro' region becoming sterically accessible. Finally, the *i*–4 carbonyl oxygen has to shift away from the nitrogen that it is normally hydrogen bonded to, as a result of the steric constrains imposed by the presence of the ring Cδ (Figure 3a). Thus, in a water-soluble protein, proline is generally considered a helix breaker.

The location and structural context of a proline within a TM protein determine whether it breaks or merely kinks helices. Von Heijne and co-workers [107–109] used glycosylation mapping to determine which residues had the greatest tendency to break helices and induce helical hairpins in membranes. When introduced such that it could break a helix near the headgroup region, proline stood out as the most hairpin-stabilizing amino acid. However, when embedded deep within a membrane, proline is easily accommodated in a helix [110], which is the default state of a hydrophobic chain at high dilution within a solvent of low dielectric. The increased local backbone flexibility introduced by the proline residue may be important for hinge-bending motions that are likely to play a functional role in catalysis and signal transduction [111,112].

Although proline lacks an amide NH with which to form a hydrogen bond, its $C\delta H_2$ protons (the δ carbon is the carbon directly bonded to the nitrogen) can form hydrogen bonds to the unfulfilled amide carbonyls at positions *i*–3 and *i*–4 [113]. This interaction mitigates the loss of stronger hydrogen bonds to amide NH groups and induces a slight kink in the helix. A database study of proline kinks in TM helices shows a mean kinking angle of $21 \pm 11^\circ$ [114]. We have computed related distributions for proline kinks in water-soluble helices versus TM helices (Figure 3c). Interestingly, we find that the distribution is sharper and favors smaller kink angles for TM helices versus water-soluble helices. Thus, the environment and/or tertiary packing constrain the helices to be straighter within membranes.

This finding prompted us to compare the degree of bending of water-soluble versus membrane-soluble helices that lack proline. This is a particularly interesting question given a recent analysis that showed that the α helices in membrane proteins tended to have more ideal α-helical ϕ/ψ angles than water-soluble helices [115]. Figure 3c illustrates histograms of the angle between the helical axes of consecutive seven-residue segments of long helices (≥14 residues) in water-soluble versus membrane-soluble proteins. The two distributions are identical within the error of analysis. The frequency distribution peaks at approximately 10°, indicating that helices that lack proline are, on average, slightly bent in both classes of structures. As discussed by Cross *et al.* [115], helices are expected to become straighter with increased refinement of a structure, which could lead to some shifting of the histograms towards lower angles. However, we expect coordinate error to be approximately the same for both water-soluble and membrane structures, suggesting that the helices in each class are similar. We also examined the radius of curvature of water-soluble versus membrane helices, and again found no significant difference between the two distributions.

Effects of removing proline residues from helices

The diagram in Figure 3c provides a comparison of the stiffness of a proline-containing helix and the stiffness of a helix lacking this residue, and can help to rationalize much of the data concerning proline mutations in the literature. We first consider the effects of mutating a proline residue to another sidechain, such as alanine, in a kinked helix within the context of a native folded protein. The mutation will be accommodated, at little thermodynamic expense, as assessed by the fact that helices lacking proline are quite frequently kinked to this extent. What might be gained in improved helical propensity in water-soluble proteins will be lost due to the need to kink the helix and weaken the NH to carbonyl amide hydrogen bonds at the site of the mutation [116]. However, the alanine mutation introduces strain in the structure, because the helix would generally prefer to be more straight, but for the tertiary interactions that force a kink. In the unfolded state, the helix would thus have a straighter conformation. Thus, the protein may have altered folding kinetics, or undergo aggregation or other misfolding events.

Bowie and colleagues [117] have mutated three proline residues in the center of bR TM helices, and determined that the crystal structure and stability were not dramatically altered. The one mutant that was able to restore the helical hydrogen bond pattern was as stable as the wild type and functional, whereas the other two were both destabilized by about 1 kcal/mol. They complemented the study with a sequence analysis of the alignments of protein families with a member of known structure. Ninety percent of the positions corresponding to a kink in the structure had 10% or more prolines in the alignment, whereas the non-kinked positions never reached that threshold. This clearly shows that proline residues are not required to kink helices, but that sites where proline residues are even slightly conserved in the aligned sequences of TM domains are very likely to be in kinked conformations across all the related proteins.

Although the removal of a proline residue appears to have a rather small effect on stability and function, it can have a deleterious effect on the kinetics and robustness of folding. For example, mutants of a heat shock transcription factor showed different kinetics of folding, with increased accumulation of an intermediate [118], suggesting that a helical proline prevented aggregation of partially folded forms. There are now indications that helical proline residues might have similar roles in the folding of TM

helices. Using a series of techniques, Wigley *et al.* [92] have studied different mutations of a proline residue in the third TM helix (CFTR-m3) of the chloride channel CFTR, a protein that undergoes inefficient maturation in the endoplasmic reticulum [119]. Mutations at P205 significantly impair correct maturation, but the small fraction of surviving protein is functional. Surprisingly, the wild-type sequence of CFTR-m3 incorporates better in SDS micelles and is more helical than leucine, glycine, serine and alanine mutants. *In vitro* translated transferrin receptor CFTR-m3 chimeras also had a higher efficiency of co-translational integration with respect to mutants. Furthermore, Ladokhin and White [120] reported that they were able to mitigate the tendency of a hydrophobic model peptide to aggregate in solution by inserting a proline residue.

Effects of introducing proline residues into helices

The introduction of a proline residue within relatively straight helices is likely to be destabilizing. Proline is rarely accommodated within straight helices in the crystal structures of natural proteins (Figure 3c), indicating that accommodating a proline in a straight helix must occur at a significant energetic cost. Thus, the mutant would either be forced into an energetically costly straight helix or it could introduce a kink, which is likely to disturb the tertiary packing. Indeed, Orzaez *et al.* [62] recently conducted proline-scanning mutagenesis of the GpA dimer, which has the interaction sequence LIxxGVxxGVxx-Txxx (x is a hydrophobic amino acid). Almost all mutations eliminated dimer formation, including mutations of the highly mutable 'x' sites, which otherwise accommodate a variety of other hydrophobic residues. Mutations near the N terminus of the consensus site (including the first leucine of the interaction motif) were much better accommodated than those near the C terminus, presumably because proline influences the conformation of residues N terminal to itself. Similarly, Langosch *et al.* [121] found that proline disrupted oligomerization of TM helices that were designed to form coiled coils in membranes.

In other contexts, proline might stabilize helix–helix packing. It has a high packing propensity in TM helices [11], indicating that it is often tightly packed in the structures of natural proteins. A direct indication of the ability of proline to be involved in TM interactions was obtained by Dawson *et al.* [76] in a selection from randomized helical interfaces with TOXCAT [31]. The main motifs observed were very rich in serine, threonine and proline residues. Pairs of proline residues at i, i+4 were also present in three of the four sequences in the selection that lacked any polar residue. Several proline patterns are also observed at high frequency in TM sequences [33,92]. Thus, when placed in the proper context, this amino acid could frequently participate in mediating helix–helix interaction.

Thermodynamic studies

One of the greatest hurdles to measuring the thermodynamics of membrane protein folding is finding conditions under which the proteins reversibly fold in a two-state process between a native state and a well-defined unfolded ensemble. For the β-sheet class of protein, the processes of membrane insertion and folding appear to be tightly coupled, so the energetics of folding can be monitored using urea denaturation, as for water-soluble proteins [122]. However, for the helical class of protein, the unfolded state consists of inserted helices that are largely non-accessible to water-soluble denaturants, thus requiring different methods of analysis. One approach involves the titration of the native protein in a structure-stabilizing micelle with the denaturing detergent SDS [123,124] or alcohols [125]. The free energy of folding in the absence of SDS is then approximated by assuming that ΔG_{fold} extrapolates linearly with respect to the mole fraction of SDS. This method has provided the first view of the energetics of folding of mutants of large membrane proteins. Its disadvantages, however, are that it is limited to proteins in micelles, the method of linear extrapolation of the free energies of folding to zero SDS concentration is an approximation, the unfolded state has not yet been well characterized from the perspective of residual tertiary structure and the assumption of a two-state process is difficult to assess.

The folding of self-associating helical bundles can be studied with an experimental approach that does not suffer from some of these limitations, although so far it has been applied primarily to relatively simple systems. In this case, folding/assembly can be studied directly by simply measuring the monomer/n-mer equilibrium, which can be manipulated by changing the peptide/detergent ratio in micelles or peptide/phospholipid ratio in bilayers. In this case, the unfolded state is a monomeric helix that corresponds well to the non-interacting helices postulated to comprise the unfolded state in the two-state folding model of Popot and Engelman [4]. For aggregates larger than dimers, the two-state nature of the overall process can be rigorously addressed by measuring the cooperativity of assembly and the concentrations of intermediate aggregation states [126]. A variety of methods can be used to monitor the degree of association (reviewed in [127]), including various fluorescence methods, analytical ultracentrifugation, CD spectroscopy and thermodynamically coupled thiol-disulfide exchange reactions [128,129]. Furthermore, a large number of membrane proteins have been cleaved into multiple fragments that nevertheless associate in membranes to adopt native functional proteins [4], allowing the extension of the method to more complex proteins when reversible folding can be established [125].

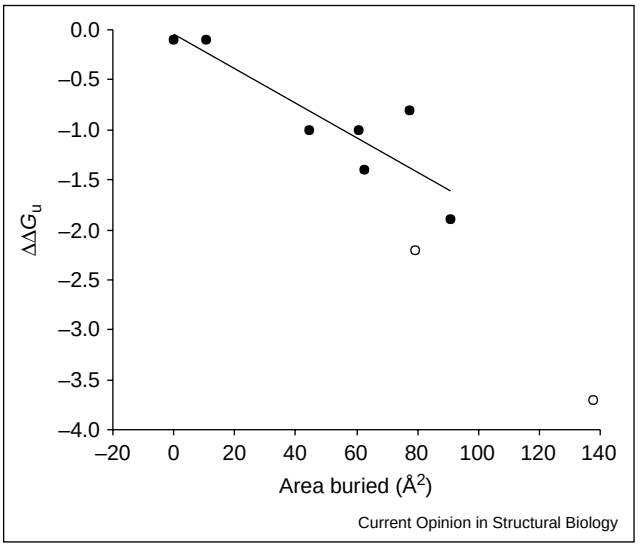
Experiments based on both types of systems are beginning to define the role of packing in the folding of

membrane proteins. With water-soluble proteins one can, for example, predict the energetic consequences of changing a buried non-polar sidechain to a smaller sidechain. A leucine to alanine mutation will 'cost' about 2–5 kcal/mol [130], depending on the extent of structural rearrangement accompanying the mutation [131]. The loss in hydrophobic effect contributes about 2.5 kcal/mol. The extent of destabilization beyond this value depends on the size of the cavity that is generated. The corresponding range of values for membrane-soluble proteins is only now beginning to be measured for a variety of systems, including GpA [132], influenza A virus M2 protein [133] and bR [123]. These studies show a range of about 0–2 kcal/mol for mutating a large hydrophobic sidechain (phenylalanine, leucine, isoleucine, methionine) to alanine, indicating that packing of buried apolar groups plays a much smaller energetic role in membrane proteins than in water-soluble proteins. This finding is consistent with the lack of a hydrophobic effect in membranes. This finding is also consistent with studies of the association of model poly-Leu and poly-Ala helices, which show weak but significant association in bilayers [134–138].

The effect of small-to-large mutations can have more drastic energetic consequences, particularly when they disrupt the intimate packing of GxxxG motifs [26,139]. On the other hand, when the large mutated sidechain can fill the aqueous pore of ion-conducting channels, they are instead tolerated or can even stabilize the structure [133].

Based on work with membrane peptides, hydrogen bonds appear to be important for stability. Thermodynamic measurements with model peptides suggest that a good hydrogen bond involving an asparagine sidechain is 'worth' about 2 kcal/mol when placed in the most apolar region of the TM helix [80]. Bowie *et al.* recently conducted an extensive analysis of one helix (helix B) of bR [123], which led to the conclusion that hydrogen bonds did not contribute to the stability of bR. Instead, they observed a weak correlation between the area of the sidechain mutated and the change in free energy upon mutation. However, it is important to ask whether the hydrogen bond is formed by a tertiary contact to a neighboring helix. For example, the hydroxyls of serine and threonines form intrahelical hydrogen bonds to a backbone carbonyl one turn up in a helix. This interaction is expected to be retained in the unfolded state, so there would be a small value of $\Delta\Delta G$ for mutation to alanine. Indeed, if one examines the thermodynamic data for the mutants that are located within the apolar region of the bilayer, only two residues, T46 and Y57, are involved in tertiary contacts with other polar sidechains. Figure 4 illustrates the thermodynamic effect of mutating the residues within the most apolar region of the bilayer, as a function of the surface area change associated with the mutation. Following Bowie's analysis, only destabilizing mutations are considered, because stabilizing large-to-small mutations presumably involves a conformational change. When the sidechains that do not form tertiary hydrogen bonds are considered as a group, a straight line is observed. They show a near-zero intercept and a slope of 17 cal/Å^2. How does this compare with the expectation from a water-soluble protein? Xu and Matthews [131] showed that the free energy change for mutation from a large residue to alanine has two components. The first is a constant −2 kcal/mol, which represents the free energy of transfer of the large apolar residue to the interior of the protein. This term is near zero for the bR mutants considered, because of the lack of a hydrophobic effect. The second term is near 20 cal/(mol Å^2) for both water-soluble and membrane proteins, and represents the loss of van der Waals interactions between the mutated sidechain and the walls of the cavity.

Figure 4



The destabilizing effect of alanine mutation in the central region of bR helices (see Faham *et al*. [123]). The two sidechains that receive a tertiary hydrogen bond in the wild-type structure (T46 and Y57, white circles) appear to be outliers from the linear fit of the remaining points. The line was calculated for the remaining residues and has a near-zero intercept (−0.05 kcal/mol) and a slope of 0.017 kcal/(mol Å^2), similar to the value of 0.022 kcal/(mol Å^2) observed in solution by Xu and Matthew [131].

The two mutants of the sidechains of bR that are involved in tertiary hydrogen bonds fall below the line for non-hydrogen-bonded residues (i.e. they are additionally destabilized) by 0.5–1.0 kcal/mol when compared to the expectation from their surface area alone [131]. Clearly, two data points are insufficient to conclude that this is a general effect, and highlights the need to obtain more data for both designed and natural systems.

If membrane proteins have a significantly lowered contribution from the burial of apolar residues and

sidechain-mediated hydrogen bonds are stabilizing by only 1–2 kcal/mol, what stabilizes their folds? One factor is that they fold with a significantly smaller loss of conformational entropy than water-soluble proteins do. The loss of backbone entropy is significantly decreased because the helices are pre-formed in the unfolded state. Furthermore, the fold of a membrane protein is highly directed by its biosynthetic insertion into a membrane, which forces the helices to lie parallel or antiparallel to one another. Finally, the loss of sidechain entropy is minimal for interfaces rich in small residues, including glycine, alanine and the β-branched amino acids threonine and valine [34,35,140].

Clearly, our understanding of the folding of membrane proteins in micelles is just beginning to emerge. An even greater challenge will be to obtain a satisfactory understanding of their folding in phospholipid bilayers [141]. Along these lines, early work with β-proteins [122], as well as studies of model [134–137] and natural [128,129] helical assemblies, has shown the role of hydrophobic matching, cholesterol content and bilayer length in defining the folding process. Work in this area will provide a particularly fertile avenue for future investigations.

Update

Two interesting papers have been published since the submission of the review. Bowie and colleagues [142] have tested how proline residues are accommodated in a TM helix by introducing 15 individual substitutions on helix B of bR. The mutations were generally destabilizing and proline residues were better tolerated near the N terminus. Deber and co-workers [143] have made a library of hairpin constructs formed by TM helices 3 and 4 of CFTR, introducing 21 individual asparagine mutations on helix 4. The altered SDS-PAGE mobility of hairpins was used to report the propensity of the mutants to form a hydrogen bond with Q207 on helix 3. The results indicate that Q207 interacts with variable strength with the 21 mutants, depending on the position of the asparagine along the main helical axis and the helical phase.

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