REVIEW

Interaction and conformational dynamics of membrane-spanning protein helices

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Abstract: Within 1 or 2 decades, the reputation of membrane-spanning α-helices has changed dramatically. Once mostly regarded as dull membrane anchors, transmembrane domains are now recognized as major instigators of protein–protein interaction. These interactions may be of exquisite specificity in mediating assembly of stable membrane protein complexes from cognate subunits. Further, they can be reversible and regulatable by external factors to allow for dynamic changes of protein conformation in biological function. Finally, these helices are increasingly regarded as dynamic domains. These domains can move relative to each other in different functional protein conformations. In addition, small-scale backbone fluctuations may affect their function and their impact on surrounding lipid shells. Elucidating the ways by which these intricate structural features are encoded by the amino acid sequences will be a fascinating subject of research for years to come.

Keywords: transmembrane helix; dynamics; interaction; assembly; membrane protein

Introduction

Integral membrane proteins comprise 25–30% of all proteins in the proteomes of organisms from every kingdom of life and thus form an inexhaustible playground for the researcher. Single-span, bitopic proteins range from growth factor receptors, adhesion proteins, SNAREs to T-cell receptor subunits, and so forth, whereas multispanning, polytopic proteins include structurally more complex receptors, transporters, and channels. Studying membrane protein structure and assembly has made it clear that interactions and dynamics of the α-helical transmembrane domains (TMDs) play a crucial role in their folding, assembly, and function. Various aspects around this topic have been covered by excellent recent reviews.[1–18]

Interactions of TMDs are experimentally investigated with a variety of biochemical and biophysical methods including gel shift assays, analytical ultracentrifugation, fluorescence resonance transfer, disulfide exchange, as well as genetic approaches with bacterial

Abbreviations: CD, circular dichroism; DHX, deuterium/hydrogen-exchange; HDX, hydrogen/deuterium-exchange; SNARE, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor; TMD, transmembrane domain.

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two-hybrid or split enzyme systems (reviewed in Refs. 13 and 18–20). TMD–TMD assembly is also suggested by the patterns of residue conservation during evolution. Specifically, TMDs of bitopic proteins are more conserved than the remainder of the protein and conservation is restricted to one side of the helix.[21] With polytopic proteins, sequence variation is higher where TMD helices face the lipid bilayer than at helix–helix interfaces,[22] a finding that has been used by several groups as a powerful constraint in molecular modeling.[23,24] Further, single-spanning membrane proteins are more tolerant to mutation in comparison to multispanning proteins, where most TMDs contact multiple helices.[25,26] Together, this reflects conservation of amino acids at the sites of TMD–TMD packing and highlights their importance for specific interaction.

Currently, we only have a rudimentary understanding of the mechanisms that ensure specificity of TMD–TMD interactions and avoidance of promiscuous ones. Known TMD–TMD interfaces from bitopic proteins often contain common interaction motifs, such as GxxxG,[27–32] polar amino acids including Asn, Gln, Asp, His,[33–35] or Trp,[19] or more complex patterns such as Ser/Thr-clusters[36] and QxxS-motifs.[37] Further, different residues and patterns can cooperate within the same TMD. For example, the GxxxG-motif can occur in tandem, like in the Alzheimer A4 protein,[38,39] be extended to an HxxxxxxGxxxG-motif in the BNIP TMD[32,40] and related sequences[41] or form FxxGxxxG-motifs.[42]

Further variation is found in the oligomerization outcome itself: transmembrane helices are capable of dimerization,[27] trimerization,[43,44] tetramerization,[45] or pentamerization.[46] Also, assembly can be homotypic or heterotypic,[14,47] certain TMDs can interact via alternate interfaces,[17] and some exhibit more than one interface in a complex, rendering it janus-headed.[48,49] The rich diversity of transmembrane helix association in terms of mechanisms and outcomes is in no doubt a consequence of their biological importance.

Like their water-soluble counterparts,[50] membrane proteins appear to exist in a hierarchy of conformational substates that cover different time scales and molecular dimensions. Rigid-body motions of individual TMDs relative to each other[51–53] are seen upon activation of bitopic and polytopic proteins and frequently underlie signal transduction after ligand binding or conformational changes leading to substrate transport. At more subtle levels, TM-helices may be subject to bending at hinge regions,[54] undergo small-scale vibrational motions of their backbones,[55–57] and exhibit side-chain rotations.[58] As the occupation of different substates, and the speed of structural transitions between them are intrinsically linked to function, membrane proteins must be stable enough to retain their structure, yet flexible enough to rapidly switch between inactive and active conformations.

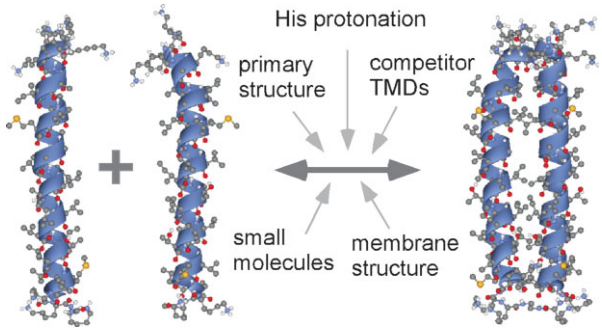


Figure 1. Factors that are known to regulate TMD–TMD interaction.

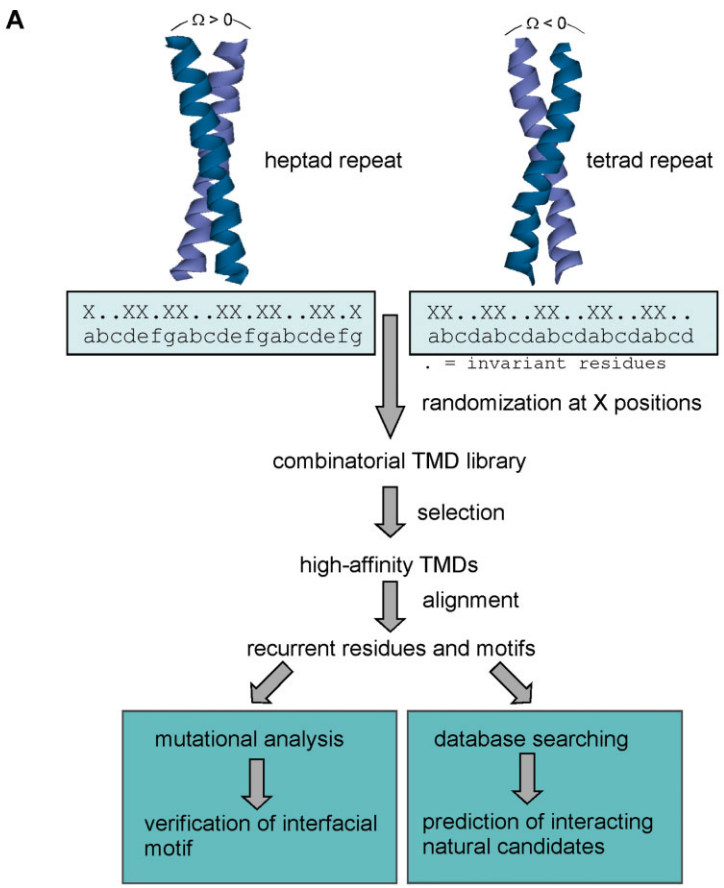
Here, we will discuss some recurrent patterns found in TMD–TMD interfaces, their energetics, the emerging concept of their dependence on sequence context, and regulation of interaction (see Fig. 1). We will then review computational approaches developed to predict these structures. Finally, we will end with a discussion on the functional relevance of the dynamics of TMD-helices and how other molecules may regulate their interactions and dynamics.

Structure of TMD–TMD Interfaces

To date, the structures of about 150 nonhomologous polytopic membrane proteins have been solved. In a simplified model, transmembrane helices cross each other either at positive crossing angles (where interfacial residues adopt an $[a..de.g]_n$ heptad repeat pattern) or at negative angles (characterized by an $[ab..]_n$ tetrad repeat)[5,29,59] [Fig. 2(A)]. Interestingly, a recent rigorous structural classification of TMD–TMD pairs from polytopic proteins revealed that about 2/3 of them fall into only four structural clusters, that is, antiparallel and parallel helices with a limited range of crossing angles that is dictated by the nature of side-chain interactions.[60] This suggests a limited conformation space for TMD–TMD pairs, as predicted based on geometrical considerations.[61] However, it has to be borne in mind that the remaining third of these pairs correspond to additional conformations with more varied crossing angles and irregularities in helix structures (mostly wide or tight helical turns that are often associated with kinks).[62] The same broad structural classification seems to hold true for TMD–TMD assemblies from bitopic proteins as indicated by high-resolution structures[30–32,63–66] and scanning mutagenesis.[40,67–71]

Energetics of TMD–TMD Interaction

Three different kinds of noncovalent interactions are thought to contribute toward stabilization of protein structure: ionic interactions, hydrogen bonds, and van der Waals interactions. Yet, one must remember that the anisotropic, hydrophobic environment of the lipid bilayer is dramatically distinct from the isotropic



B

interfacial motif:	variant aa	invariant aa	reference
pa..Ga..Ga..T	G, A, V, L, I, S, T, P, A	L	105
.[S]p..aG..aG..a	G, A, V, L, I, S, T, P, A	A	105
....F..G...G....	A, C, F, G, I, L, M, R, S, T, V, W	A	42
.[S]p..[S]p[H].....G...G	all natural residues	L	41
....SS..SS..T	A, T, S, F, V, L, I, P	L	36
W.....W.....W.	all natural residues	A	137

p = polar (S, T)
a = aliphatic (L, I, V)
s = small (G, A, S)
. = residues outside consensus motif

Figure 2. Approaches and outcomes in screening combinatorial libraries for high-affinity TMDs. (A) Outline of library construction and screening. (B) Recurrent motifs as identified from different libraries where different interfacial residue patterns had been randomized with different sets of amino acids on different invariant host backgrounds. Ω, helix/helix crossing angle; aa, amino acid. The presence of GxxxG motifs in high-affinity TMDs suggests that the corresponding helix–helix pairs have negative crossing angles, even though a heptad repeat pattern underlying left-handed pairs had been randomized. In other words, parts of the heptad pattern can potentially form interfaces of right-handed structures.

hydrophilic setting in which water-soluble proteins reside. Hence, one cannot readily extend our considerable knowledge regarding the forces that hold water-soluble proteins together into the membrane milieu. For example, hydrophobic collapse, a major driving force to water-soluble protein folding, most likely cannot play a major role in the stabilization of membrane proteins.

Could the opposite, that is, hydrophilic collapse, be the force that holds membrane proteins together? In this "reverse micelle" topology, the protein shields polar residues from the apolar membrane by sequestering them in its core. Subsequently, any electrostatic interaction (salt-bridges and H-bonds) that takes place between the shielded hydrophilic residues is predicted to be highly favorable because of the low dielectric environment of the lipid bilayer. Uncertainty exists whether a reverse micelle topology is statistically significant amongst known membrane protein structures, because polar residues are uncommon in

the core of membrane proteins.[72] Yet, as elaborated later, even in instances where polar residues are found in the core of membrane proteins, their contribution toward protein stability is debatable.[73] It is noteworthy, however, that polar substitutions are the most common mutations in membrane proteins that cause disease.[16]

Because salt-bridges are scarce in membrane proteins, researchers have attempted to gauge the importance of H-bonds toward stability. A consistent picture does not arise yet. For example, several studies have shown a substantial contribution by H-bonds formed by Asn residues on the dimerization of designed bitopic proteins[33,34,67,74] (further discussion on the participation of polar residues in oligomerization is presented later). In contrast, recent studies have shown only modest stabilization of a designed bitopic membrane-spanning leucine zipper[75] or of the polytopic bacteriorhodopsin[73] by side-chain H-bonds. Finally, the location of polar residues in membrane proteins has been used by several groups to rotationally position a transmembrane helix. This is based on the argument that the polar side chains will interact more favorably with the core of the protein, in comparison to the hydrophobic lipid bilayer.[76,77]

In addition to polar side chains, several groups proposed that the C_α—H group is capable of participating in hydrogen bonding.[78] In other words, the marginal polarity of the C_α proton might be sufficient to serve as an H-bond donor in a highly hydrophobic environment. However, experimental assessment of the strength of this H-bond did not converge to a single answer. Specifically, the effect upon stability of a single C—H···O=C bond in bacteriorhodopsin was estimated by mutagenesis in detergent micelles to be insignificant.[79] In contrast, FTIR spectroscopy in lipid bilayers was able to measure directly the enthalpy of a similar H-bond in glycophorin A to be 0.88 kcal/mol.[80]

Taken together, one is faced with a conundrum: On the one hand, it is possible to engineer H-bonds that contribute significantly toward transmembrane protein stability,[33,67,68,74,81] as expected from electrostatic arguments. Yet on the other hand, such electrostatic interactions do not seem to be prevalent in membrane proteins.

Several different reasons were brought forth[73,82] to account for the above conundrum: (i) strong electrostatic interactions may be deleterious to protein function because of their potential lack of specificity. (ii) Such strong interactions may hinder conformational flexibility that may be essential to protein function. (iii) Finally, precise geometric positioning may be required for optimal stabilization of H-bonds. Thus, electrostatics may not universally stabilize TMD–TMD interactions, but rather it will cooperate with van der Waals forces, aromatic interactions, and so forth, in ways that depend on the individual case as discussed in the sections later.

Modeling Integral Membrane Assembly

Helical membrane proteins are far more accessible to the protein modeler in comparison to their water-soluble counterparts. This is due to the fact that the lipid bilayers dramatically reduce the number of degrees of freedoms that a protein can adopt. Thus, one can attempt to exhaustively search the conformational space of a transmembrane helix bundle, whereas an exhaustive search of water-soluble configurations is at this point exceedingly difficult. Moreover, the topology of helical membrane proteins can normally be ascertained with some certainty by hydropathy analysis. In contrast, determining the topology of a water-soluble protein requires tedious structure elucidation. Thus, it should be of no surprise that approaches to model transmembrane helix–helix interactions are common in the community. Finally, a recent study has shown that knowledge-based modeling methods that have been developed for water-soluble proteins, work equally well on membrane proteins.[83] Thus, one can model membrane proteins with methods that have been developed for water-soluble proteins, as well as methods that are uniquely suited to model membrane helical bundles.

It is beyond the scope of this report to present a comprehensive review of membrane protein modeling. However, it might be instructive to mention one category of modeling methods that is unique to membrane proteins that relies on exhaustive searching, and has been shown to be particularly useful. Brunger and coworkers have pioneered an approach to model membrane proteins based on global-searching molecular dynamics.[84,85] In this method, a helical bundle is exhaustively permutated by iteratively rotating and tilting the helices relative to the bundle axis. The stability of each conformer is then tested by a short molecular dynamics trajectory in vacuum. Clustering of all the conformations is undertaken to determine if modeling of a particular region of structure space has been particularly successful. One can analyze symmetric homo-oligomers by adjusting the rotation of all the helices concomitantly or hetero-oligomers by combinatorial rotations. In practice, although it is possible to exhaustively search the conformation space of a homo-oligomer, it remains to be seen how effective this procedure is in the analysis of hetero-oligomers The aforementioned approach has been shown to be useful in predicting several plausible structures.[86–90] One then uses experimental data, such as mutagenesis, to select the "correct structure."[84,85] Alternatively, silent substitution modeling can be used to select the correct structure based on the premise that "silent mutations," as identified by sequence alignment, might be able to discriminate between the correct structures and outliers.[91]

More recently, the aforementioned approach has been extended to analyze different helical bundles by molecular dynamics simulation in hydrated lipid bilayers.[92] It remains to be seen if analyzing structures in membranes are preferable to a more exhaustive search in vacuum. Finally, Kruger and Fischer[93] have recently presented a similar approach of exhaustive geometric searching. In their approach, the authors first conducted molecular dynamics simulation in hydrated lipid bilayers of individual helices. Subsequently, the configuration space was searched exhaustively by assembling helical bundles and assessing the energetics of each bundle.

Recurrent Motifs in High-Affinity TMD–TMD Interfaces

The TMD–TMD interface of the homodimeric glycophorin A, an erythrocyte protein of unknown function, has been the first to be investigated in detail.[27,28,94–97] Its structure gives rise to a negative crossing angle as implied by molecular modeling[85,98] and confirmed by nuclear magnetic resonance studies in detergent[30] and membranes.[31] This interaction is dominated by a GxxxG motif that is central to the TMD–TMD interface.[27,28,99–103] The glycophorin A TMD–TMD interaction is apparently driven by a complex mixture of attractive forces and entropic factors.[104] The GxxxG motif may drive assembly by the formation of a flat helix surface that allows for multiple van der Waals interactions to form. In addition, the entropy loss upon association is considered minimal for Gly and the neighboring Val residues.[105] Moreover, the Gly residues reduce the distance between the helix axes and thus may facilitate hydrogen bond formation between their C_α-hydrogens and the backbone carbonyl of the partner helix.[106] The GxxxG motif was identified as such when the residue spacing between both Gly residues was found to be critical and GxxxG induced homomerization of model TMDs.[100]

The early work on glycophorin A TMD assembly was particularly rewarding because the GxxxG motif and degenerate versions thereof (designated "small-xxxsmall" or "GxxxG-like" with Gly exchanged for Ala, Ser, Cys, etc.) were found in many other cases later, including syndecans,[70,107] members of the BNIP family,[32,40,108] protein tyrosine phosphatases,[109] viral envelope proteins,[110] growth factor receptors,[64,111] integrins,[14,112–115] and the Alzheimer precursor protein.[38,39,116] These motifs are also important in helix–helix interfaces of polytopic proteins that prefer Gly, Ala, and Ser.[60,117–121] It is clear, however, that numerous TMDs interact without involvement of the GxxxG motif, as exemplified by SNARE proteins,[69,122–124] E-cadherin,[125] the erythropoietin receptor,[68,126–128] cytochromes,[129] and many others.

The mechanisms underlying TMD–TMD interactions are explored in a systematic *ab initio* approach by selection of self-interacting TMDs from combinatorial libraries holding randomized TMD sequences.[105,130] Selection of high-affinity TMDs requires an experimental system where their interaction results in a selectable phenotype. The ToxR transcription activator system has been developed for this purpose[28] and exploits the fact that self-interaction of ToxR-embedded TMDs within the inner membrane of expressing *E. coli* reporter strains enhances the expression of chloramphenicol resistance. The ToxR system exists in two versions used for library screening for homotypic interactions, TOXCAT[96] and POSSYCCAT.[130] It has been modified to investigate heterotypic interactions in a dominant-negative fashion.[41,131,132] The beauty of the library screening approach is that interfacial consensus motifs can be identified *ab initio* by alignment of selected sequences and comparison to unselected ones. These motifs can be characterized in detail by mutational analysis to uncover the structural basis of helix–helix interaction. Moreover, database searching with these motifs leads to testable predictions of similar motifs in natural membrane proteins. The outcome of individual screens depends on whether tetrad or heptad motifs are randomized, on the hydrophobicity of invariant amino acids, and on the complement of codons used for the variant ones. Figure 2(A) illustrates the general strategy, whereas Figure 2(B) summarizes the results obtained so far.

In one such study, randomization of a tetrad repeat pattern yielded high-affinity GxxxG motifs in over 80% of all isolates,[105] thus underpinning the role of this motif in TMD–TMD interactions. Indeed, database searching identified the GxxxG motif as the most prevalent pairwise motif in TMDs.[42,133,134] Overrepresentation of GxxxG relative to statistical expectation demonstrates that its presence supports protein function in evolution.

If randomization is done without codons that encode Gly, SxxSSxxT and SxxxSSxxT motifs emerged. Mutational analysis of these motifs indicated that their contribution to TMD–TMD interaction is based on multiple hydrogen bonds between these hydroxylated residues.[36]

In other library screens, GxxxG of high-affinity TMDs were frequently associated with Phe or His, depending on the randomization strategy. Phe was frequently associated with GxxxG motifs and has a strongly stabilizing role at the i-3 position, thus yielding FxxGxxxG motifs. This motif, and a number of derivatives with different Phe/Gly spacings, is overrepresented in TMDs of natural bitopic membrane proteins suggesting their functional relevance.[42] One candidate TMD identified by the database search corresponds to the Vesicular Stomatitis Virus G-protein, and mutational analysis confirmed the relevance of the consensus motif for its self-interaction.[42] Interaction of this TMD has also been demonstrated by mass spectrometry to persist in the gas phase if the corresponding peptide is ionized from the α-helical

state.[135] The role of the GxxxG part of the FxxGxxxG motif might be to orient the Phe residues such as to promote aromatic–aromatic interactions. Alternatively, the first Gly of GxxxG could interact with the Phe residue of the partner helix via a C_α—H···π interaction known to be prevalent in soluble protein cores.[136] Albeit weak, these C_α—H···π interactions could be stabilized by the low dielectric environment of membranes as discussed earlier.

Enrichment of His residues was seen in another library screen that yielded high-affinity TMDs preferentially displaying Gly, Ser, and/or Thr residues at positions i-4 and i-1 relative to His. The sequences with the highest affinities also contained a C-terminal GxxxG, which results in a [G/S/T]xx[G/S/T]HxxxxxxGxxxG consensus pattern.[41] Mutational analyses confirmed the importance of these residues in homotypic interaction. Probing heterotypic interactions indicated that His residues interact in *trans* with hydroxylated residues suggesting that hydrogen bonds and possibly aromatic interactions stabilize the interface. Reconstruction of minimal interaction motifs on an oligo-Leu sequence supported the idea that His is part of a hydrogen-bonded cluster that may be brought into register by a distant GxxxG,[41] whereas isolated His residues support the assembly of the model TMD much less efficiently.[34] This exemplifies one case where precise geometric positioning may be required for optimal stabilization of hydrogen bonds. Database searching yielded only few candidate TMDs holding this motif one of which corresponds to the previously well-investigated BNIP3 TMD. BNIP3 is a Bcl-2 family proapoptotic protein that initiates hypoxia-induced cell death. The BNIP3 TMD forms a homodimer characterized by the motif SHxxAxxxGxxxG[40,108] and its NMR structure confirmed these interfacial residues in the right-handed pair.[32] The BNIP3 TMD–TMD interface thus corresponds to one variant of the consensus motif identified in a library screen.

Yet another library screen yielded TMDs where Trp residues prevail at *g* positions of the randomized heptad motif. Mutation of Trp residues reduced self-interaction and grafting Trp residues onto artificial TMDs strongly enhanced their affinity.[137] A contribution of aromatic residues is also implied by the overabundance of WxxW and YxxY motifs in bacterial TMDs and mutational analysis of one candidate TMD that belongs to the cholera toxin secretion protein EpsM confirmed that WxxW, YxxW, WxxY, YxxY, and single Trp residues support its self-interaction.[138] A stabilizing role of aromatic–aromatic interactions in the order F > Y ≈ W was seen in a model study that also suggested that cation-π interactions between aromatics and Arg, Lys, or His residues strongly enhance TMD–TMD affinity.[139] Apart from stabilizing noncovalent TMD assembly, aromatic side-chains also contribute to the folding stability of membrane proteins through interactions with the lipid head-group regions, as exemplified by the *E. coli* outer membrane protein OmpA.[140]

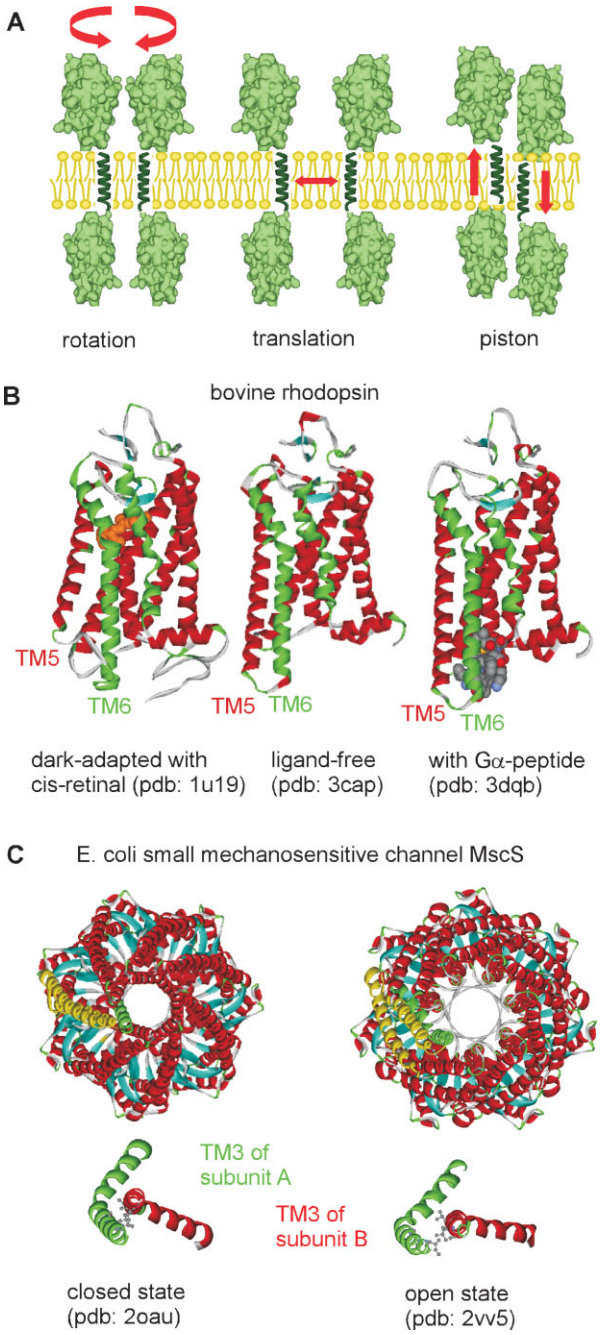
Context Dependence of Interfacial Motifs and the Evolution of Membrane Protein Function

Despite the observation that simple motifs, such as GxxxG, are part of many TMD–TMD interfaces, it is clear that the mere presence of such motifs does not reliably predict high-affinity interaction. This is exemplified by the fact that GxxxG is highly effective within the contexts of oligo-Met and oligo-Val sequences,[100] but not within a number of randomized TMDs[42] or the M13 major coat protein TMD.[141] Screening combinatorial TMD libraries for high-affinity sequences yielded GxxxG motifs whose relative positions and nearest neighbors depended on whether invariant Leu or Ala residues were used.[105] Also, the interaction energy of the glycophorin A TMD varies over a wide range after mutation of the sequence surrounding GxxxG.[103] Similarly, GxxxG present within the erbB2 receptor TMD lies outside the closely packed part of the helix–helix interface.[64] It thus appears as if the precise packing within a given interface modulates the role of GxxxG. This is not too surprising given the fact that 12.5% of all TMDs within nonredundant databases of bitopic proteins contain GxxxG.[42,134] To avoid promiscuous homo- and heterotypic interactions between these TMDs, GxxxG motifs have to be placed within appropriate structural contexts where they may enter long-range communication with other residues[142] and high-affinity TMDs holding GxxxG may be regarded as islands in sequence space. Screening combinatorial TMD libraries has identified some of these islands by showing that GxxxG can form high-affinity interfaces with appropriately spaced Phe[42] or clusters of His and polar/small residues[41] as mentioned earlier.

Similar arguments apply to polar residues (Asn, Asp, Gln, Glu, His) that drive association of model TMDs.[34,35,67,143] Because ~25% of all bitopic protein TMDs contain at least one of these residue types, their existence would be expected to induce unspecific assembly in the membrane.[144] That this danger is a real one, is highlighted by a number of disease-causing mutations where polar residues may constitutively activate function by causing TMD–TMD interaction. For example, the *neu* tyrosine kinase receptor is activated by a substitution of V664 within its TMD for Glu[145] that appears to induce permanent receptor dimerization by interhelical hydrogen-bond formation.[146] Likewise, mutating S498 of the thrombopoietin receptor TMD to Asn rendered this receptor constitutively active,[147] and mutation of T617 to Asn within the granulocyte colony-stimulating factor receptor TMD, as found in patients with acute myeloid leukemia, conferred growth factor independence[148] (for

more complete lists of disease-causing TMD mutations, see Refs. 16 and 149). On the other hand, polar residues did not induce TMD–TMD interaction in a number of other natural proteins.[144,150]

Taken together, it appears that evolution of TMD–TMD interfaces, which are associated with beneficial gains of protein function, depends on coevolution of critical sequence hot spots, such as GxxxG or polar residues, and of surrounding residues. This context dependence may ensure that high-affinity interfaces evolve much slower than anticipated on the mere basis of the codon statistics of the residues that make up the hot spots.



Rigid-Body Motions of TMDs in Dynamic Membrane Proteins

Apart from stable TMD–TMD interactions, as in protein oligomerization, many TMDs enter dynamic associations that are subject to regulation by ligands, other proteins, or the membrane proper. In bitopic proteins, TMDs may interact reversibly by translational movement within the bilayer plane, rotate relative to each other, or undergo even piston motions [Fig. 3(A)]. These motions frequently transmit a ligand-binding event at extracellular domains across the bilayer to intracellular domains, thus activating a variety of signaling cascades (reviewed in Refs. 16 and 17). A few model proteins will be discussed here to illustrate the point.

Reversible interactions involving translational movement are proposed to regulate the adhesive function of integrins.[154,155] There, heterotypic TMD–TMD interactions between a set of α and β subunits—as implied by biochemical and computational studies[86,89,112,156] as well as by a recent NMR structure—[157] are displaced in favor of homotypic interaction[71] during activation.[158] It is the reversibility of TMD–TMD interactions that make integrin function controllable by the addition of exogenous TMD peptides. In an elegant combination of computational and experimental approaches, novel TMD peptides were designed that compete with integrin heteromerization in a sequence-specific way, and thus activate the protein.[132,159] Similar approaches have been developed for other membrane proteins (reviewed in Refs. 14 and 16).

Rotation of TMDs relative to each other is a concept that appears to supersede the more traditional idea of ligand-induced dimerization of growth factor receptors. There is now substantial evidence that these receptors can exist as preformed dimers that are

Figure 3. Dynamics of membrane-embedded protein domains. (A) The activation of bitopic proteins upon binding of soluble ligands to extracellular domains has been proposed to involve the reorientation of transmembrane helices relative to each other about their long axis, reversible association/dissociation, and piston movements. (B) A comparison of X-ray structures of bovine rhodopsin and opsin reveals that TM5 elongates and moves closer to TM6 in the ligand-free and Gα-peptide (shown as space-filling representation) associated states when compared with the dark-adapted form containing cis–retinal (shown in orange).[51,151,152] TM6 and TM7 are shown in green for better orientation. (C) A comparison of closed and open states of the small mechanosensitive channel MscS from *E. coli* indicates a large rearrangement of TMDs upon channel activation.[153] The representations of the full structures permit a view down the pore; TM1 and TM2 of subunit A are in yellow and TM3 is in green for better orientation. The blow-up underneath the full structures shows the rearrangement of TM3 from subunits A and B after channel activation. Interfacial residues are in gray.

stabilized by TMD–TMD interactions. Receptor activation seems to involve TMD rotation in response to ligand-binding to extracellular domains, in case of erythropoietin,[160] epidermal growth factor,[161] and growth hormone[162] receptors. Interestingly, the arrangement of TMDs can also be influenced by direct binding of hydrophobic ligands. For example, the thrombopoietin receptor was activated by a synthetic compound that required a TMD His residue.[163,164] Also, modeling studies suggest that the TMD of the erbB2 tyrosine kinase is able to rotate between two dimerization motifs, thereby controlling the activity of the protein.[165]

Changing the electrostatics between TMDs is an another way to change their relative orientation. The homotetrameric M2 protein from influenza A forms a proton channel that is blocked by the anti-viral drug amantadine[166] and is activated by lowering the pH. Its TM-helices cross each other at positive angles as indicated by earlier functional,[167] biochemical,[168] and modeling[169] work. Recently, high-resolution structures of M2 have been solved,[65,66] and molecular modeling[170] suggests that His protonation promotes channel gating although how exactly a pH change opens the pore remains unknown. Recently, linear and 2D-IR spectroscopic studies have provided evidence that is consistent with a rotation of the helices about their long axis upon pH change.[171] This rotational change is on the order of one amino acid register and may provide a molecular picture of channel gating.

Activation of polytopic membrane proteins frequently involves rearrangement of TMDs within multihelical bundles, which we illustrate here by discussing some recent findings. For example, G-protein coupled receptors (GPCRs) exist in equilibrium between antagonist-bound inactive and agonist-bound active states, where diffusible ligands bind within the TM-helix bundle. Recent work has produced a number of GPCR structures that mostly represent the inactive conformation, as exemplified by adrenergic receptors crystallized in the antagonist-bound state.[81,172–174] New structures of the retinal pigment rhodopsin provide structural information on the kind of conformational changes associated with protein activation [Fig. 3(B)]. Although earlier rhodopsin structures[151] contain cis–retinal and thus represent the inactive, dark-adapted form, new structures were obtained in the ligand-free state[152] or bound to a peptide from the G-protein transducin.[51] The new structures are distinguished from the retinal-bound one, in that TM6 is tilted outward, whereas the TM5 helix is more elongated and close to TM6. Apparently, binding of the transducin peptide has no structural effect above the one seen upon removal of retinal. These changes may also occur upon rhodopsin activation and it is likely that active and inactive states of other GPCRs also correspond to alternate arrangements of the TM-helix bundle. Depending on whether antagonists or agonists are bound within the bundle, the spatial arrangement the TMDs is thought to switch from inactive to active.[175,176]

Apart from ligand-binding, conformational changes can be subject to modulation by the surrounding bilayer tension. Recent crystallographic[153] and spectroscopic[177] results indicate large-scale movement of TMDs of the prokaryotic small mechanosensitive MscS channel upon activation [Fig. 3(B)]. A close inspection of the interface between the pore-forming TM3 helix [blow-up in Fig. 3(B)] reveals that a Gly-rich helix surface connects to an Ala-rich surface. Upon activation, the relative orientation of the TM3 helices changes, yet the interface is largely maintained. Thus, the accumulation of small residues within this interface may render it stable, yet flexible enough for reversible channel activation.

In addition to these examples, rearrangements of TM-helices have also been seen after activation of transporters[178,179] and voltage-dependent[180] or ligand-gated[181] channels.

The examples cited here nicely illustrate how TMD arrangements can be regulated by ligand-binding, competitor TMDs, His protonation, and membrane tension. The assembly of many bitopic and polytopic membrane proteins is also regulated by lipid composition and bilayer width as documented by a wealth of data; this topic is discussed in other excellent reviews.[12,19,182–186]

Backbone Dynamics of Transmembrane Helices

Apart from rigid-body motions, TM-helix backbones experience local and transient unfolding reactions, or conformational fluctuations, which still is a largely unexplored area in membrane protein research.[19] One way to monitor these fluctuations is hydrogen/deuterium-exchange experiments where transient openings of amide hydrogen bonds give rise to successive exchange of hydrogens for deuterium (HDX) or vice versa (DHX), depending on the experimental design. Membrane-embedded protein domains are not readily accessible to the catalytically active hydroxyl ions and thus undergo only limited exchange depending on the protein and on experimental conditions. For example, only little exchange was reported for the fd coat protein TMD in detergent micelles[187] and the EmrE multidrug transporter in a membrane,[188] whereas significant numbers of amides exchanged with the HIV-1 virus Vpu protein TMD,[189,190] the phospholemman TMD,[191] the SliK potassium channel,[192] and WALP model helices.[193,194] In a few cases, the majority of amides exchanged even in the membrane-embedded state as exemplified by the influenza hemagglutinin TMD[195] and lactose permease.[192] The latter finding is in line with the known conformational flexibility and solvent access via the transport pore of this polytopic transporter.[196] That pore-forming proteins undergo much more efficient HDX is supported by the results

obtained with the membrane-spanning influenza M2 TMD.[197] There, complete exchange was seen and exchanges were located to one face of the helix that represents the water-exposed part of a flexible channel-forming helix.[198]

Although HDX or DHX experiments do not report the kinetics of individual unfolding events, infrared spectroscopy has recently been able to probe the dynamics of a membrane helical bundle in time regimes that have been unapproachable by other methods.[55,199] Using a series of 1-$^{13}C{=}^{18}O$ labeled TM peptides, 2D-IR spectroscopy was able to measure the homogeneous and inhomogeneous linewidths and to correlate these with peptide structure. Although the homogeneous linewidths were insensitive to the label position, the inhomogeneous ones varied as a function of the labeled site. For example, residues in contact with the aqueous environment exhibited larger inhomogeneous linewidths relative to residues that are in the protein–lipid interface. Taken together, this study presented the first one of the picosecond dynamics of a membrane protein.[199]

Little is currently known about the potential biological significance of these vibrational TMD motions. It seems clear, however, that a functional role of helix dynamics can only be subject to evolutionary fine-tuning, if it depends on primary structure. None of the aforementioned studies has addressed the sequence dependence of TMD backbone dynamics in a systematic manner. So, how can TMD sequence influence backbone dynamics? The stability of an α-helix mainly depends on burial of hydrophobic surface upon folding, on van der Waals contacts between side-chain atoms, and on the extent of side-chain entropy loss upon folding (reviewed in Ref. 200). In soluble proteins, Leu and Ala residues rank among the best helix-promoters, whereas Ile, Val, Gly, and Pro destabilize helices (reviewed in ref. 201). Although the destabilizing effect of Gly is attributed to its inability to enter side-chain/side-chain interactions and to a strong main-chain entropy loss upon folding, Pro cannot form a hydrogen bond to its i-4 residue and its side-chain clashes with the side-chain of the preceding residue. The mechanism of helix destabilization by Val and Ile is discussed more controversially. The buried hydrophobic areas of Leu, Ile, and Val in a host helix correlate well with their impact on its stability,[202,203] suggesting a stabilizing role of the hydrophobic effect and/or van der Waals interactions. On the other hand, experimental helix-forming tendencies of Leu, Ile, and Val also correlated well with the entropy loss upon helix formation.[204,205] Although these issues have not been investigated intensively in transmembrane helices, there is mounting evidence that Ile and Val also enhance the local TM-helix backbone dynamics although if they did not globally destabilize certain hydrophobic guest helices.[206,207] A functional advantage of Ile/Val accumulation was originally suggested by their overrepresentation within the TMDs of soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptors (SNAREs)[208] and fusogenic viral envelope proteins.[209] Indeed, the TMDs of various fusogenic proteins have been shown to support outer leaflet mixing of docked membranes.[210–213] In line with this, synthetic peptides harboring the hydrophobic cores of SNARE TMDs drive liposome–liposome fusion *in vitro*,[208,214] and thus mimic basic aspects of the fusogenic function of full-length SNAREs.[215,216] The dynamics of SNARE TMD helices was studied by recording DHX kinetics in isotropic solution, where all amide deuteriums are exposed to solvent. This revealed subpopulations of amide deuteriums within each peptide that exchanged with rate constants in a way that depended on sequence,[56] as did fusogenicity.[208] Thus, backbone dynamics appears to be connected to fusogenic function. Inspired by the apparent connection of Ile/Val-content, fusogenicity, and flexibility of SNARE TMDs, a set of low-complexity membrane-fusogenic TMD-peptides, termed LV-peptides, was designed *de novo*. The hydrophobic core sequences of LV-peptides are composed of helix-promoting Leu and helix-destabilizing Val residues at different ratios, as well as Gly and Pro residues in some variants.[217] The fusogenicity of LV-peptides indeed increases with an increasing Val/Leu ratio[217] and also requires charged terminal residues.[218] LV-peptides exhibit sequence-specific exchange rates that correlate with fusogenicity, thus corroborating the link between backbone dynamics and lipid mixing. Further, LV-peptides having Val residues concentrated at peripheral or central domains of the hydrophobic core, respectively, suggest that dynamic domains close to the helix termini are more relevant for fusogenicity than central domains.[57] The local dynamics of the helix backbone may therefore affect the structure of the surrounding lipid bilayer and thus contribute to initiation of membrane fusion, which is consistent with the membrane-perturbing activity of an LV-peptide.[219] Further, completion of fusion by inner leaflet mixing appears to depend on SNARE TMD–TMD interaction,[214] which illustrates how different structural features of TMDs may influence the functional interaction of integral membrane proteins with the surrounding lipid bilayer.

In conclusion, as in any scientific endeavor, investigating something seemingly as simple and clearcut a subject, as the structure of transmembrane helices, turns out to resemble looking through a microscope while switching to lenses of ever higher magnification.

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