Folding and Stability of α-Helical Integral Membrane Proteins

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Contents

1. Introduction

Integral membrane proteins are embedded in a water-solvated lipid bilayer that presents contrasting features in an anisotropic, chemically heterogeneous environment. A transmembrane protein interacts with the aqueous solvent, with an interfacial membrane region that contains a variety of polar, zwitterionic, or charged lipid headgroups, and with a central, hydrophobic membrane core ∼20 Å thick composed primarily of hydrocarbon tails.[1] This chemical complexity of native membranes makes identifying the factors that determine the folding and stability of membrane proteins quite daunting from both conceptual and experimental considerations. The instability of membrane proteins when subjected to detergent solubilization and the dependence of membrane protein stability and function on specific amphiphilic environments has severely hampered overexpression and purification efforts, and this bottleneck has restricted progress in biophysical and biochemical studies of membrane proteins. Nevertheless, the significant advances being made in the field of membrane protein folding, together with the healthy and exciting rate at which new membrane protein structures are being determined, show that these systems are beginning to yield under the combined pressure of a variety of approaches.

What are the sequence and structural determinants of the stability of membrane proteins? What are the folding pathways and kinetic barriers for the insertion of polypeptides into membranes? Complete answers to these questions may be as complicated, diverse, and subtle as membrane composition itself, but the current state of knowledge of membrane protein folding and stability suggests that some general principles and broad themes are already available. Many reviews on this subject have been presented.[2−7] Here, I review the results of genetic, cell biological, biochemical, and biophysical studies of α-helical integral membrane protein folding and stability with an emphasis on connecting the results from different types of experiments in the context of the current thermodynamic formalisms. Studies of lytic peptides (reviewed in refs 8−10) and membrane fusion (reviewed in refs 11−17) have been largely excluded, studies of β-barrel membrane proteins (reviewed in refs 18 and 19)

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Kevin R. MacKenzie was born in Ithaca, NY, and grew up in Montreal, Canada. He received a B.Sc. in Biochemistry from McGill University and a Ph.D. in Molecular Biophysics and Biochemistry from Yale University in the laboratory of Donald Engelman. After postdoctoral work at Yale, he joined the Department of Biochemistry and Cell Biology at Rice University, where his laboratory has two main research interests: the structure and specificity of transmembrane helix–helix interactions and the structural basis for calcium signaling.

have been excluded except where comparison or contrast provides compelling insights into α-helical proteins not otherwise available, and studies that are of a primarily computational or bioinformatics nature (reviewed in refs 20−22) have been excluded except when they directly address other data essential to the arguments presented. These omissions, and errors of omission of a less systematic nature, are the consequence of the structure of this review and the limitations of the perspective of the author.

2. Conceptual Framework

2.1. The Two-Stage Model: A Useful Simplification for α-Helical Membrane Proteins

Understanding membrane protein function, stability, and folding has been hindered by a paucity of structural information for membrane proteins that is only slowly being alleviated,[23] but even before the sequence (let alone structure) of a membrane protein was known, amino acid analysis revealed the differences in overall polarity of soluble and membrane proteins.[24,25] The importance of the hydrophobic effect in the organization and stability of biological material[26,27] suggests that the folding of membrane proteins may differ considerably from that of soluble proteins and that even limited amounts of structural information about membrane proteins and lipid bilayers could reveal factors that influence the stability of membrane proteins.[28] In 1990, when the atomic structures of two bacterial reaction centers[29−33] and the near-atomic resolution structure of bacteriorhodopsin[34] were known, along with diffraction based structures for the gel phase[35,36] but not yet the fluid phase[1] of phosphatidylcholine membranes, Popot and Engelman presented a "two-stage model"[37] for the folding of the most abundant class of membrane proteins, those that span the bilayer as α-helices. Based on refolding and reassociation experiments with bacteriorhodopsin[38−43] and the predicted enhancement of hydrogen bonding interactions in the hydrophobic interior of a lipid bilayer, they hypothesized that "the final structure in the transmembrane region results from the accretion of smaller elements (helices), each of which has reached thermodynamic equilibrium with the lipid and aqueous phases before packing".[37] This hypothesis, which forms the central tenet of the two-stage model for membrane protein folding, is represented schematically in Figure 1.

Popot and Engelman based their idea on estimates of the behavior of the type of peptide known to span membranes

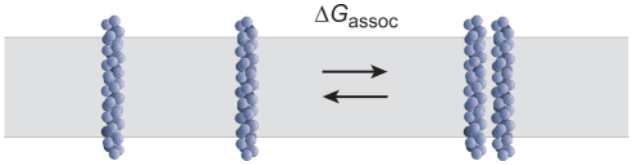

Figure 1. The two-stage model for α-helical integral membrane protein folding proposed by Popot and Engelman.[37] Stretches of predominantly hydrophobic amino acids exist as independently stable α-helical transmembrane spans that may associate laterally within the membrane to form bundles of helices. The free energy of association may be influenced by loops between the membrane spans, by packing, hydrogen bonding, or charge-pair interactions between the helices, by the binding of ligands or prosthetic groups, by lipid−protein or lipid−lipid interactions, and by the properties of the bilayer. Adapted with permission from ref 37. Copyright 1990 American Chemical Society.

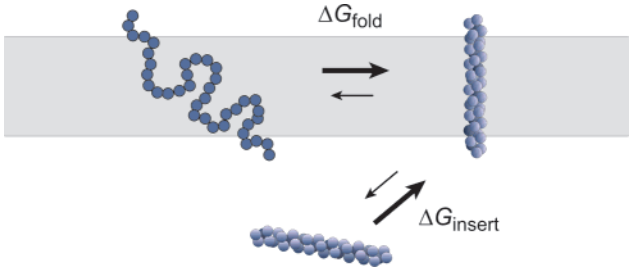

Figure 2. The transbilayer hydrophobic helix as a folding domain. The helix can neither unfold within the membrane, due to the cost of breaking hydrogen bonds in an apolar environment, nor leave the membrane, due to the hydrophobic effect. Adapted with permission from ref 37. Copyright 1990 American Chemical Society.

as helices: stretches of about 20 amino acids with predominantly hydrophobic side chains.[44−49] They considered three possible fates for such a peptide arranged as a transbilayer helix (see Figures 1 and 2): the α-helix solvated in lipids might leave the membrane to become an α-helix solvated in water, it might unfold within the bilayer to a random coil solvated in lipids, or the transbilayer α-helix might associate with other α-helices. The first two scenarios would involve unacceptably large thermodynamic penalties: removal of the helix from the membrane would be opposed by the energetic cost of transferring hydrophobic side chains from lipid to water, whereas unfolding into a random coil within the bilayer would be opposed by the high energetic cost of breaking backbone hydrogen bonds in a water-poor, low-dielectric environment. Accordingly, the helix can neither leave the membrane nor unfold within it, and the formation of a transmembrane helix can be thought of as essentially irreversible. Thus, Popot and Engelman reasoned, most hydrophobic spans could probably be thought of as α-helices, or associations thereof, once they were inserted into a membrane.

This scheme deliberately ignores the complexity of just how the protein might become inserted into the membrane and neglects any other potentially accessible states for the peptide (such as lying in the interfacial region of the bilayer), but the result of this major simplification is that individual transmembrane helices can be thought of as *independently stable domains*. While allowing that "a number of other circumstances can be imagined, such as the stabilization of an otherwise unstable helix or extended segment through a hairpin link to a stable helix",[37] the authors suggested that for the majority of cases the straightforward expectation of independently stable helices might hold and that these helices

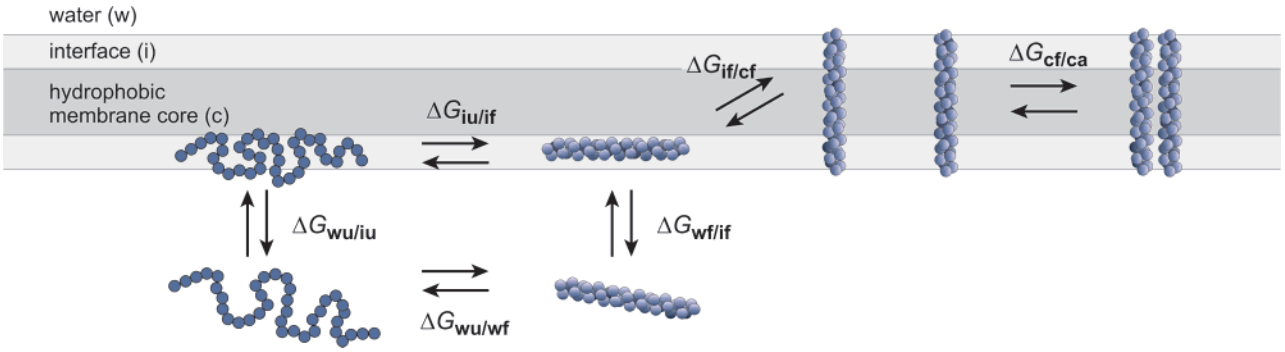

Figure 3. The interface-focused thermodynamic framework for membrane protein folding proposed by White and Wimley.[2,55] A polypeptide chain can partition among three distinct environments: water (w), the membrane interfacial region (i), or the membrane hydrophobic core (c). In these environments, the chain may either lack regular secondary structure and be unfolded (u) or be folded (f) such that it achieves a hydrogen bonded secondary structure (represented here as an α-helix). A peptide that is in a transbilayer configuration may associate (a) with other membrane spans. The free energy change of a peptide going from one state to another is identified using subscripts for the conformational or oligomeric state of the peptide as well as the environment in which each species is located. Adapted with permission from ref 2. Copyright 1999 by Annual Reviews.

would be capable of interacting with one another through lateral associations within the bilayer.

Experimental support for the two-stage model (at the time) included the observations that lateral association of protein fragments could regenerate functional bacteriorhodopsin,[38,40,41] that these fragments could form a native two-dimensional crystalline bacteriorhodopsin purple membrane lattice,[42] and that this refolding could occur for fragments that had been first separately reconstituted into lipid bilayers and then mixed.[43] More examples of "split" integral membrane proteins that reassemble functionally are discussed in section 4.5, but perhaps the broadest base of support for these ideas is that the net hydrophobicity of a stretch of residues in a polypeptide chain is a good predictor of transmembrane domains[44−49] even though in polytopic helical bundle proteins the transmembrane helices make extensive contacts with one another as well as with lipids. Despite (or more likely because of) its simplicity, the two-stage model and its hypothesis of independently stable helices have had a strong influence on membrane protein folding research.

The two-stage model focuses attention on how protein sequence and lipid composition might modulate the lateral interactions between transmembrane α-helices. However, the thermodynamic arguments set up by the model eliminate both the hydrophobic effect and the formation of α-helical hydrogen bonds as factors that can stabilize helical bundles relative to lipid-solvated helices, since these energy terms are already accounted for in the formation of the independently stable transmembrane helices. Thinking about the stability of membrane proteins in terms of the two-stage model allows one to recast the question "How do membrane proteins fold?" as the separable questions "What are the sequence determinants and lipid requirements for the formation of stable transbilayer helices?" and "What aspects of protein sequence and lipid composition drive or inhibit interactions between transmembrane helices?" Asking and answering these questions has generated a great deal of information about the sequence and structural and lipidic determinants of the stability of membrane proteins, which has been recently reviewed in several contexts.[5,7,50−54]

2.2. The Membrane Interfacial Region: Hydrophobicity Meets Hydrogen Bonding

Although the two-stage model provides a tidy conceptual framework within which to consider many important aspects of membrane protein folding, its focus is rather narrow. A broader perspective is required when the question "How do membrane proteins fold?" is rephrased as "How does the sequence of diphtheria toxin enable it to convert from a soluble protein to membrane spanning helices?" In going from a soluble state to a transmembrane configuration, a protein will pass through the membrane interface and may well adopt different conformations, either as transiently populated intermediates along the folding pathway or as species that can be isolated under certain experimental conditions; consideration of only the transmembrane helical states of the two-stage model clearly will not suffice. White and Wimley[2,55] suggested using a thermodynamic cycle that included folded and unfolded states both in water and at the membrane interface, in addition to inserted helices and assembled bundles, to analyze experimental thermodynamic data for the partitioning, folding, insertion, and assembly of polypeptides into transmembrane α-helical bundles (Figure 3). They took a boot-strapping approach, starting with thermodynamic measurements in small peptides, to quantifying the influence of the membrane/water interfacial region of the bilayer upon protein conformation and have developed a strong argument that this approach can provide insights into membrane protein folding.[2,55]

For a set of hydrophobic tripeptides (Ala-X-Ala-*O*-*tert*-butyl), Jacobs and White found that the calculated hydrophobicity[56] correlated well with their measurements of the free energy of peptide partitioning into dimyristoyl phosphatidylcholine bilayers.[57−59] However, the dependence of partitioning on sequence varied only about half as much as the calculated free energy change from hydrophobicity. The tryptophan version of this peptide, which interacted with membranes most tightly, was located predominantly at the membrane interfacial region between the aqueous phase and the hydrocarbon phase.[59] Since these short tripeptides did not self-associate or aggregate in either water or the bilayer interface, they are a model for an extended chain that is not forming backbone hydrogen bonded secondary structure. Accordingly, the free energy values from these peptide partitioning experiments represent a measure of $\Delta G_{wu/iu}$ (the free energy change for transferring an unfolded peptide from water to the membrane interface; see Figure 3) for the different guest amino acids tested.

Notwithstanding the behavior of these short peptides, the capacity to form secondary structure may be expected to

strongly influence the association of hydrophobic peptides with membranes. When Li and Deber measured the helicity of a series of model membrane peptides containing different uncharged amino acids, they found that the rank order of helix formation in detergent micelles and in lipid bilayers correlated with side chain hydrophobicity and depended upon the hydropathy of the peptide sequence under consideration.[60−62] In particular, residues that had been shown to destabilize α-helices in soluble proteins[63] (such as glycine or isoleucine) actually supported helix formation in membrane-like environments as readily as their close homologues (alanine or leucine, respectively). These results provided strong evidence that the partitioning of hydrophobic peptides into membrane environments is coupled to helix formation and hydrogen bonding.

This interesting observation highlights a distinct problem: experimental partitioning may correspond to more than one step in Figure 3, so the free energy changes resulting from altering amino acid sequence or lipid composition might therefore be attributed to any of the steps along the thermodynamic cycle. White and colleagues addressed this formal possibility by undertaking a systematic study to quantify the per-residue partitioning free energy of unfolded peptides from water to the interface, $\Delta G_{wu/iu}$. They measured partitioning from water to octanol[64] and from water to the membrane interfacial region of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers[65] for model di- to hexapeptides and a host−guest pentapeptide series; analysis of these data yielded whole-residue transfer free energy scales. These peptides were designed and analyzed to ensure that the species under study correspond to aqueous and interfacial *unfolded* peptides, and thus the scales represent exclusively partitioning and not folding ($\Delta G_{wu/iu}$ and not $\Delta G_{iu/if}$).[65] The measured transfer free energies from water to large unilamellar vesicles of POPC are about half the magnitude observed for transfer to octanol, suggesting that partitioning into the membrane interface is driven by the hydrophobic effect but not as strongly as would be expected for transfer to an entirely apolar environment. By analysis of the length series and host−guest series, the authors calculated the effective cost of partitioning the peptide bond into the interfacial region of a membrane as 1.2 ± 0.1 kcal mol^{-1}, or about as much as transferring a charged side chain. This experimental value agrees remarkably well with an estimate from electrostatics computations[66] and with a value determined empirically for optimal identification of transmembrane domains from protein sequences.[67] The transfer free energies from water to POPC[65] correspond to a whole-residue scale for transferring an unfolded peptide from water to the bilayer interface ($\Delta G_{wu/iu}$ in Figure 3). Because the portion of this transfer free energy that corresponds to the cost of transferring the peptide bond to the membrane interface would be reduced by hydrogen bond formation, the formation of secondary structure at the interface can be expected to contribute substantially to the partitioning of peptides into membranes.

The whole-residue interfacial hydrophobicity scale of Wimley and White (WW interfacial scale)[65] provides a quantitative basis for predicting the partitioning of unfolded peptides into the membrane interface based solely on their sequences—indeed, based on their amino acid *compositions*, since there are no context-dependent terms in this scale. The validity of this approach has been demonstrated by testing the predictive power of the scale. Ladokhin and coauthors analyzed partitioning of the membrane-active peptide indolicidin,[68] which does not self-associate or form a hydrogen-bonded secondary structure in either membranes or water and whose behavior should therefore be well described by the whole-residue interfacial hydrophobicity scale ($\Delta G_{wu/iu}$). For partitioning into neutral POPC membranes, the experimental free energies of sequence variants of indolicidin (which covered a range of 6 kcal mol^{-1}) were remarkably well predicted by the WW interfacial scale, with the correlation between calculated and experimental free energies having a slope of unity.[68] This suggests that the reduction of interfacial partitioning free energy by about half relative to octanol partitioning holds for this 13 amino acid peptide as well as for the pentapeptide series used to establish the scale. By contrast, transfer free energies from host−guest experiments of Shin and co-workers using a 25 amino acid peptide that corresponds to the presequence of yeast cytochrome *c* oxidase correlate well with previously measured octanol/water partitioning free energies, with no evidence for attenuation of the magnitude of the hydrophobic effect.[69] This peptide and the guest variants adopt little regular secondary structure in solution or in neutral lipid bilayers as measured by CD, although the wild-type peptide forms measurable helix in negatively charged bilayers and in detergents;[70] perhaps this peptide lies deeper within the membrane interface and thus experiences a more hydrophobic environment.

The quantitative prediction of the effects of sequence changes on the partitioning of indolicidin into POPC contrasts sharply with attempts to predict the much stronger partitioning of the positively charged indolicidin peptide into negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) membranes in the same work.[68] While the rank order of the effects is well predicted, the correlation with the WW scale exhibited a slope of 0.3. This lack of additivity corresponds in magnitude to an effective reduction of the net charge on the peptide by 1 for each 3 kcal mol^{-1} of favorable hydrophobic transfer free energy and suggests that predicting partitioning behavior of charged peptides at interfaces will require a basis for estimating nonadditivity. There may also be components of the free energy that depend on the sequence context and not just the amino acid composition—for instance, partitioning may be affected by the relative proximity of charges to one another.

In the context of the scheme proposed in Figure 3, the *partitioning* of an unfolded peptide into the bilayer interface due to the hydrophobic effect ($\Delta G_{wu/iu}$, from the WW scale) can drive *folding* of that peptide in the interface ($\Delta G_{iu/if}$) because of the increased favorable free energy associated with forming a hydrogen bond in the interfacial region compared to water. This partitioning−folding coupling can be considered quantitatively if $\Delta G_{iu/if}$ can be measured. Ladokhin and White used partitioning experiments of monomeric melittin and D-amino acid variants[71] to estimate the per-residue free energy of formation of a helical hydrogen bond in the interfacial region of the membrane at about −0.4 kcal mol^{-1}, while analysis of the folding of a hexapeptide indicated that the corresponding value is about −0.5 kcal mol^{-1} for a β-sheet hydrogen bond.[72] Other estimates of the per-residue free energy for helical hydrogen bond formation range from −0.14 kcal mol^{-1} from studies of magainin 2 amide[73] to −0.25 kcal mol^{-1} from studies of the influenza virus hemagglutinin fusion peptide.[74]

End effects also need to be considered when partitioning peptides into membranes. Hristova and White have recently measured the free energy associated with partitioning the C-terminal carboxyl (free or amidated) and the N-terminal amino group (free or acetylated) of an unfolded peptide into bilayers or octanol from water.[75] Combining these results with the WW scale allows a detailed thermodynamic accounting of how the sequence of a peptide modulates its association with the interface of a bilayer. How do these findings of the interfacial approach inform or influence research on membrane protein folding?

The boot-strap approach of Wimley and White has currently yielded a reasonably quantitative understanding of the thermodynamic relationships between the unfolded soluble state, the interfacial unfolded state, and the interfacial folded state of a peptide from its sequence using the whole-residue interfacial scale for $\Delta G_{wu/iu}$ and the estimates of $\Delta G_{iu/if}$ in phosphatidylcholine membranes.[2] Because the transfer free energies for the ionizable side chains are large and unfavorable, these residues can effectively oppose or abolish insertion into the interface; by contrast, aromatic residues strongly favor interfacial partitioning. While the transfer free energies from host−guest experiments will not account for any specific interactions between side chains of different amino acids in a particular peptide sequence, these contributions are expected to be small for unfolded peptides and the partitioning of a peptide is expected to be well described by a per-residue hydrophobicity that varies only with amino acid composition. Because the contribution of main chain hydrogen bond formation ($\Delta G_{iu/if}$) is comparable in magnitude to the whole-residue interfacial transfer free energy ($\Delta G_{wu/iu}$) for uncharged residues, which make up the majority of membrane spanning sequences, secondary structure formation and the net unfolded partitioning free energy will significantly affect association of peptides with membranes. The "interfacial-yet-unfolded" state of most peptides may be sparsely populated because of the effects of partitioning−folding coupling, but it nevertheless provides an excellent reference or virtual state for considering the factors that drive folding since its free energy depends on composition but not sequence.

Extending these methods[76] and measurements to other lipids and arriving at a more complete understanding of the role of electrostatics will broaden the predictive value of this approach and test its limits. The power of the formalism is evident; the open question is the extent to which composition will be sufficient, or details of the sequence context will be needed, to predict energetics. Measuring the variation of $\Delta G_{wu/iu}$ and $\Delta G_{iu/if}$ with sequence context (for instance, the ability to form salt bridges[77]) is beginning to provide the answers to this question and will extend the utility of the scales. The straightforward expectation is that predicting the behavior of folded species is more likely to require sequence context information than predicting the behavior of unfolded species: differences in the partitioning of peptides having similar $\Delta G_{wu/iu}$ but different sequences can be attributed to differences in the free energy of folding in the interface. The existing scales and their extensions can be used to help dissect the modes of action of amphipathic antimicrobial peptides,[8,10,78−80] designed membrane-active peptides,[81,82] and viral fusion peptides.[13,14,83−85] Most importantly, the detailed description of the thermodynamics of interfacial folding provides the point from which to boot-strap to the next level, understanding the free energy of insertion of folded species across the membrane, $\Delta G_{if/cf}$, so that a thermodynamic description of all the states on the folding pathway of a membrane protein may be obtained. It is expected that sequence will be a critical factor (and amino acid composition will not suffice) to explain the thermodynamics of transmembrane helix insertion (see section 3). Similarly, the manner in which lipid composition modulates interfacial peptide binding may differ strongly from how lipids influence the thermodynamics or kinetics of peptide insertion.

2.3. Thermodynamics as an Organizing Principle

The thermodynamic formalisms of Popot and Engelman[37] and of Wimley and White[2] provide a conceptual framework, based on and bounded by physicochemical principles, within which researchers can design experiments and consider the general implications of their results. These formalisms, as schematically represented in Figure 3, also provide an organizational structure to this review. Section 3 reviews current understanding of the determinants of stable transmembrane helix formation by presenting biophysical, biochemical, and biological experiments that test the roles of peptide hydrophobicity, peptide length, lipid composition, lipid acyl chain length, and the presence of polar, helix-breaking, or charged residues on incorporation of peptides across bilayers. The section closes with a discussion of recent analyses of biological translocon-mediated insertion of transmembrane domains and the dependence of this insertion process on amino acid sequence. Section 4 presents the experimental basis for our current understanding of helix−helix interactions in membranes from experiments demonstrating how amino acid sequence and lipid composition modulate lateral interactions between biological and designed transmembrane helices in detergents, in model bilayers, and in membranes of living cells; the emphasis is on understanding the formation of oligomeric bundles of single span transmembrane helices. Section 5 provides an overview of studies on the sequence and lipid determinants of thermodynamic stability of the best-characterized polytopic helical membrane proteins, including bacteriorhodopsin and diacylglycerol kinase. Biophysical analyses of the insertion folding pathways of nonconstitutive helical membrane proteins and of the kinetic refolding of helical integral membrane proteins from detergent- or urea-denatured states into mixed micelles and bilayers are presented in section 6, as are experiments that explore aspects of co- and posttranslational folding in biological systems.

3. Forming Stable Transmembrane Helices

A long-standing goal in the field of protein folding is to predict structure from sequence; one small step toward that goal is the ability to identify transmembrane domains within proteins. Local amino acid composition is generally sufficient for this prediction: stretches of about 20 amino acids with predominantly hydrophobic side chains have long been known to likely correspond to the membrane-spanning regions of integral membrane proteins.[44−49] The growing availability of genome sequences has allowed biases and correlations in the sequences of helical transmembrane spans to be noted,[86−89] and the increase in experimental topology data for membrane proteins,[90,91] the ready availability of such topology data,[92] and the dozens of membrane protein structures at atomic resolution[23] have enabled a refinement of methods for predicting the existence, positions, and

interactions of transmembrane domains[22,67,93] from sequence information. However, as the database of membrane protein structures grows, the revealed architectural diversity of helical membrane protein structures challenges the generality of such approaches: White points out[94] that the structures of the ClC chloride channel[95] and the KvAP voltage gated potassium channel[96] could not be predicted by identifying canonical transmembrane spans and assembling them into bundles. Although the simple approach of scanning sequences for segments of strong hydrophobicity may not identify all elements that can be functionally assembled into a membrane protein, more refined approaches may eventually decode how protein sequence determines the energetics of interactions between protein and membrane. Examining the forces and factors at work in the formation of independently stable transmembrane spans provides one way of challenging simple approaches to membrane protein folding and identifying the limits of their predictive power.

3.1. Sequence Length and Hydrophobicity

The strong hydrophobicity of the segments of proteins that span the membrane as α-helices was first noted from a small amount of sequence and structural information.[44−47] But are the membrane-anchoring properties of these hydrophobic sequences dependent on their context, or can they function as transmembrane domains independent of their location within a protein? Experiments with proteins translated in the presence of endoplasmic reticulum membranes (microsomes) revealed that a sequence corresponding to a biological transmembrane domain placed at different positions within a heterologous secreted protein could convert it into an integral membrane protein,[97] suggesting that the information specifying a halt to translocation through the membrane is contained locally in the sequence that remained anchored in the lipid bilayer. Similar studies in the *Escherichia coli* inner membrane showed that 16 hydrophobic residues (four repeats of Leu-Ala-Leu-Val) are sufficient to anchor an otherwise secreted protein,[98] identifying hydrophobicity as the primary characteristic of the "stop-transfer" sequence. These proteins and most constitutive α-helical membrane proteins are inserted into membranes cotranslationally by proteinaceous machinery resident in these biological membranes, the Sec61 (or SecYEG) translocon (reviewed in refs 52 and 99−102).

How does protein sequence affect or determine the formation of a transbilayer helix? In particular, how hydrophobic does a sequence have to be to incorporate into bilayers as a transmembrane domain in vitro? A model peptide with a hydrophobic stretch of 24 leucines flanked by a pair of lysines is able to incorporate into a series of phosphatidylcholine (PC) membranes,[103] and the amide protons of its membrane span are extremely resistant to exchange.[104] This is consistent with the strong hydrophobicity of leucine in all thermodynamic scales; on the other hand, polyalanine stretches appear to be poised near the minimum hydrophobicity needed to form a transbilayer helix. The peptide Lys_2-$Ala_{24}Lys_2$ could be incorporated into dry phosphatidylcholine bilayers in a transbilayer conformation, but hydration of that system causes the peptide to partition to the membrane surface and to the aqueous phase, resulting in rapid exchange of all amide protons.[105] Solid-state ^{15}N chemical shifts of a labeled alanine residue indicated that peptides $Lys_3Ala_{14}Leu_4$-Lys_3 and $Lys_3Ala_{16}Leu_2Lys_3$ were stably inserted across hydrated, oriented membranes, whereas $Lys_3Ala_{18}Lys_3$ and $Lys_3Ala_{17}LeuLys_3$ initially adopted primarily transbilayer conformations but suffered degradation of their NMR signals over a period of weeks.[106] Results for biological insertion of a single span are similar: for spans of 21 residues composed of only alanine and leucine, Kendall and colleagues found that a minimum of five leucines were needed for *E. coli* to incorporate the protein into the inner membrane through its translocation machinery.[107] This threshold is predicted[67] using the WW octanol scale,[2,64] which also indicates that peptides composed of alternating leucines and alanines will be stable in membranes (−0.75 kcal mol^{-1} transfer free energy per repeat). Studies of peptides that contain alternating leucine and alanine residues,[108−111] discussed in section 3.2, show that these peptides are indeed sufficiently hydrophobic to form stable transmembrane helices.

Biological transmembrane domains are often flanked by charged residues; through interactions with the aqueous and interfacial region of the bilayer, these could modulate the stability of the transbilayer orientation of a helical span. A role of flanking charges in inducing or preventing association of transbilayer helices has also been noted: predominantly polyleucine peptides flanked by $LysAsp_3$ or LysAsp exhibit self-association in the lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) that can be reduced by lowering the pH, while flanking Lys_2 prevents dimerization at neutral pH.[112] Heteromeric lateral association between the $LysAsp_3$-flanked peptide and the Lys_2-flanked peptide in DOPC at neutral pH indicates that ionic interactions between flanking charges of transmembrane peptides can induce helix−helix association.[112] The preference of tryptophan for the membrane interfacial region,[113] the ability of tryptophan and tyrosine to affect the anchoring of membrane spans,[114,115] and the propensity for aromatic residues to be found at the membrane interface in transmembrane proteins[86,116] suggests that aromatic residues may have a role in anchoring membrane spans, as discussed in sections 3.2 and 3.4. However, the demonstration that an uncharged peptide containing only leucine and alanine (and a fluorophore) can assume a stable transmembrane orientation shows that flanking or anchoring charged, polar, or aromatic residues are not absolutely required for in vitro integration into bilayers.[117,118]

What minimum and maximum lengths of hydrophobic sequences will function as transmembrane domains in biological systems? Work in bacterial and eukaryotic systems has shown that quite short sequences can form transmembrane domains if they are very hydrophobic: nine leucines can suffice in microsomes,[119] while 11 leucines are needed in *E. coli*.[107] Synaptobrevin, which inserts its C-terminal tail into membranes posttranslationally, can be anchored by as few as 12 leucines.[120] On the other hand, while hydrophobic stretches of 40 or even 50 residues[121] span the microsomal membrane just once, domains longer than 26 residues seem to kink or bend such that lumenal glycosylation sites are brought close to the membrane surface,[122] and a single proline in the middle of long hydrophobic spans can enable the sequence to form a helical hairpin and cross the membrane twice.[121] Thus, the general composition and length of peptides needed to span bilayers is roughly consistent with the hydrophobicity and thickness of the lipid bilayer.

3.2. Hydrophobic Mismatch and Interfacial Anchoring

The accommodation of a wide range of apolar transmembrane lengths by the translocon machinery leads to the issue

Table 1. Sequences of Model Peptides Used to Study the Interaction of Transmembrane Helices with Lipids under Conditions of Hydrophobic Mismatch

peptide name	sequence	apolar span[a] (Å)
pLeu(D11)	acetyl-$K_2\underline{GL_7DLWL_9}K_2A$-$NH_2$	30
pLeu11	acetyl-$K_2\underline{GL_5}\overline{WL_5}K_2A$-$NH_2$	18
pLeu15	acetyl-$K_2\underline{GL_7}\overline{WL_7}K_2A$-$NH_2$	24
pLeu19	acetyl-$K_2\underline{GL_9}\overline{WL_9}K_2A$-$NH_2$	30
pLeu21	acetyl-$K_2\underline{GL_{10}}\overline{WL_{10}}K_2A$-$NH_2$	33
pLeu23	acetyl-$K_2\underline{GL_{11}}\overline{WL_{11}}K_2A$-$NH_2$	36
WALP13	acetyl-GWW$\underline{(LA)_3L}$WWA-ethanolamide	10.5
WALP16	acetyl-GWW$\underline{(LA)_5}\overline{W}$WA-ethanolamide	15
WALP19	acetyl-GWW$\underline{(LA)_6L}$WWA-ethanolamide	19.5
WALP21	acetyl-GWW$\underline{(LA)_7L}$WWA-ethanolamide	22.5
WALP23	acetyl-GWW$\underline{(LA)_8L}$WWA-ethanolamide	25.5
KALP16	acetyl-GKK$\underline{(LA)_5}\overline{K}$KA-ethanolamide	15
KALP23	acetyl-GKK$\underline{(LA)_8L}$KKA-ethanolamide	25.5
KALP31	acetyl-GKK$\underline{(LA)_{12}L}$KKA-ethanolamide	37.5
$(LA)_{12}$	acetyl-$K_2\underline{(LA)_{12}}K_2$-$NH_2$	36
L_{24}	acetyl-$K_2\underline{L_{24}}K_2$-NH_2	36
P_{16}	acetyl-$K_2\underline{GL_{16}}K_2A$-$NH_2$	25.5
P_{24}	acetyl-$K_2\underline{GL_{24}}K_2A$-$NH_2$	37.5

[a] The expected length of the hydrophobic region of the peptide is calculated using 1.5 Å per residue for the underlined portions of the spans.

of hydrophobic mismatch:[123,124] if the hydrophobic thickness of a lipid bilayer and the hydrophobic length of an α-helix do not match, what structural rearrangements of lipid, protein, or both take place, at what energetic costs, and with what functional consequences? The lipids in the membrane could respond to mismatch with helices by adopting a more or less extended acyl chain conformation, thus effectively adjusting the thickness of the bilayer. Responses of the protein to hydrophobic mismatch could include lateral association of the helices to minimize their exposure to bilayer or water or alteration of the polypeptide backbone conformation; in cases of positive mismatch, where the transmembrane span is overly long, the helix may tilt, while in the case of negative mismatch the side chains of polar residues may "snorkel" to extend their polar ends to the aqueous interface. This section outlines how biophysical experiments addressing positive and negative mismatch between model peptides and lipid bilayers have provided insight into how single-spanning hydrophobic domains interact with bilayers (reviewed elsewhere[125−127]).

London and colleagues have explored hydrophobic mismatch using fluorescence methods and a series of model peptides.[128,129] They showed, using lipids of different acyl chain lengths and model hydrophobic peptides that could interconvert between surface-associated and transbilayer structures, that the energetics of insertion could be modulated by hydrophobic mismatch and cholesterol content.[128] Transbilayer insertion of the polyleucine-based peptide pLeu(D11) (see Table 1) across fluid state phosphatidylcholine membranes is optimal for acyl chain lengths of 18 or 20 carbons; the thicknesses of the hydrocarbon core of these bilayers (~28 or 31.5 Å, respectively) correspond closely to the length of the hydrophobic span of pLeu(D11) as an α-helix (30 Å).[128] For lipids with acyl chains longer than 20 carbons, transbilayer insertion of the peptide becomes less favorable, presumably due to the energetic cost of burying the flanking residues in the thicker membrane: the free energy of transbilayer insertion is reduced by 1−1.5 kcal mol^{-1} per two-carbon increase in chain length.[128] With the pLeu series of model peptides, a short hydrophobic stretch of 11 leucines was shown to be surface-associated with a moderately thick membrane (DOPC), while spans of 15, 19, and 23 leucines were capable of inserting across the bilayer.[129] Thus, hydrophobic mismatch can significantly affect the free energy of the transbilayer conformation of a membrane spanning sequence, and one response of the peptide can be to partition into the membrane interface rather than assume a transbilayer structure. While the shorter pLeu peptides are largely monomeric upon transmembrane insertion, the 23 residue span shows evidence of a stronger tendency to undergo lateral association, demonstrating that mismatch can influence helix−helix interactions.[129] By contrast, lateral association of very similar polyleucine-based model peptides was shown to be influenced modestly by the length of the hydrophobic membrane span but to increase systematically with increased thickness of the lipid bilayer, including conditions of strong negative mismatch.[130]

Killian and colleagues have used artificial membranes composed of either lipid mixtures or pure lipids together with the WALP series of alternating leucine−alanine model peptides (see Table 1) to study the effects of hydrophobic mismatch between peptide and bilayers. The extent of positive or negative mismatch decreases the amount of peptide that can be stably incorporated into bilayers,[131] with excess peptide forming aggregates.[111,132] WALP peptides that are incorporated into phosphatidylcholine bilayers encourage the formation of nonlamellar lipid phases in a manner that is consistent with mismatch effects[111,125,133] and with a role for the flanking tryptophans of the WALP peptides.[113] Even at very low peptide/lipid ratios, WALP peptides also strongly influence the lamellar-to-isotropic phase transition of mixtures of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG) in a way that correlates with hydrophobic mismatch.[134] KALP variants of the WALP peptides bearing charged anchoring residues instead of tryptophan (see Table 1) also promote formation of nonlamellar phases of PE and PE/PG lipids in a similar manner[135] and can be incorporated into bilayers to a greater extent than the WALP homologues.[127] A tryptophan-specific effect on the ability of a transmembrane model peptide to induce nonlamellar phase has been seen for mixtures of WALP peptides, unsaturated PCs, and cholesterol: for this lipid composition, peptides flanked by histidines or arginines cannot induce the isotropic phase.[136] Thus, peptide flanking sequences and hydrophobic mismatch can have interdependent effects on the ability of peptides to be incorporated into bilayers and on the behavior of lipid/peptide mixtures.

While many of the experiments described above were designed to test the effects of hydrophobic mismatch, other factors certainly underlie the behavior of peptide−lipid mixtures, and hydrophobic mismatch does not always explain the effects of peptides on lipids. For instance, polyleucine peptide P_{24} (see Table 1) interacts with phosphatidylcholine lipids in a way that is modulated by acyl chain length[103] but exhibits chain-length independent peptide−lipid interactions with phosphatidylethanolamine lipids.[137] Most dramatically, the effects of polyleucine peptide L_{24} (see Table 1) and variants on the fluid to hexagonal phase transition temperature of phosphatidylethanolamine go *against* hydrophobic mismatch predictions.[138]

At peptide-to-lipid ratios that support lamellar phases, it is expected that hydrophobic mismatch may cause alterations to the bilayer thickness.[123] Gramicidin, which has a hydro-

phobic thickness corresponding to WALP16, thickens 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) bilayers by 1.3 Å and thins 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers by 2.6 Å when incorporated at a 1:10 mole ratio.[139] Protein-free membranes reconstituted from lipid extracts are as much as 5 Å thicker or thinner than the biological membranes from which the lipids were derived, suggesting that integral membrane proteins modulate biological membrane thicknesses.[140] While incorporation of either L_{24} or $(LA)_{12}$ model peptides (see Table 1) perturbs the orientational order of lipid acyl chains in liquid-phase bilayers as measured by ^{2}H NMR[141,142] in a manner consistent with altering the spatial extent of the lipid chain to minimize hydrophobic mismatch, it is not clear to what extent these changes would resolve the mismatch. Long WALP peptides are inferred by ^{2}H NMR quadrupolar splittings to thicken membranes by as much as 1.4 Å, and short WALP peptides can thin bilayers by 0.4 Å,[143] but these differences are modest compared to the extent of mismatch and therefore indicate that the peptides have minimal effects on bilayer thickness. This conclusion is supported by direct X-ray diffraction measurements of oriented bilayers showing that WALP13, -16, and -19 do not affect the thickness of membranes made from C12, C13, or C14 saturated phosphatidylcholine lipids.[110] Single membrane-spanning helices therefore appear to have a minimal ability to modulate the overall thickness of pure lipid bilayers.

Another response to positive hydrophobic mismatch is for peptides to tilt, effectively burying more of their hydrophobic surface area in the membrane hydrophobic core. Circular dichroism and ^{15}N solid-state NMR showed that a series of peptides with alternating leucines and alanines flanked by two lysines on each end were able to incorporate across bilayers estimated to be 14 Å too thin or 4 Å too thick, and mismatch systematically affects the ^{15}N chemical shifts in a manner consistent with slight helix tilts.[144] While the degree of mismatch strongly affects the amount of WALP peptides that are incorporated into membranes, attenuated total reflection Fourier transform infrared (ATR-FTIR) measurements show that those species incorporated into PC bilayers exhibit quite similar tilts.[131] A solid-state ^{2}H NMR method using site-specific alanine labels shows that WALP19 exhibits the same small tilt of about 4° away from the membrane normal in lipids having acyl chains ranging from 12 to 18 carbons.[145] For the WALP23 peptide and the same range of lipid acyl chain lengths, a small but systematic increase in peptide tilt is seen for increasing mismatch, but the maximum tilt of about 8° in di-C12:0-PC is still much too small to resolve the mismatch.[146] A peptide flanked by lysines also exhibits increasing tilts with increasing positive mismatch, but while the range of tilt is larger than that of the WALP species, suggesting that tryptophan anchors the peptide differently from lysine, it is still very modest ($<12°$).[147] The inhibitory effect of tryptophan on the tilt of the peptide can be overcome by making the peptide more hydrophobic: polyleucine peptides flanked by tryptophan or lysine residues and each containing a single labeled alanine (at one of four consecutive positions in the middle of the membrane span) both tilt as much as 11° in di-C12:0-PC.[147] Interestingly, the peptides tilt toward a different face of the helix for tryptophan or lysine anchored peptides, but the direction of tilt is only slightly modulated by the polyleucine or alternating leucine−alanine nature of the hydrophobic membrane span. The development of methods for studying helical tilts of peptides in ways that minimize the number of labeled synthetic peptides required[146,148,149] suggests that the exploration of the effects of mismatch on the orientation of biological single membrane spans is feasible. However, at least for the case of the WALP series, while hydrophobic mismatch can deter peptide integration across lipid bilayers, such peptides have little effect on bilayer thickness and mismatch only very slightly perturbs the orientation of peptides that are successfully incorporated. Minimal tilts in response to large positive mismatch suggest that tilting is more energetically costly, perhaps in terms of the required lipid conformations, than other possible outcomes resulting from mismatch.

By contrast, solid-state NMR data of peptides in oriented multilayers indicate that the uniformly ^{15}N-labeled transmembrane domain of Vpu does tilt in response to positive hydrophobic mismatch, from 18° in a bilayer made of C18 lipids to 27°, 35°, and 51° in bilayers made from lipids with tails of 14, 12, and 10 carbons respectively.[150] The differences between the strong mismatch-dependent tilts observed here and the minimal tilts seen for the WALP and KALP peptides discussed above may result from the manner in which the lipid/peptide complexes are prepared or from the level of sample hydration. The differences may simply reflect the different sequences of the peptides under study, but two major differences in the lipids used in these studies should also be noted. The Vpu work uses a mixture of lipids, 9:1 phosphatidylcholine/phosphatidylglycerol, which may facilitate lipid rearrangements needed to accommodate peptide tilting; however, in some of these samples, the phosphatidylcholine lipid used is ether-linked, unlike the WALP and KALP studies. Thus, transmembrane helices can tilt, under some circumstances, in response to positive hydrophobic mismatch.

Although several lines of evidence indicate that the helicity of model peptides is largely unperturbed by mismatch,[103,131,145] some slight distortions do occur[108,151] and the possibility that biological membrane spans experiencing positive or negative mismatch may undergo rearrangements of their secondary structure cannot be formally excluded. If hydrophobic mismatch is not very unfavorable, peptides that are too long for the hydrophobic core of the bilayer could simply extend through the interfacial region, or into the solvent. This seems to be the case for bilayer-incorporated KALP peptides (see Table 1), where hydrogen/deuterium exchange data indicate that the additional hydrophobic residues present in longer peptides are almost fully susceptible to amide proton exchange and are thus exposed to aqueous environments at least transiently.[127] By contrast, WALP peptides compensate for positive mismatch in a way that largely protects backbone amides from exchange: there are seven additional amide protons in WALP23 compared to WALP16, but only one or two of these exchange quickly when the peptides are inserted in DMPC bilayers.[152] The dependence of protection from amide exchange on the location of the flanking tryptophan residues[152] is consistent with the interfacial anchoring role of trytophan in glycosylation mapping studies of model transmembrane domains inserted into microsomes[114] and with observations that tryptophan partitions preferentially to the membrane interface.[59,113] However, the precise physical basis for the protection of the amides in WALP peptides from exchange is not clear, given that WALP23 does not increase the thickness of thin bilayers[143] and the peptide does not exhibit

significant tilt,[146] as described earlier in this section. It may be that a local accommodation of lipid structure in the immediate vicinity of the peptide helps resolve the mismatch problem and protect the peptide backbone amides from exchange, as suggested by molecular dynamics simulations[153] (reviewed elsewhere[154]). Although the hydrophobic core of the DMPC bilayer is about 24 Å thick, corresponding to a helix length of 16 residues, WALP16, KALP23, and KALP31 peptides in DMPC bilayers each protect only about eight amide hydrogens from exchange at long times, while WALP23 protects 14 amides within a leucine−alanine core of 17 residues.[127] This protection of additional amides may indicate that the tryptophan-anchored peptides make minimal excursions away from their time-averaged positions and conformations in the bilayers, whereas the lysine-flanked peptides could sample a greater range of depths. This anchoring behavior can be explained by the partitioning free energy of tryptophan as expressed in the WW interfacial scale:[55,65] two tryptophans at the same end of the hydrophobic span of WALP23 favor the interface over water by a combined 3.7 kcal mol^{-1} and thus strongly resist being pushed into the aqueous layer, while partitioning a leucine−alanine dipeptide into the aqueous phase would require only 0.4 kcal mol^{-1}.

The current state of knowledge regarding the response of proteins and bilayers to hydrophobic mismatch is far from exhaustive, and studies involving model peptides, biological membrane spans, polytopic proteins, and especially mixtures of lipids will yield additional insight into this subject. Information available to date suggests that for single spans, even conditions of extreme mismatch have little effect on peptide tilt or on bilayer thickness. Unless the flanking residues partition into the membrane particularly well or the hydrophobic spans are extremely long, single spans may simply extend out of the hydrophobic core or perhaps even the interface region of the bilayer. The indications that peptide self-association or aggregation seems to be increased by positive or negative mismatch suggest that the increased free energy associated with protein−lipid mismatch can be lowered by eliminating protein−lipid contacts through formation of peptide−peptide contacts. Thus, hydrophobic mismatch may be expected to impact oligomerization, folding, and conformational changes of helical integral membrane proteins.

3.3. Polar Residues and Proline

A putative transmembrane span containing an internal polar or charged residue should still be able to assume a stable transmembrane configuration if the other residues in the span are sufficiently hydrophobic. London and colleagues have shown that polyleucine helices bearing a serine, asparagine, lysine, or aspartic acid residue in the middle of the span are capable of achieving a transbilayer orientation in DOPC membranes, although the buried ionizable residues can induce changes to other orientations under extremes of pH.[155] Using a dual fluorescence quenching assay for distinguishing surface-associated and transbilayer configurations,[156] Caputo and London have determined the effects of making substitutions for one or two of the leucine residues near the middle of a polyleucine span bearing an internal tryptophan reporter. In DOPC membranes, single substitutions of hydrophobic, polar, and charged residues did not affect the transbilayer incorporation of the peptide, nor did the substitution of two prolines.[157] When these same peptides were tested in thicker 1,2-dierucoyl-*sn*-glycero-3-phosphocholine membranes, peptides with single substitutions of hydrophobic or slightly polar residues were accommodated in transbilayer orientations, while single proline and charged amino acid substitutions and the double proline substitution showed some surface association; mutations to adjacent like charges (Lys-Lys or Asp-Asp) abolished transbilayer insertion.[157] The ionization state of aspartic acid residues within the membrane span directly determines the orientation of the peptide: at high pH, the surface-associated form prevails, while at low pH, the protonation of the side chains allows the peptide to insert in a transbilayer state.[158]

It should be noted that the cost of burying a positively charged residue within the membrane may be alleviated by "snorkeling" of a long side chain to make favorable interactions with lipid phosphates, as suggested by glycosylation mapping experiments,[159] analysis of protein sequences and structures,[160] and molecular dynamics simulations.[161] Since snorkeling may have very little free energy cost,[135,162] this can provide an adaptation of a membrane span to apparent hydrophobic mismatch (section 3.2).

While a general strong inhibitory effect of charged side chains on insertion across the bilayer is to be expected based on the WW scale, the role of proline in the formation of transmembrane helices is more subtle. The covalent structure of the imino acid proline results in steric restriction of accessible backbone torsion angles and has been shown in soluble proteins to strongly oppose helix formation,[63] although there is a propensity for prolines at the N-terminal ends of helices in soluble proteins.[163,164] Given the importance of satisfying hydrogen bonds within the membrane environment and the potential helix-breaking or kink-inducing nature of this amino acid, proline sites in hydrophobic membrane protein spans and in intact membrane proteins are of considerable interest.

Deber and colleagues investigated the secondary structure of the transmembrane span of the insulin receptor, along with a variant bearing a double mutation of a Gly-Pro pair to Ala-Ala that had previously been shown to increase internalization of the receptor. Consistent with the strong helix-forming tendency of alanine, the mutant peptide exhibits greater helicity than wild-type in detergent and is more resistant to thermal denaturation; it also seems to self-associate more strongly.[165] By contrast, a proline to glycine mutation isolated in a mutagenesis screen of the IKe coat protein destabilizes the helical secondary structure of the detergent-solubilized peptide in favor of β-structure at high temperature;[166,167] it appears that the proline helps define the amino-terminal end of the membrane-spanning helix.[168] Circular dichroism measurements of a series of model hydrophobic peptides showed that proline was capable of supporting helix formation in detergents, in lipids, and in organic solvents.[169] These data indicate that the presence of a proline in a membrane span can either block or enhance the formation of helical structure, presumably in a way that depends on the sequence context, the lipidic environment, or both. Prolines could also provide conformational flexibility or heterogeneity, as seen in solid-state NMR studies of a synthetic peptide corresponding to the M1 segment of nicotinic acetylcholine receptor studied in hydrated bilayers.[170]

The effect of prolines on transmembrane domain positioning has also been explored in glycosylation mapping experiments (see Figure 4), where constructs with linkers of different lengths separating a single lumenal glycosylation

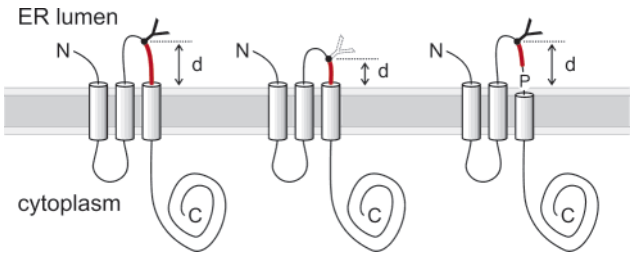

Figure 4. Glycosylation mapping method of von Heijne and colleagues.[172] Modification of a lumenal glycosylation site (black dot) by the oligosaccharyl transferase (not pictured) depends on the distance (d) of the site from the membrane. Sites that extend sufficiently far from the membrane are glycosylated (bold Y, at left), while sites that are too close to the membrane surface because of internal deletions in the linker (red line) between the membrane span and the glycosylation site are not modified (outlined Y, center). Constructs bearing linkers too short to allow glycosylation can be made accessible if a proline substitution is made in the first two turns of the polyleucine membrane span, causing local unraveling of the helix in the interfacial region of the membrane and increasing the apparent extent of the membrane span. Adapted with permission from ref 172. Copyright 1998 Academic Press (Elsevier Ltd).

site from the hydrophobic span are translated in the presence of microsomes to map the position of the glycosylation site, and thus the hydrophobic span, relative to the location of the active site of the oligosaccharyl transferase.[171,172] The effects of single proline replacements within a 20 or 23 residue polyleucine stretch on the availability of the adjacent glycosylation site depends strongly on the positions of the prolines within the hydrophobic span: substitutions within the first two turns of helix make the glycosylation site more accessible, while substitutions deeper in the transmembrane span behave like the original polyleucine construct.[172] Although interpretation of precise distances is complicated, it is clear from these studies that a proline in the middle of a membrane span (and thus in the hydrophobic core of the membrane) does not change the effective helix length, while a proline near the end of the span (and thus in the membrane/water interfacial region) causes local unraveling of the transmembrane helix. Similar experiments showed that when lysine and aspartic acid are placed three or four residues apart in the context of a polyleucine membrane span, such that they could form a salt bridge, the transmembrane domain inserts more deeply into the membrane than when the charged residues are one or two residues apart.[173] These experiments report on the physical behavior of biological transmembrane spans in the complex membrane of the endoplasmic reticulum, supporting and extending the structural and energetic expectations available from model systems and from first principles.

Another potential role for proline in α-helical membrane protein folding has been identified by analysis of the third transmembrane span of cystic fibrosis transmembrane conductance regulator (CFTR), where the mutation Pro205Ser blocks maturation of the full-length channel. A peptide corresponding to the wild-type transmembrane domain is much more helical in detergent than when Pro205 is replaced by glycine, alanine, serine, or leucine;[174] the mutants appear to form aggregates with β-structure. Chimeric constructs translated in the presence of microsomes and tested for topology show that the mutants incorporate into microsomes only half as well as wild-type; similarly, wild-type synthetic peptide is readily reconstituted into synthetic lipid bilayers as a helix, but the mutant peptides can only be incorporated in a transmembrane configuration if they are treated with organic solvents.[174] This example suggests that, as seen previously for soluble proteins,[175] an amino acid that prevents formation of alternate conformations can stabilize a membrane spanning domain.

3.4. The Translocon

Most eukaryotic helical integral membrane proteins become integrated into the membrane of the endoplasmic reticulum through a cotranslational process that involves the Sec61 translocon, a membrane-embedded protein-conducting pore.[102,176] Proteins are targeted for translocation and insertion by hydrophobic stretches in their primary sequence, which together with the charges of flanking residues control the topology of integration into the membrane.[99] The machinery and mechanisms by which this process is directed are the subject of intense investigation, as reviewed elsewhere.[52,100,101,177−179]

Experiments testing the determinants of topology showed that charged residues on either side of hydrophobic signal sequences affect the insertion of the protein so as to orient the more positively charged flanking region on the cytosolic side of the membrane (the "positive-inside rule").[180,181] Although the positive-inside rule can be used to control and design bitopic and polytopic protein topology,[182] conflicting positive-inside signals for hydrophobic spans within a single polytopic membrane protein can result in the exclusion of one of the spans from the *E. coli* membrane,[183] and simple models for topology where the orientation of the first hydrophobic span determines the topology of all subsequent spans[184] are not adequate to describe all membrane protein insertion events.[185] However, the behavior of the translocon is not determined by the sequence of the polypeptide substrate alone. Tuning the anionic phospholipid content of the *E. coli* membrane was shown to affect both insertion and topology in a way that was modulated by altering the number of positively charged residues flanking the membrane span,[186] strongly suggesting that the translocon-mediated insertion process was sensitive to interactions between charged residues and charged lipids. Sequence changes at the middle of a hydrophobic span can alter topology in remarkable ways: placement of a proline near the middle of a 31 residue polyleucine span (but not a 30 residue span) can convert a single membrane span into a polytopic "helical hairpin".[187] The propensities of different residues to promote the integration of helical hairpins, and thus turn formation, have been quantified; only phenylalanine and the large aliphatic residues have no tendency to promote turns.[187−189]

Based on biochemical evidence including cross-linking, glycosylation, and membrane integration data, Rapoport and colleagues have proposed that the translocon mediates membrane integration by allowing the polypeptide that is passing through the protein pore to equilibrate with lipids, partitioning into the bilayer or staying associated with the pore based on its hydrophobicity.[190,191] White suggested that thermodynamic formalisms for membrane protein folding might be combined with information about translocon-mediated processes to explain membrane protein structure, folding, and stability.[94] This proposal has been dramatically fulfilled by recent work published by von Heijne, White, and colleagues[192,193] (and reviewed elsewhere[194]) relating translocon-mediated biological membrane protein insertion data to thermodynamic models and firmly establishing the usefulness of relating simple quantitative models for peptide/membrane interactions to the translocation process.

Previous work with the eukaryotic[119] and prokaryotic[107] translocons had demonstrated minimum lengths and hydrophobicities (relative number of leucines or alanines; see section 3.1) needed for translocon-mediated integration into membranes. Extending this approach, von Heijne and colleagues generated dozens of membrane spans composed of leucine and alanine in different proportions, tested them for membrane integration as part of a polytopic protein, treated the measured fractional integration as an apparent association constant, and calculated apparent free energy differences between the inserted and noninserted states.[192] Working with transmembrane spans having a small number of leucines in the context of polyalanine poised the system near the integration threshold, allowing the authors to quantify fractional integrations precisely and accurately using a glycosylation assay (see Figure 5). Ten different constructs containing two leucines spaced symmetrically in the 19 residue membrane span opposed integration by about 0.7 kcal mol^{-1} on average, an analogous set containing three leucines opposed integration by about 0.1 kcal mol^{-1}, and a set containing four leucines favored integration by about 0.5 kcal mol^{-1}. The effects of these substitutions are essentially additive. Increasing the leucine content of membrane spans that also contained polar substitutions enabled the authors to keep the system poised near 50% integration efficiency. Comparison of apparent transfer free energies for constructs that differed from one another by single or double amino acid substitutions allowed the authors to construct an apparent free energy scale (ΔG_{app}) for the amino acid dependence of partitioning a transmembrane domain from the translocon into the membrane of the endoplasmic reticulum, which they termed a base biological hydrophobicity scale.

The biological hydrophobicity scale correlates extremely well with the WW whole-residue octanol/water transfer free energy scale,[64] with the most significant outliers being tryptophan and proline. Since the WW octanol scale gives a better correlation with the data than the interfacial scale, the observed "partitioning" between secretion and membrane integration corresponds to transfer from an aqueous environment to a strongly hydrophobic environment. This is consistent with the idea that the protein threaded through the translocon resides in an aqueous environment and partitions into the hydrophobic core of the membrane bilayer.

Notably, the positional effects for the hydrophobic residues tested are generally small (±0.2 kcal mol^{-1}) but are as large as 1.4 kcal mol^{-1} for proline, with placement of proline in the middle of the span favoring integration the least.[192] This same spatial dependence is expected for transferring a proline-containing hydrophobic helix from water into a bilayer. Proline replacements at the N-terminal side of the helix are more favorable for integration than those at the C-terminal side, consistent with the observed locations of proline in membrane spans.[195] Strong positional effects on integration were also seen for model transmembrane spans containing an arginine: the experimental ΔG_{app} varied as much as 2.2 kcal mol^{-1}, depending on the position of the arginine within a 19 residue span.[193] When the base biological hydrophobicity scale alone is used, the predicted ΔG_{app} for the S4 helix from the voltage-gated potassium channel (which contains four arginines) is +3.9 kcal mol^{-1}, whereas the experimental value is +0.5 kcal mol^{-1}. However, including the position-specific terms yields a predicted ΔG_{app} of +0.9 kcal mol^{-1}, which is in excellent agreement with the experimental value.[193]

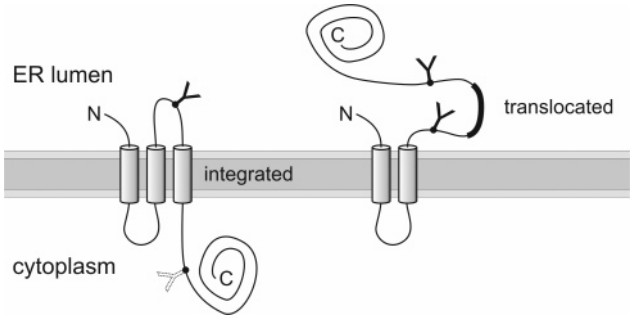

Figure 5. Glycosylation assay of Hessa et al.[192] for determining fractional integration of membrane spans. Integration of the third membrane span leaves one glycosylation site (black dot) in the cytoplasm (left), while translocation of the sequence allows both sites to be glycosylated. Unmodified, singly, and doubly modified species are resolved by SDS−PAGE and quantified. Adapted with permission from *Nature* (http://www.nature.com), ref 192. Copyright 2005 Nature Publishing Group.

The apparent free energies for integration of membrane spans with the composition $Leu_4Ala_{13}Ser_2$ vary over a range of 0.9 kcal mol^{-1} in a manner that is well explained[192] using the hydrophobic moment,[47] which is a measure of the asymmetric distribution of hydrophobic residues wrapped onto an ideal helix. The analysis shows that when polar residues cluster on one face of the helix, the span integrates less well compared to when the polar side chains face in different directions.[192] Membrane spans containing two lysine or asparagine residues integrate least well when the polar residues are separated by six intervening residues, thus placing them on the same face of a helix. The hydrophobic moment, which quantifies the amphiphilic nature of a helical region, can also account for much of the variance in the apparent transfer free energies of the classes of constructs containing only leucine and alanine in the same proportions but in different sequence arrangements.

Spacing effects seen for tryptophan are quite interesting: two tryptophans in the middle of the membrane span strongly oppose insertion, while positioning the residues at the edges of the span strongly favors insertion (the difference is as much as 1.4 kcal mol^{-1}).[192] Tyrosine exhibits a similar pattern, but phenylalanine is almost as indifferent to residue spacing as leucine. These effects mirror the membrane interfacial partitioning behavior of these amino acid side chains,[59,113] the observed statistical preference of these residues for membrane interfaces,[196] and the effects of these residues on the depth of inserted spans in membranes.[114]

Taken together, these observations overwhelmingly support the hypothesis that the integration of transmembrane helices into the endoplasmic reticulum membrane is related to the ability of the sequence to partition into lipid bilayers. These results illustrate the power of applying quantitative apparent free energies to a complex biological process such as the insertion of a transmembrane helix into a membrane from the translocon. It seems that supplementing the base biological hydrophobicity scale with position-dependent terms may suffice to explain the tendency of natural biological sequences[193] to insert as transmembrane helices via the translocon. Both the position-independent apparent free energy terms (the base biological hydrophobicity scale) and the position-dependent terms described to this point can be readily related to physical parameters: the base biological scale correlates with hydrophobicity, the position-dependence of many residues correlates with the hydrophobic moment, and the positional effects of the aromatic residues correlates

with their propensity to partition to the membrane interface. Thus, it appears that the rules employed by the biological translocon machinery in "deciding" between translocation and membrane integration are largely encoded in the composition and the precise sequence of the protein that determine the physicochemical properties of the translocating polypeptide chain. Other aspects of translocon function, including the tendency for turn or helical hairpin formation[187−189] and the topology of insertion of a signal-anchor sequence,[197] may also be explained by amino acid propensities or apparent free energy scales, as discussed further in section 6.3. Other protein translocation machinery may respond similarly to protein sequences: strong hydrophobicity of membrane spans favors the direct membrane incorporation of transmembrane proteins imported into mitochondria via the TIM23 complex (the so-called "stop-transfer" pathway), whereas moderate hydrophobicity or the presence of proline residues in the membrane-spanning domains favors the transfer of the membrane protein to the mitochondrial matrix and subsequent export/insertion.[198]

Relating these apparent free energy terms to the thermodynamic picture that has emerged from the interfacial approach to membrane protein folding is not immediately straightforward. Does the translocon truly sample the energetic difference between the translocated state and the integrated state? Even if one blurs the distinction between free energy of partitioning and apparent free energy, the base biological hydrophobicity scale is not easily related to the thermodynamic cycles in Figure 3. The integrated state of the helix clearly corresponds to a species that is folded and in the core of the membrane, but the translocated state could be either folded or unfolded in the aqueous lumen of the ER, or even lying on the lumenal surface of the ER membrane; thus, the base biological hydrophobicity scale could correspond to $\Delta G_{wf/cf}$, $\Delta G_{wu/cf}$, or $\Delta G_{if/cf}$. This means that the observed partitioning (and its position-dependence for certain amino acids) may arise from effects on the folded polypeptide in the membrane interface or on the free energy of the folded or unfolded aqueous polypeptide. However, the slope of 1.07 for the correlation between the base biological hydrophobicity scale and the WW water−octanol partitioning free energy[192] indicates that the biological scale covers the full expected range of hydrophobicity (as opposed to the reduced hydrophobic effect seen for the WW interfacial scale; see section 2.2), suggesting that the scale approximates partitioning from water to the hydrophobic core of the membrane. The intercept for this correlation is displaced from zero, with the biological scale shifted by 0.5−0.6 kcal mol^{-1} (per residue) in favor of the aqueous state compared to the octanol scale;[192] because each scale was zeroed using empirical boot-strapping approaches, the source and meaning of this difference is not clear. The magnitude of the difference can, however, be appreciated when summed over the 20 residues of a membrane span: if the biological scale were shifted to match the zero of the octanol scale, the "corrected" base biological hydrophobicity scale would over-predict membrane integration by 10 kcal mol^{-1} of apparent free energy.

Since the zero point of the octanol−water scale was arrived at by analyzing short peptides, one might choose to adjust it to match the biological scale; however, the excellent predictive value of the octanol−water scale in identifying transmembrane spans[67] would be compromised by this adjustment. Since the water−octanol scale includes a per-residue cost for desolvating the polypeptide backbone upon partitioning into the membrane[55,67] (see section 2.2), changing this contribution would change the scale offset, but theoretical considerations and computation[66] both suggest it is unlikely that peptide integration into a bilayer should carry a *larger* energetic cost for desolvating the polypeptide backbone than water−octanol transfer. Differences in the number of hydrogen bonds in the integrated species compared to the translocated species could also result in an offset between the scales, but it is difficult to see how the transmembrane span would be *less* folded than the translocated sequence, as needed to account for the sign of the scale offset, because the membrane is expected to drive hydrogen bond formation. Although the physical basis for this offset is not clear at present, the boot-strapping approaches based on the partitioning of model peptides and on the analysis of membrane integration data have advanced our understanding of the energetics of peptide−bilayer association to the point where detailed questions about the sequence dependence of protein behavior in vivo and in vitro can be addressed in a context where the fundamental ground rules are agreed upon.

4. Lateral Interactions between Transmembrane α-Helices

The two-stage model indicates that the stability of a helical transmembrane protein can be understood by conceptually separating its folding into two steps: the formation of independently stable transmembrane helices and the lateral association of these helices into bundles.[37] Perhaps the best case in which to apply the two-stage thinking is the association of single membrane spans into oligomers. The presence of loops in polytopic proteins and of intramembranous ligands were acknowledged in the original two-stage model to have some influence on folding,[37] and updates to the model have provided explicit discussion of these factors (and of the involvement of peripheral domains in the folding process).[3,51] The formation of oligomeric complexes of single membrane spans avoids these additional complicating factors, placing the simplifying principles of the model on their firmest possible standing.

When monomeric peptides associate into an oligomer, peptide−lipid contacts are lost with the release of lipids to the bulk phase and peptide−peptide contacts are made. The equilibrium between transmembrane helix monomer and oligomer will be determined by the balance of enthalpic and entropic terms that govern helix−helix, helix−lipid, and lipid−lipid interactions; some of these terms will clearly depend on protein sequence (and not merely the amino acid composition), while others must vary with the identity of the lipids. This section of the review will focus on the sequence, structural, and lipid determinants that stabilize lateral interactions between single membrane spans, with the goals of identifying the forces and factors that mediate the lateral interactions between helices and of demonstrating the utility of the two-stage model in analyzing such systems. Many biophysical, biochemical, and biological methods have been developed to study the oligomerization of single membrane spans in detergents, in synthetic bilayers, and in the membranes of living cells. Section 4.1 introduces most of these methods in the context of a review of papers exploring the homodimeric association of the transmembrane domain of human glycophorin A, the most thoroughly studied example of the two-stage model.

4.1. Glycophorin A: A Two-Stage Model Protein

Glycophorin A, the major sialoglycoprotein from human red cell membranes, has been the subject of biochemical investigations for more than three decades. Selective extraction of glycophorin A from red cells using lithium diiodosalicylate showed that the amino terminal domain of the protein is the carrier of epitopes for MNO blood type[199] while the C-terminal domain associates with the membrane.[200] Purified and reconstituted glycophorin A[201,202] exhibits the same topology as the native protein.[203] The determination of the 131 amino acid sequence of glycophorin A by peptide mapping and sequencing represented the first primary structure of an integral membrane protein.[204] The transmembrane region of glycophorin A binds tightly to phosphoinositides during purification,[205] and full-length glycophorin A affects the properties of lipids when incorporated into membranes,[206,207] with an interaction preference for phosphatidylcholine over phosphatidylserine.[208−210] About 10 amide hydrogens of glycophorin A transmembrane peptide reconstituted into phosphatidylcholine bilayers are resistant to chemical exchange over many hours.[211]

4.1.1. Sequence-Specific Transmembrane Helix Dimerization

The property of glycophorin A that has been of most interest in the protein folding field is its self-association: glycophorin A forms a detergent-resistant complex that migrates primarily as a dimer under conditions of SDS−PAGE.[212] This self-association of glycophorin A was used to map the homodimerization domain to a tryptic peptide that includes the hydrophobic membrane-spanning domain.[212] Interestingly, the sequence of the membrane span is almost entirely hydrophobic, containing only four polar residues: Thr74, Thr87, Ser91, and Tyr92. Dimerization is abolished by chemical modification of a methionine within the membrane span[213] and can be reproduced by a synthetic peptide.[214]

Exploration of the sequence-dependence of glycophorin A dimerization was made possible through heterologous expression. Lemmon, Engelman and colleagues showed that in-frame fusion of a C-terminal fragment of glycophorin A with staphylococcal nuclease, a monomeric soluble protein that is readily expressed in *E. coli*, yielded a chimeric protein that exhibits the same self-association properties as full-length native glycophorin A.[215] Addition of a peptide corresponding to the transmembrane region of glycophorin A to samples of chimera that migrate as homodimers on SDS−PAGE results in the formation of chimera/peptide heterodimers.[215] Importantly, the production of this chimeric protein in *E. coli* allowed the introduction of sequence changes by site-directed mutagenesis.[215] Using this approach, Lemmon and colleagues generated more than two hundred single point mutations of the 23 residue transmembrane domain and measured the dimerization phenotypes of these mutant proteins using SDS−PAGE[216] (Figure 6). Although time-consuming, this saturation mutagenesis approach provides a wealth of essentially unbiased data for identifying important trends in the effects of sequence changes on dimerization. While ionizable or strongly polar substitutions at any position are almost always disruptive, hydrophobic substitutions reveal a pattern of sensitive and insensitive positions that cluster on one face on an α-helix and implicate a seven residue motif—Leu75, Ile76, Gly79, Val80, Gly83, Val84, and Thr87—as participating in sequence-specific

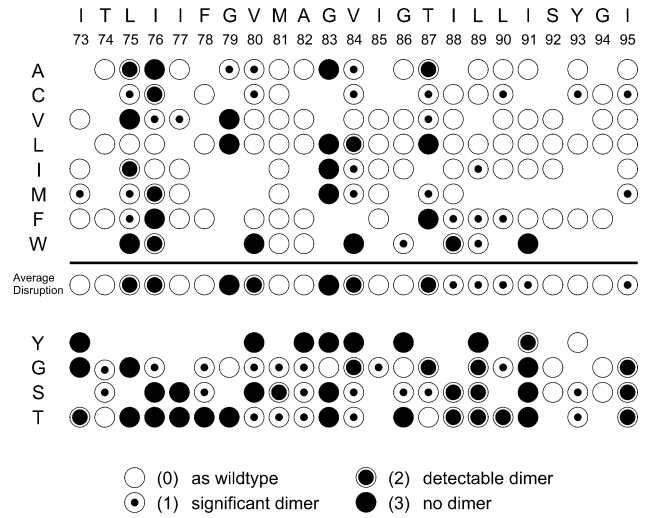

Figure 6. Effects of hydrophobic and slightly polar point mutations on glycophorin A transmembrane domain dimerization. Each mutant was given a phenotype based on the amount of dimer seen in SDS−PAGE (see legend). The average effects of mutations at each position were determined by averaging the numeric score for each observed mutant. Adapted with permission from ref 216. Copyright 1992 American Chemical Society.

dimerization.[216] Because the same substitutions at other leucine, isoleucine, glycine, and threonine residues within the membrane span do not influence dimerization, the disruptive effects of the hydrophobic mutations are consistent with sequence- and structure-specific effects on the dimer interface and are not simply a consequence of amino acid composition. The effects of these mutations most probably reflect their impacts on the details of helix−helix interactions, although the possibility that they modulate helix−lipid interactions cannot be formally excluded.

In the data set generated by Lemmon and colleagues,[216] motif sites showed differential sensitivity to substitutions, but at least one hydrophobic substitution at each motif site was able to completely abolish dimerization on SDS−PAGE. The dimerization of each mutant was classified as "wild-type", "significant dimer", "detectable dimer", or "no dimer". Because the effects of different substitutions at a *single* site often spans a range of phenotypes, the authors chose to also report and interpret an average of the effects of hydrophobic mutations at each site; polar substitutions were excluded from the average because they showed no site-specific tendencies. Based on the "average disruption", the relative importance of the motif sites is Gly83 > Gly79 ≈ Thr87 ≈ Leu75 ≈ Ile76 ≈ Val84 > Val80. Only tryptophan substitutions at Val80 and Val84 are fully disruptive, while all other motif sites have at least one aliphatic substitution that gives no dimer, suggesting that not only Val80 but also Val84 may be less critical for dimerization than the other motif residues. The highly disruptive potential of tryptophan is borne out by its effect at Ile91 (outside the motif but on the same face of the helix), which is the only fully disruptive mutation that is not contained in the motif.[216] (Note, however, that tryptophan substitutions at two sites in the middle of the helix but on the opposite face from the motif (Met81 and Ala82) give constructs that dimerize as wild-type.) The importance of the seven residue motif identified from the average disruption effects was experimentally confirmed by the demonstration that the motif residues are sufficient to confer dimerization in the context of a polyleucine membrane span.[217] Still, the highly varied effects of hydrophobic

substitutions at the individual motif sites, which are suppressed in the averaging process, suggest that the specificity of the self-association of the transmembrane domain of glycophorin A is not fully explained by the sequence and stability of the wild-type motif alone.

The insights available from the saturation mutagenesis study of the glycophorin A transmembrane domain derive in large part from the considerable number of mutants studied. While some sites in the transmembrane domain give similar results for all observed hydrophobic substitutions (any change at Gly83 abolishes dimerization, while changing Met81 to alanine, cysteine, valine, leucine, isoleucine, or tryptophan does not alter dimerization), many sites show more complex patterns that are appreciated only by the comparison of different mutants. The authors note that "with the exception of Gly83...it is possible to mutate any position in the transmembrane domain of GpA and still retain at least some propensity for dimerization".[216] Thus, any single point mutation may not suffice to characterize how a particular site contributes to helix−helix interactions.

Saturation mutagenesis therefore reveals the intricacies of the sequence dependence of glycophorin A dimerization, albeit at a considerable cost of time and effort. One suggestion for rapidly mapping the important region of such a dimer interface with a minimum of constructs is alanine insertion mutagenesis: insertion of a residue into a transmembrane span will alter the relative orientation of sequences to either side of the insertion, resulting in the interruption of any helical "face" that spans the insertion site. While this approach mapped the C-terminal side of the motif precisely, intermediate phenotypes obtained from insertions of alanine at the N-terminal flank suggested that the first two motif residues are less critical than the identity and spacing of the C-terminal portion of the motif.[218]

Polar substitutions identified in the saturation mutagenesis study of Lemmon and colleagues are almost always fully disruptive and often give rise to monomers of altered mobility on SDS−PAGE.[216] These effects were interpreted as influencing the nature of the detergent/protein complex with the introduced polar side chain probably interacting with the surface of the micelle. Such mutations may therefore affect dimer stability either by altering helix−helix interactions or by affecting the stability of the monomeric transmembrane α-helix (or by some combination of these effects). While the stability of the monomer can be ignored in the formalism of the two-stage model because of the influence of the bilayer, mutations may affect the stability of the helical monomer in detergents, especially if strongly polar residues or proline are introduced (as discussed extensively in section 3.3). By contrast, individual hydrophobic substitutions are not expected to affect stability of the monomer and their effects may therefore be interpreted in the context suggested by the two-stage model: such mutations influence dimerization by affecting helix−helix or helix−lipid interactions. Lemmon and colleagues are careful to point out that the physical basis for the effects of any particular mutation cannot be discerned from their data alone,[216] but the mapping of critical sites in the mutational data to one face strongly implicates that face in helix−helix interaction.

It is worth noting that placement of a stop codon at position 95 of the chimera abolishes dimerization, although this is more than two turns of helix from the dimerization motif.[215] As with the polar substitutions, it seems likely that the truncation of the membrane span and elimination of the flanking charges may alter the association of the hydrophobic span with the micelle. Deletion mutations within the C-terminal end of the glycophorin A transmembrane span also disrupt dimerization,[219] and a series of constructs with polyleucine hydrophobic spans of different lengths bearing a minimized interaction motif exhibit different extents of self-association when subjected to electrophoresis in alkyl sulfates of different chain lengths:[220] an 18-residue membrane span that dimerizes strongly in dodecyl sulfate is entirely monomeric in tetradecyl sulfate.[220] These indications that dimerization can be modulated by the "hydrophobic mismatch" of transmembrane span and the lipidic media may be manifestations of effects of mismatch on the stability of the helical monomer or may be caused by modulation of helix−helix interactions by the detergent.

Interestingly, mutations to proline are well tolerated at Leu75 and Phe78 and at both ends of the glycophorin A hydrophobic span,[221] suggesting that in some sequence contexts a proline within a detergent micelle can be accommodated in the (presumably) helical dimer. The tendency for glycophorin A to better tolerate proline at its N-terminal side is consistent with integration propensities from experiments with the translocon[192] and the statistical propensity for prolines to be involved in capping the N-terminal ends of helices in soluble proteins,[163,164] although the tendency for prolines to induce certain relative orientations of helices when inducing a kink[195] may also determine which positions can accept proline substitutions while maintaining dimerization. The position-specific effects are striking, but it is not clear whether the disruptive effects of proline at other positions within the span[221] result from effects on helix−helix packing or on the stability of helical monomer, or both. For residues that uniformly disrupt dimerization, such as strongly polar or charged substitutions, it is tempting to apply a thermodynamic scale such as that of Wimley and White[65] to explain the disruption as a partitioning of the helical folded monomer into the micelle interface.

4.1.2. Structural Basis for Dimerization

The wealth of qualitative information about the sequence dependence of glycophorin A dimerization in detergents obtained through mutagenesis studies, and the hypotheses that these data generated about the basis for transmembrane helix−helix interactions, provided considerable impetus to understand the structure of the dimer and to characterize its self-association in membranes. Analyses of FTIR data[222] and of a combination of CD, FTIR, and solid-state NMR data[223] indicate that the membrane spanning region of glycophorin A is α-helical and oriented roughly perpendicular to the membrane surface. Fluorescence resonance energy transfer experiments showed that the GpA membrane-spanning peptide self-associates in bilayers as dimers and not higher order oligomers or aggregates.[224] Rotational resonance solid-state NMR experiments using specifically labeled peptides identified close intermonomer contacts between Val80 methyl and Gly79 carbonyl carbons, as well as between Val84 methyl and Gly83 carbonyl carbons,[225] suggesting a "ridges-into-grooves" packing for the dimer structure. Additional solid-state NMR experiments have further defined the interface in lipid bilayers.[226−228]

The solution NMR structure of a dimeric 40 residue fragment of glycophorin A determined in detergent micelles provides an atomic-resolution view of the structural features underlying specific dimerization[229] (see Figure 7). The

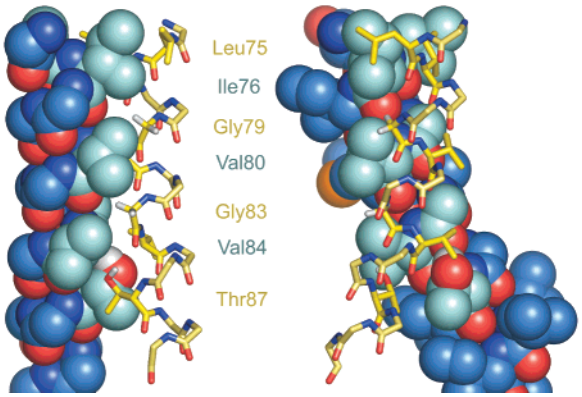

Figure 7. Representation of the helix−helix interface of the glycophorin A transmembrane dimer (PDB 1AFO). Intermonomer packing of backbone and side chain atoms of glycophorin A helices is revealed by juxtaposing a blue space-filling monomer with a yellow stick model. The lefthand dimer depicts only backbone and interfacial atoms; the righthand view is rotated 90° about the vertical (dyad) axis and all side chain atoms of the blue monomer are portrayed. Labels are at the level of the Cα atoms in both views and are colored to match the atoms closest to the viewer in the lefthand dimer. The hydrogen of the Thr87 γ hydroxyl is shown in all models, making an intramonomer hydrogen bond with the carbonyl of Gly83. The CαH atoms of Gly79 and Gly83 are shown in the stick models to indicate the geometry of interhelical CαH··· O hydrogen bonds.[234]

structure reveals a right-handed crossing of helices (as had previously been predicted by Fourier analysis of the mutagenesis data[216]) that exhibits close intermonomer packing of motif residues against one another with the two motif glycines allowing backbone−backbone contacts in a ridges-into-grooves manner[230] similar to that predicted from solid-state NMR data.[225] A very similar structure had been predicted by a modeling approach that treats the sequence as forming straight α-helices.[231] Mutagenesis work had also highlighted the importance of these two glycines: the only substitution at Gly79 that supports dimerization is alanine, while any substitution at Gly83 (even alanine) completely disrupts the dimer.[216] The relative effects of glycine-to-alanine substitutions at these two sites can be rationalized from the structure, since packing around Gly83 leaves no room for an additional methyl group while the region around Gly79 could accommodate the substitution with only a minor clash.[229] The only polar side chain in the interface, Thr87, does not form intermonomer hydrogen bonds as had been predicted from one modeling study[232] but is hydrogen bonded to a backbone carbonyl on the same helix. (Solid-state NMR data suggest that Thr87 may form an interhelical hydrogen bond to the carbonyl oxygen of Val84 in bilayers,[227] but a computational study indicates that the side chain makes intramonomer hydrogen bonds.[233]) The possible formation of intermonomer hydrogen bonds between glycine α hydrogens and carbonyl oxygens was noted by a subsequent analysis.[234] A simple modeling approach that introduced mutant side chains into the wild-type NMR structure using rotamers[235] showed that of the 15 hydrophobic substitutions identified by Lemmon and colleagues as completely disruptive,[216] only one (Ile76Ala) did not cause a serious intermonomer clash. Thus, steric incompatibility is the strongest destabilizing influence that can be easily accessed by changing the sequence of the protein. With the caveat that the effects of these mutations and the experimental NMR structure were obtained in detergents, and not in membranes, the sequence-specific dimerization of glycophorin A appears to be qualitatively explained by intermonomer packing and weak hydrogen bonding interactions of motif residues displayed on straight, ideal helices.

4.1.3. Energetics of Transmembrane Helix Dimerization

The two-stage model provides an excellent conceptual framework in which to consider the determinants of lateral helix−helix interactions within membranes, but making and interpreting thermodynamic measurements in these systems is neither simple nor straightforward. Sedimentation equilibrium analytical ultracentrifugation provides one route to the energetics of helix−helix interactions[236,237] and has been extensively applied to glycophorin A and sequence variants,[238−241] but this method requires the use of detergents and thus may obscure contributions of protein−lipid or lipid−lipid interactions that would be present in a membrane; effects of bulk bilayer properties such as lateral pressure are also lost. Fluorescence resonance energy transfer has been used to probe the monomer−dimer equilibrium of glycophorin A in a variety of detergents, and indeed both the free energy and the enthalpy of association depend on the identity and concentration of the detergent.[242,243] Fluorescence methods have also been applied to measuring free energies of helix−helix interactions in bilayers[244−246] although not for glycophorin A. DeGrado and colleagues have presented a method for quantifying helix−helix interaction energies through coupling of protein cross-linking to aqueous thiol−disulfide equilibria that, like fluorescence methods, can be used in detergents[247] or in membranes.[248] The unfolding free energies of polytopic membrane proteins (discussed in section 5) have been measured by varying the amount of a denaturing detergent in a mixed micelle system and monitoring the fraction of unfolded protein.[249−252] Thermodynamic parameters are extracted from the concentration dependence of oligomerization in pure detergent by considering the mole fraction of protein relative to micellar detergent;[253] similarly, in cases where a protein is being unfolded by a denaturing detergent, the micellar mole fraction of this detergent is the important parameter.[254] Quantitative measurement of the thermodynamics of membrane protein folding and oligomerization, combined with structural information, can be used to explain the observed sequence dependence of transmembrane helix−helix interactions.

The close agreement between the dimer interface identified by saturation mutagenesis[216] and the interface in the NMR structure[229] shows that disruptive mutations map predominantly to the dimer interface, which suggests that sequence changes that directly alter helix−helix contacts and interactions modulate self-association. However, explaining the sequence specificity of the lateral association of glycophorin A requires an appreciation of the energetic effects that mutations can have on dimerization. Such measurements have been made for the chimeric protein in detergents using analytical ultracentrifugation under conditions where the density of the detergent micelles matches that of the solvent.[236] Fleming and colleagues have presented analytical ultracentrifugation data and thermodynamic analyses for wild-type glycophorin A,[241] for a series of single alanine substitutions,[240,241] for multiple substitutions at each residue in the dimer interface,[238] and for a series of double alanine mutants.[239] These data provide a basis for a quantitative thermodynamic understanding of the lateral interactions between transmembrane α-helices.

The demonstration that single alanine substitutions in the glycophorin A transmembrane domain exhibit $\Delta\Delta G_{dimer}$

values from −0.5 to +3.2 kcal mol^{-1} quantifies the sequence specificity of dimerization[240] that was qualitatively apparent from the saturation mutagenesis data in SDS−PAGE.[216] Alanine substitutions away from the dimer interface have little or no effect on dimerization, while alanine substitutions at motif residues destabilize by between +0.4 kcal mol^{-1} (Val80Ala) and +3.2 kcal mol^{-1} (Gly83Ala). The authors show that the energies from ultracentrifugation correlate with the qualitative phenotypes previously reported from gels;[216] the rank ordering of the site-specific effects of alanine in the two methods is roughly the same. A correlation is also seen with in vivo measurements of association[240] (discussed further in section 4.1.4), suggesting that the thermodynamic measurements of the effects of mutations on dimerization in detergents parallel the sequence dependence of dimerization in membranes. Characterization of more than 20 additional single point mutants consisting of (mostly aliphatic) substitutions at the motif positions[238] extended the overall range of the effects of mutations only slightly (−0.5 to +3.8 kcal mol^{-1}). Thus, no mutations strongly stabilize the glycophorin A dimer, in contrast to findings for the helical bundle membrane proteins diacylglycerol kinase[255] and bacteriorhodopsin[252] (discussed in section 5.1). However, the range of effects seen at each position reveals significant differences between motif sites in how mutations affect the energetics of dimerization.

At Gly83, all aliphatic substitutions including alanine are strongly disruptive ($\Delta\Delta G_{dimer}$ of +3.1 to +3.8 kcal mol^{-1}), confirming the critical importance of this residue to glycophorin A dimerization.[238] Large aliphatic substitutions at Gly79 also result in strong disruption ($\Delta\Delta G_{dimer}$ ranging from +2.6 to +3.6 kcal mol^{-1}), while Gly79Ala is destabilized by only 1.7 kcal mol^{-1} compared to wild-type, consistent with the more moderate phenotype for this substitution from SDS−PAGE.[216] Mutations at Thr87 exhibit a wide range of effects, with $\Delta\Delta G_{dimer}$ varying from +0.8 to +3.2 kcal mol^{-1}. Mutations at any of these three motif positions can destabilize the dimer by more than 3 kcal mol^{-1}, but while *any* mutation at Gly83 has this dramatic effect, one mutation at Gly79 has an intermediate effect on dimerization, while two changes at Thr87 affect self-association only mildly.

By contrast, the substitutions examined at the other four motif positions destabilize the dimer by no more than 1.8 kcal mol^{-1}, or about as much as the *least* destabilizing substitution at Gly79. Substitutions at Leu75 ($\Delta\Delta G_{dimer}$ of +1.4 to +1.7 kcal mol^{-1}) and Val84 ($\Delta\Delta G_{dimer}$ of +0.8 to +1.6 kcal mol^{-1}) have rather uniform, moderately destabilizing effects. Certain substitutions at Ile76 ($\Delta\Delta G_{dimer}$ of 0.0 to +1.8 kcal mol^{-1}) and Val80 ($\Delta\Delta G_{dimer}$ of −0.4 to +1.8 kcal mol^{-1}) can be as destabilizing as at the previous two positions, but other mutations do not disrupt the dimer at all or can indeed slightly stabilize the interaction.

To explain the effects of these mutations, Fleming and colleagues modeled the side chains of mutant sequences in the context of the backbone structure of the wild-type sequence to generate model structures; these models were then scored for changes in favorable occluded surface area, unfavorable occluded surface area, and side chain rotamer entropy.[238] Regression analysis against 23 experimental $\Delta\Delta G_{dimer}$ values (eight mutants with severe steric clashes and two additional outliers were excluded) yielded best-fit coefficients that were able to explain about 80% of the variance in the experimental data. This excellent agreement suggests that helix−helix packing and steric interactions at the dimer interface can explain most of the energetic effects of mutations on dimerization, and the coefficients indicate that 1 Å^2 of favorable occluded surface corresponds to approximately 40 calories of favorable free energy of interaction. Examining the contributions of the various empirical terms to the calculated stability of a given mutant reveals how the interplay of these factors determines dimer stability.[238]

Insight into the basis of dimerization is also gained by examination of the excluded data points and outliers in this fitting process. Those mutations that give strong steric clashes, including any substitution at Gly83, were removed from the fitting process described above[238] because they were skewing the fit. These mutations are all strongly disruptive, and the combination of the thermodynamic data and the structural rationale for the effects of the substitutions argues for steric clashes as a means of defining the sequence specificity of interactions between transmembrane helices. Interestingly, however, these mutant proteins can still form measurable amounts of dimer even at detergent-to-protein ratios of 1000:1. These substitutions cannot be compatible with the structure of the wild-type dimer due to severe steric clashes, so the structural basis for association of this class of mutant is not clear. Thus, the exclusion of these mutants from the structure-based analysis on statistical grounds is further supported by the recognition that the contributions to helix−helix interactions for these variants cannot arise from the same interface used to model the other substitutions.

The outliers Ile76Gly and Ile76Leu are predicted by the structure-based calculations to be highly destabilizing (about +2.5 kcal mol^{-1}) but are experimentally determined to be only slightly destabilizing (0.9 and 0.7 kcal mol^{-1}, respectively). The thermodynamic measurements are in good agreement, however, with the qualitative data from SDS−PAGE,[216] where Ile76Gly is slightly disruptive and Ile76Leu dimerizes as wild-type. As with the mutations that generate large clashes, the observed extent of dimerization of these outliers cannot be rationalized using the wild-type solution NMR structure. Because the NMR structure is consistent with the majority of the experimental thermodynamic data, it provides a reasonable approximation of the relevant dimeric state of the wild-type protein (although inaccuracies at certain regions within the structure may be responsible for some of the discrepancies between model and experimental energies). However, the comparatively low but still measurable dimerization of mutants that introduce strong clashes in the wild-type interface (Gly83Ile) and the strong dimerization of mutants that lose important packing interactions (Ile76Gly) probably indicate that alternate interfaces are being formed.

Doura and Fleming have also compared the free energy changes of single alanine substitutions at each of the seven motif residues of glycophorin A[240,241] to the complete set of pairwise combinations of motif double alanine substitutions.[239] The 21 double mutants destabilize the dimer by as little as 0.1 kcal mol^{-1} or more than 4.8 kcal mol^{-1}. The precision of the measurements (errors of about 0.2 kcal mol^{-1}) permits a thermodynamic cycle analysis to assess additivity of the alanine double substitutions, which reveals that most double mutants are as stable as or slightly more stable than the sum of the single mutations. However, five double mutants are at least 1 kcal mol^{-1} more stable than the sum of the single mutants (negative thermodynamic coupling), while two double mutants are much less stable than the sum of the single mutations (positive coupling of

2.4 kcal mol^{-1} or more). Examining these thermodynamic cycles reveals the complexity of the interactions that support glycophorin A dimerization.

The single mutations Leu75Ala and Val84Ala reduce dimer stability by about 1.3 and 1.0 kcal mol^{-1}, respectively,[240,241] while the double mutant Leu75Ala/Val84Ala is only 0.1 kcal mol^{-1} less stable than wild-type. The single point mutations show that each residue contributes to stability, but the double mutant indicates that compensatory effects can actually restore dimerization. A very different result is seen when Leu75Ala and Thr87Ala are examined in the double mutant cycle. The single mutation Thr87Ala reduces dimer stability by about 0.9 kcal mol^{-1}, which is essentially indistinguishable from the effect of Val84Ala described above.[240] Although Leu75Ala/Val84Ala is as stable as wild-type, the double mutant Leu75Ala/Thr87Ala gives no detectable dimer whatsoever![239]

If taken in isolation, the wild-type level of dimerization exhibited by the double mutant Leu75Ala/Val84Ala could suggest that neither of these two residues is important for dimerization. However, the catastrophically disruptive combination Leu75Ala/Thr87Ala shows that these two residues act in concert to stabilize the dimer; another study has also noted synergistic effects of residues at positions 75 and 87 in promoting dimerization.[256] Of course, both qualitative and quantitative data for single point mutations at Leu75 and Val84 also indicate that these sites can contribute significantly to dimerization.[216,238] It should be noted that since these pairs of residues do not interact across the dimer interface or within a single helix, the structural basis for the compensatory or synergistic effects is not at all clear. These analyses reveal not only the complexities of interactions in the glycophorin A transmembrane dimer but also the danger associated with interpreting the results of double (or multiple) mutants in the absence of data for each of the single mutants. The question of whether these mutants self-associate with a wild-type-like interface remains open, but because the changes are large-to-small mutations, no clashes would prevent the wild-type interface from forming.

4.1.4. Biological Assays of Helix–Helix Association

Relating measurements of the folding or stability of a membrane protein in detergents to the behavior of the protein in lipid bilayers is a major challenge for the study of membrane protein folding. Examination of the sequence dependence of glycophorin A helix−helix interaction energetics in membranes has been made possible by various biological assays. Langosch and colleagues developed the ToxR system[257] for measuring lateral interactions between helices in the inner membrane of *E. coli*. This assay is based on the properties of ToxR,[258] a membrane-spanning transcriptional activator[259] that can increase expression from the *ctx* promoter 100-fold in *E. coli*.[260] In-frame fusion of the ToxR cytoplasmic domain to a transmembrane span of interest and to a periplasmic maltose binding domain permits expression of a chimeric protein that is directed to the inner membrane, with its ToxR domain in the cytoplasm. This chimera activates the *ctx* promoter in a manner that depends on dimerization of the transmembrane span, driving expression of the chromosomal reporter gene β-galactosidase[257] (see Figure 8). A similar assay subsequently developed by Russ and Engelman called TOXCAT[261] uses a plasmid-borne reporter construct in which the *ctx* promoter drives expression of chloramphenicol acetyltransferase (CAT); dimerization-induced expression of CAT can be quantified in lysates or used to select interacting sequences from libraries based on bacterial resistance to chloramphenicol. Langosch and colleagues have also developed a selectable version of their assay, termed POSSYCCAT, which uses a chromosomal copy of the CAT gene.[262]

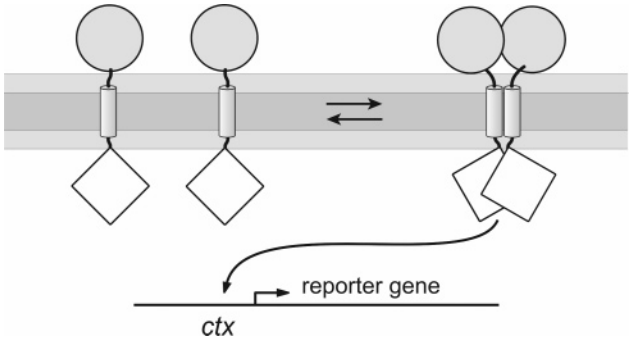

Figure 8. Schematic for ToxR-based assays of transmembrane helix−helix association. Interactions between transmembrane domains (cylinders) brings the ToxR DNA binding domains (diamonds) together, enabling them to bind the *ctx* promoter and drive expression of a downstream reporter gene.[257] The maltose binding protein domain (circle) helps the fusion construct insert into the cell membrane and provides controls for assaying topology. Adapted with permission from ref 257. Copyright 1996 Academic Press (Elsevier Ltd).

In these assays, reporter gene expression is quantified using activity assays of cleared cell lysates, and the effects of mutations in the transmembrane region on the level of reporter gene expression are interpreted as effects on the extent of dimerization of the construct. All of these assays depend on controls to ensure that the chimerae are inserted in the membrane in the correct orientation and that sequence changes are not altering the amounts of expressed or membrane-inserted chimerae. Significant background levels of reporter gene expression in the absence of the membrane-inserted chimera lower the sensitivity of the assay and complicate the interpretation of the data. Nevertheless, these methods (and others[263−265]) have been shown to provide at least the rank order of oligomer stability for transmembrane domains (and their sequence variants) in real membranes.

Langosch and colleagues used glycophorin A as a positive control to characterize their ToxR transmembrane helix homodimerization assay and performed an alanine scanning mutagenesis on their construct.[257] Mutations at interfacial residues showed varying effects on reporter gene expression, with Gly83Ala exhibiting the largest drop (about a 4-fold decrease). The rank order of the phenotypes roughly correlates with the SDS−PAGE data available at that time[216] and the thermodynamic data that have been reported since.[240,241] Because the strongly disruptive mutation Gly83Ala gives only a 4-fold decrease in signal, whereas the same mutation lowers the association constant by a factor of 150 in the ultracentrifuge[238] ($\Delta\Delta G$ of +3.1 kcal mol^{-1}), it appears that the ToxR assay is only weakly sensitive to sequence changes. The difference between measured $\Delta\Delta G$ values and ToxR signals could be caused by the monomer−dimer equilibrium being pushed very far toward dimer in the membranes of *E. coli* by high effective protein concentration, the favorable lipidic environment, or both. However, the approximate linear correlation seen between the sedimentation equilibrium data and the ToxR data for the alanine point mutants[240] suggests that the wild-type ToxR fusion protein is not overwhelmingly dimeric in the *E. coli* membrane. The

source of the apparent low sensitivity could also be high level background reporter gene expression: although the reporter strain used shows endogenous β-galactosidase activity corresponding to only about 5% of the signal measured for the optimal glycophorin A construct, most membrane-anchored constructs seem to give significant basal reporter gene expression[266] even if their transmembrane domains do not interact by other measures.

The TOXCAT assay has also been used to characterize the sequence dependence of self-association for glycophorin A.[261] As for the ToxR case, the effects of mutations correlate with the disruptive effects in SDS−PAGE[216] and in the ultracentrifuge.[240,241] The reported error bars for the TOXCAT data are somewhat smaller and the range of the reported effects is somewhat larger than for ToxR: the strongly disruptive mutation Gly83Ile reduces the chloramphenicol acetyltransferase reporter gene signal by a factor of 12, while this mutation lowers the association constant by a factor of 300 in the ultracentrifuge[238] ($\Delta\Delta G_{dimer} = +3.5$ kcal mol^{-1}). Single point mutations that reduce association only slightly in detergents, such as Thr87Ala or Ile76Leu ($\Delta\Delta G_{dimer} = +0.8$ and $+0.7$ kcal mol^{-1}, respectively[238,240]) decrease the TOXCAT signal by 35−40%, demonstrating that (as in the ToxR assay) the fusion protein is far from completely associated in the native membrane. Although TOXCAT appears to provide a bit more discriminating power than ToxR, both assays have limited effective ranges (especially compared to the biological system on which they are based[260]) that can make it difficult to reliably rank order mutants and thus to determine whether mutations have similar or different effects in detergents and in membranes. Nevertheless, aspects of the sequence dependence of glycophorin A dimerization have been studied using these assays.

Brosig and Langosch analyzed the relative contributions of the motif residues of glycophorin A to dimerization using the ToxR assay to determine whether the minimal motif in detergents corresponds to the minimal motif in membranes.[266] The seven residue motif grafted into a polymethionine or polyvaline membrane span gave a reporter signal that was as strong as wild-type glycophorin A, although this signal was only about 3-fold larger than polymethionine alone. The pair of motif glycines in the context of polymethionine gave a signal that was almost as strong as wild-type glycophorin A, while the polyvaline host sequence required two or three additional residues from the motif to give near wild-type reporter gene expression.[266] These results confirmed the critical importance of the two motif glycine residues.[216,229,231]

Russ and Engelman showed that while hydrophobic substitutions have similar effects under conditions of SDS−PAGE and TOXCAT, polar substitutions away from the dimer interface that are disruptive in SDS−PAGE still support dimerization in TOXCAT.[261] This confirms the hypothesis previously used to exclude polar substitutions from the averaging process in the SDS−PAGE saturation mutagenesis study:[216] polar substitutions at or away from the glycophorin A dimer interface disrupt the detergent-solubilized dimer by destabilizing the helical monomer. By contrast, and as expected from the two-stage model, the same glycophorin A sequence variant is locked into a helical structure when in a bilayer and dissociates only if the mutation in question affects lateral interactions between helices.

Addition of synthetic glycophorin A peptides has been shown to disrupt the dimerization signal of a ToxR−GpA construct in *E. coli*.[267] A peptide containing the wild-type glycophorin A sequence, or a substituted version with an intact dimerization motif, can inhibit the ToxR signal over background by almost 50%, while a scrambled version of the peptide that binds membranes as well as wild-type has no effect on the ToxR signal of cells.[267] The sequence-specific nature of the effect suggests that the exogenous peptide inserts into the *E. coli* inner membrane and disrupts ToxR−GpA dimerization by formation of ToxR−GpA/peptide heterodimers, but the complexity of the biological assay system allows other explanations. It is noteworthy, however, that several groups have independently reported the ability of exogenously supplied transmembrane peptides to interfere with the biological function of the protein from which their sequence is derived,[268−273] as discussed in section 4.4.

4.2. Helix–Helix Interaction Motifs

In cases where the two-stage model is largely correct and canonical transmembrane helices associate into higher order structures within the membrane, then the specificity of helix−helix interactions will be determined by the side chains displayed along the α-helix. The potential for useful applications of this structural idea has been demonstrated by the ability of a computational search algorithm to correctly identify the structure of the glycophorin A dimer[229,231] and by the success of structure-based approaches to predict the effects of point mutations on glycophorin A dimerization.[235,238,241] Structure prediction for bundles of single spanning helices may require very little external information,[274] although the limitations of assuming that helices are canonical must be acknowledged.[94] Simplifying matters one more step, by abstracting the determinants for lateral interactions between helices into sequence motifs, seems similarly straightforward and is supported by the example of the strong dimerization of a construct carrying the glycophorin A motif residues in the context of a polyleucine helix.[217] However, it is important to recall that every glycophorin A interfacial residue except Gly83 has at least one substitution that allows retention of significant dimerization. Accordingly, several mutations at any given position are needed to demonstrate the *lack* of involvement of that residue in association. The complexity of the glycophorin A system as evidenced by strong positive or negative coupling between motif sites[239] should also encourage a conservative approach to the identification of such motifs. Senes and DeGrado have recently reviewed the roles of sequence motifs in transmembrane helix−helix interactions.[5]

4.2.1. GxxxG and Variants

Russ and Engelman identified the GxxxG motif as the most significant feature of a set of more than 100 highly dimeric transmembrane domains identified with a TOXCAT selection scheme[261] (described in section 4.1.4) from two libraries of approximately 10^7 possible sequences.[275] This motif corresponds to the spacing of the critical dimerization motif glycines in glycophorin A (Gly79 and Gly83) whose importance was identified by saturation mutagenesis[216] and confirmed using the ToxR assay in vivo.[266] A statistical analysis of membrane span sequences revealed that the GxxxG spacing is the most over-represented pairwise spacing of residues in transmembrane helices compared to random expectation.[89] The combination of these two observations—that the motif is over-represented in genomes and can support

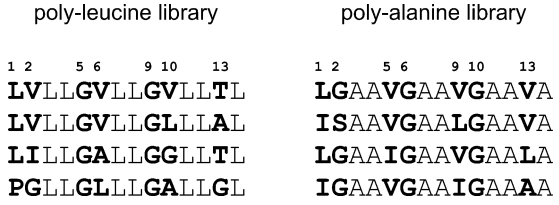

Figure 9. Examples of strongly self-associating transmembrane spans isolated by Russ and Engelman[275] using TOXCAT selection of random libraries. While most sequences from each library contain the GxxxG dimerization motif, those in a polyleucine background have the motif at positions 5 and 9, while those in a polyalanine background have the motif at positions 6 and 10. Only 1 of 49 sequences from the polyleucine library has a glycine in position 6 without also having a GxxxG at positions 5 and 9; only three glycines occur at positions 5 or 9 in the 72 sequences from the polyalanine library.[275]

strong helix−helix interactions—suggests that many (but not all) GxxxG pairs in transmembrane helices will be involved in forming specific intramembranous structures. The role of the sequence motif GxxxG in membrane protein structure and stability has been reviewed recently.[5,276]

In the strongly dimerizing clones identified by Russ and Engelman from a polyleucine context, the glycophorin A motif was not found (presumably because the large random library was not fully sampled), but one sequence that differed from the seven residue motif at just a single position was identified[275] (see Figure 9). However, nine clones differed from glycophorin A at two sites, 21 clones differed at three sites, 13 clones differed at more than three sites, and two clones had no residues other than the GxxxG in common with the glycophorin A motif.[275] Thus, the library data of Russ and Engelman demonstrate that GxxxG-containing sequences that differ very substantially from glycophorin A can mediate tight dimerization in *E. coli* membranes.[275] The nonrandom distribution of residues in the selected libraries[275] suggests that there is some specificity to the flanking sequences that help support GxxxG-mediated dimerization. The strong dependence of the position of the GxxxG motif on the identity of the flanking, nonrandomized residues (see Figure 9) shows that the self-association is not a function only of the residues targeted for selection. Note that although the library data indicate that these sequences interact tightly, they cannot say whether the various GxxxG containing sequences interact with the same backbone geometry and helix crossing angle as one another.

Although many sequence contexts can apparently support dimerization of a GxxxG motif, it is equally clear that dimerization of the GxxxG motif within the glycophorin A transmembrane domain is sensitive to single point mutations at *each* of the other five motif residues.[216,238,240,241] Indeed, single point mutations that keep the GxxxG motif of glycophorin A intact still modulate stability over a range of 3.7 kcal mol^{-1} or a factor of 400 in association constant, and most of these thermodynamic stabilities can be rationalized using a structure-based approach[238] that assumes that the dimer backbone geometry remains unchanged from the wild-type NMR structure.[229] However, the evidence from 21 double alanine mutants for strong thermodynamic coupling between sites at opposite ends of the glycophorin A dimer interface[239] argues that the effects of multiple mutations may not be as easily interpreted in structural terms. This suggests that the simplified GxxxG motif may be compatible with more than one dimer structure.

The effects of multiple mutations in the context of a GxxxG motif have also been studied by generating transmembrane domains based on the M13 sequence, which contains a GxxxG motif. The native M13 sequence, or variants bearing mutations corresponding to one, two, three, or all five of the remaining motif residues from glycophorin A, were assayed for self-association using SDS−PAGE and TOXCAT.[256] This work was based on a sequence and structural alignment between modeled M13 and experimental glycophorin A structures, and the authors explain synergistic effects of introducing Leu75 and Thr87 into the M13 sequence with a structural model in which steric interactions at the N-terminal end of the motif modulate GxxxG-mediated packing to permit or deny intermonomer hydrogen bonding by the Thr87 side chain.[256] Formation of such an intermonomer hydrogen bond is consistent with solid-state NMR structural measurements of wild-type glycophorin A peptides in bilayers[227] but not with recent electrostatic calculations based on this structural model[233] or with the wild-type solution NMR structure in detergent.[229] Recently published $\Delta\Delta G_{dimer}$ values for glycophorin A mutants, where Thr87Ala (+0.9 kcal mol^{-1}) and Thr87Ser (+0.8 kcal mol^{-1}) disrupt dimerization to the same extent, also argue against side chain hydrogen bonding contributing substantially to dimerization.[238] Because potential structural rearrangements are subtle and because of the possibility that mutations may cause different effects in lipid bilayers than in detergents, high(er) resolution structural data for GxxxG motifs in different sequence contexts and in different lipidic environments may be needed to further dissect the similarities and differences between these dimer interfaces.

Close packing of helices at a GxxxG motif may also allow the formation of Cα−H···O or Cα−H···O═C hydrogen bonds. After ab initio calculations showed that interaction energies of as much as −3.0 kcal mol^{-1} could result from such hydrogen bonds,[277,278] a survey of membrane protein structures identified nearly 150 potential backbone to side chain or backbone to backbone hydrogen bonds involving Hα atoms, including several such contacts in the structure of glycophorin A.[234] Arbely and Arkin used FTIR of lipid-reconstituted glycophorin A peptides to measure a small difference between the asymmetric stretch frequencies of a deuterium labeled glycine at position Gly79 in the context of a wild-type sequence and a nondimerizing mutant (Gly83Ile); from a previously established empirical correlation, this frequency difference suggests that the Cα−H···O═C hydrogen bond contributes −0.9 kcal mol^{-1} to glycophorin A dimerization.[279] Mutations that change the packing at the GxxxG motif only subtly might be able to modulate this interaction energy substantially. However, when Bowie and colleagues mutated Thr24 in bacteriorhodopsin, whose side chain oxygen was similarly implicated as an acceptor of an interhelical Cα−H···O hydrogen bond from an alanine, their measurements showed that replacing the threonine with alanine or serine is slightly stabilizing while valine is slightly destabilizing.[280] A recent computational study[233] using molecular mechanics and an implicit model for the membrane[281] to calculate interaction energies for the neutral groups that contain the putative hydrogen bond donors and acceptors seems to reconcile these results. In these calculations, the contacts identified in glycophorin A (and many other membrane helix−helix interactions) are stabilizing, while those identified by similar geometrical criteria at Thr24 of bacteriorhodopsin are not, primarily because of the

spatial arrangement of the donor Cα and its amide N with respect to the acceptor oxygen. Putative hydrogen bonds involving a glycine donor are also seen, on average, to be about twice as energetically favorable as those involving other amino acids.[233]

The high incidence of the GxxxG motif in transmembrane domains has led to the examination of the role of such motifs in assembly and biological function of many single-spanning membrane proteins. The ability of hepatitis C envelope glycoproteins E1 and E2 to form noncovalent heterodimers after being cleaved from a single polyprotein is abolished by alanine insertions in the transmembrane domain of E1 that interrupt either a GxxxG motif or other portions of the transmembrane domain.[282] The membrane spans of subunits *e* and *g* of the yeast F_1F_o ATPase contain GxxxG motifs that appear to influence the functional assembly and oligomerization state of the ATP synthase complex in vivo.[283−285] Mutation of subunit *e* motif glycines to alanine or leucine results in loss of subunit *g* from the complex and the loss of dimers and oligomers of the complex.[283] Subunit *g* can be chemically cross-linked to subunit *e*, and mutation of glycines in a GxxxG motif in the transmembrane domain of subunit *g* affects the assembly and function of the ATP synthase complex.[284,285] These data suggest that heterodimerization of these two domains might be mediated by these GxxxG motifs, but because subunits *g* and *e* can still be cross-linked to one another when the GxxxG motif of subunit *g* has been mutated, the authors suggest that other interactions may also help to support the association of these subunits with another as yet unidentified protein.[284,285] Interestingly, cross-linking of the subunit *e* homodimer increases when the GxxxG motif of subunit *g* is disrupted;[284] there may be alternate interaction possibilities for these transmembrane spans.

Tandem GxxxG motifs can drive helix−helix interactions. The secreted protein toxin VacA from *Helicobacter pylori* that inserts spontaneously into eukaryotic membranes contains an N-terminal hydrophobic span that can mediate self-association in the TOXCAT assay.[286] This span contains three tandem GxxxG motifs composed of glycines at positions 14, 18, 22, and 26; Gly14 and Gly18 are important for self-association in TOXCAT whereas Gly22 and Gly26 are not.[287] The proline residue at position 9 is dispensable for self-association in the TOXCAT assay but is required, as are Gly14 and Gly18, for the ability of the full-length toxin to form anion selective channels and disrupt the integrity of the membrane of cultured cells.[287] A computational model for the structure of hexameric VacA suggests that the architecture of this pore is similar to the heptameric pore MscS from *E. coli*.[288] The single transmembrane domain of BNIP3, a pro-apoptotic member of the Bcl-2 super-family, has been shown to support sequence-specific dimerization in the TOXCAT assay and in detergents.[289] Mutations at motif residues in a tandem AxxxGxxxG sequence disrupt dimerization, and a pair of flanking polar residues is also important to dimerization.[289]

GxxxG motifs have also been implicated in the function, assembly, and targeting of polytopic membrane proteins. The first membrane span of the α factor receptor from *Saccharomyces cerevisiae*, a G-protein coupled receptor, contains a GxxxG motif that helps to mediate oligomerization and cell-surface expression of GFP- and YFP-tagged receptors in vivo.[290] Transmembrane domain interactions are also important for the assembly of the γ-secretase complex, which generates intramembranous proteolytic events important in Notch signaling and implicated in familial Alzheimer's disease.[291,292] Two tandem GxxxG motifs in the fourth membrane span of APH-1 (one of four components of the γ-secretase complex) are important to assembly of the complex,[293] although other interactions may help support assembly and these residues may be dispensable for activity once assembly has occurred.[294] Tandem GxxxG motifs (including variants where glycine is replaced by alanine) have also been observed at transmembrane helix−helix interfaces in the crystal structures of the polytopic proteins aquaporin-1[295] and the glycerol facilitator[296] although the contributions of these motifs to assembly and stability of these proteins is not known.

On the other hand, not all GxxxG motifs drive strong helix−helix interactions. Equilibrium analytical ultracentrifugation of a chimeric protein encoding the transmembrane domain of the CCK4 oncogene shows that the purified protein exhibits no tendency to self-associate in detergents except in the limit where micelles are insufficiently abundant and the proteins are stochastically co-sedimenting.[297] Sedimentation equilibrium studies also show that the transmembrane domains of ErbB receptors do not homo- or hetero-oligomerize in detergents,[298] although contrasting results using the TOXCAT assay,[299] the ToxR assay,[300] and a biological inhibition strategy[273] suggest that these sequences do support interactions within membranes. Similarly, transmembrane spans that contain GxxxG-like motifs may interact through other residues: the sixth transmembrane domain of *E. coli* YjiO was identified as a moderately strongly associating transmembrane domain by screening a genomic library, but a GxxxA motif present in the transmembrane domain appears to be unimportant for self-association, while the substitution of bulky flanking residues significantly decreases oligomerization.[264]

4.2.2. Polar Residues

Hydrogen bonding between transmembrane helices involving strongly electronegative atoms has been explored extensively using protein design approaches. Work from the DeGrado and Engelman laboratories showed that the presence of an asparagine residue in a hydrophobic transmembrane span can drive self-association of the transmembrane domain.[301,302] A single asparagine converts a chimeric protein monomer into a protein−protein dimer on SDS−PAGE; an asparagine also dramatically increases the TOXCAT dimerization signal of a hydrophobic span.[302] A designed hydrophobic peptide bearing an internal asparagine forms trimers on SDS−PAGE, and FRET measurements showed that these trimers also form in nonionic or zwitterionic detergents.[301]

Sedimentation equilibrium data for a designed host peptide with a series of guest residues showed that aspartic acid or glutamine stabilized trimer formation by about 1.6 kcal mol^{-1} relative to alanine, glutamic acid or asparagine stabilized the trimer by about 1.0 kcal mol^{-1}, and slightly polar residues did not appreciably affect trimer stability.[303] While a host sequence of 19 leucines provides weak dimerization signal in TOXCAT (25% of wild-type glycophorin A), a single asparagine, aspartic acid, or glutamic acid guest residue at position X in the sequence Leu_7XLeu_{11} increases self-association more than 4-fold.[304] Histidine also has a strong effect on oligomerization, while slightly polar residues serine, threonine and tyrosine have modest effects.[304] Differences between the reported oligomeric states of these peptides and chimeric proteins may reflect differences in the sequence

context within which the polar residues reside, but the detergent used can also influence the order of the oligomer.[305] Systematic asparagine substitutions demonstrated that the position of the polar residue within the micelle influences the contribution to stability: asparagine at positions expected to be buried in the middle of the micelle strongly stabilizes the trimer, while asparagines at the micelle/water interface do not.[306] A similar positional dependence was also seen in ToxR studies and SDS−PAGE analysis of a polyleucine sequence hosting a single asparagine.[307]

DeGrado and colleagues have also shown that the specificity of lateral interactions between transmembrane helices can be modulated by protein design of either the transmembrane domain or flanking aqueous domains. While a designed membrane span with a single asparagine forms a mixture of dimers and trimers, addition of coiled-coil aqueous extensions previously shown to favor either a dimer or a trimer specifically stabilized one form over the other.[308] A membrane spanning peptide lacking an aqueous extension but having two transmembrane asparagines also forms a specific trimer,[306] and solid-state NMR structural characterization of this species indicates that a leucine expected to participate in the trimer interface shows restricted motion.[309] This interplay between stability and specificity of intramembranous and juxtamembranous portions of helical membrane proteins indicates that lateral associations between transmembrane domains can be coupled to the folding of soluble domains in ways that can be controlled by rational design.

Because interactions between strongly polar residues can drive oligomerization of transmembrane domains, it is important to understand how such interactions may be controlled to prevent nonspecific association in vivo. Sequence context has been shown to affect the self-association of biological sequences that contain strongly polar side chains in the transmembrane region. The transmembrane domain of BNIP3 dimerizes tightly in membranes and in detergents, and polar residue His173 is critical to this interaction; a slightly polar residue, Ser172, is also critical to dimerization, consistent with the possibility that these residues might participate in intermonomer hydrogen bonding.[289] However, small-to-large point mutations on the same face of the helix strongly disrupt the dimer in both SDS−PAGE and TOXCAT, suggesting that a particular backbone arrangement supported by GxxxG motifs allows the favorable hydrogen bond to form. This interpretation of the limited data from the BNIP3 system is based in part on the marked disruptive effects of residues that introduce steric clashes in the glycophorin A interface.[216,229,235,238] However, polar side chains and the residues that flank them do not always show the same importance to self-association. Engelman and colleagues tested the dimerization propensity of four biological transmembrane domains containing a strongly polar amino acid and showed that the glutamine in the transmembrane domain of Tnf5 was critical for tight self-association in TOXCAT, while the other three sequences associated weakly or not at all.[310] The slightly polar residues in the Tnf5 transmembrane domain, unlike Ser172 of BNIP3, are dispensable for dimerization.[310] It is possible that the sequences flanking the glutamine of Tnf5 provide unique packing interactions that stabilize the dimer or the sequences that flank the polar residues in the other spans may prevent close approach of the hydrogen bonding groups.

A selection scheme developed by DiMaio and colleagues to identify transmembrane domains capable of binding and activating the platelet-derived growth factor receptor β (PDGFβ receptor, a receptor tyrosine kinase) has yielded insight into the role of polar residues in promoting functional interactions in the membranes of living cells.[311] The homodimeric bovine papillomavirus protein E5 interacts with the PDGFβ receptor and activates it, causing cell transformation, in a way that depends on a glutamine within its otherwise highly hydrophobic transmembrane domain.[312−314] From a random library that retains the previously identified glutamine but randomizes 15 flanking hydrophobic amino acids, approximately 10% of the sequences were able to dimerize and activate the PDGFβ receptor. Statistical analysis of the position-dependent differences between transforming and nontransforming sequences suggests that, relative to the glutamine at position 17, positions 18, 20, 24, and 28 are most important, with position 18 favoring leucine and the other three favoring valine.[311] These positions map to one face of an α-helix and suggest that sequence context for the polar glutamine modulates steric interactions between helices. When the glutamine site is included in the randomization, only 1% of clones are transforming, and polar residues are under-represented in the transforming sequences.[315] Many constructs containing one or two polar residues are nontransforming. A single glutamine, glutamic acid, or aspartic acid is seen in most transforming membrane spans, with one-third of these polar residues occurring at position 17 and the remainder on the same face of the helix. However, almost one-quarter of the transforming membrane spans have no polar residues, indicating that intermonomer hydrogen bonds are not necessary for functional interactions and further supporting the importance of favorable packing.[315] While interpretation of these results is complicated by the possibility that functional interactions may depend on transmembrane homodimerization of E5, binding of E5 to the PDGFβ receptor, or both, it is clear from these libraries that polar residues can drive functional interactions only in certain sequence contexts.

Slightly polar side chains can cooperate to drive transmembrane domain self-association. Analysis of strongly associating sequences selected by TOXCAT from a library biased toward right-handed interactions but without permitting the use of glycine revealed motifs involving serines and threonines such as **X**SxxSSxxT or S**X**xxSSxxT (x is leucine); interestingly, the boldfaced residues **X** are compatible with proline.[316] Single point mutations of any of the serines (to alanine) or threonines (to alanine or valine) in the transmembrane sequences LAxxSSxxSSxxT or LSxxSPxxSSxxT dramatically reduce the dimerization signal, indicating that the association is stabilized by contributions from each of these hydrogen bonding residues. Mutations of proline to alanine or glycine are also strongly disruptive;[316] the presence of proline may affect helical geometry,[195] free a carbonyl group to accept a hydrogen bond more strongly, or both. An analysis of membrane protein structures suggests that isolated serines or threonines may also contribute to specificity and stability of helix−helix interactions through hydrogen bonds.[317]

Inappropriate interhelical hydrogen bond formation by polar side chains introduced by mutations may be the molecular basis for some disease states.[318] Deber and colleagues have expressed a fragment of the cystic fibrosis transmembrane regulator chloride channel (CFTR; discussed in section 6.3) containing membrane spans 3 and 4 as well as the connecting loop[319] and have shown that mutation

L_{16}	LLLLLLLLLLLLLLL
AZ2	LLAALLALLAALLALL
	a de ga de g
$L_{18}A_6$	LLLLLLAALLALLAALLALLLLLL
L_{24}	LLLLLLLLLLLLLLLLLLLLLLLL

Figure 10. Leucine zipper related sequences. Langosch and colleagues used span L_{16} and AZ2 in the ToxR in vivo assay;[322] Renthal and Velasquez used peptides $L_{18}A_6$ and L_{24} (flanked by two lysines on either side) for in vitro analyses.[323]

Val[232]Asp in the fourth membrane span that affects CFTR function in vivo results in formation of a hydrogen bond between Asp232 and Gln207 of the third membrane span that stabilizes formation of a helical hairpin.[320] Noncovalent higher order association of the fourth membrane span bearing mutation Val232Asp depends on the flanking hydrophobic sequence,[321] showing that strong helix−helix interactions mediated by hydrogen bonding can be modulated by sequence context.

4.2.3. Leucine Zippers

Aliphatic side chains can contribute to the stability and specificity of lateral association of transmembrane helices through favorable van der Waals interactions; they can also confer specificity by blocking potentially favorable contacts through steric clashes. Hydrophobicity, which is an important consideration for the folding of soluble proteins and for the formation of stable transmembrane helices, is not a contributing factor to these lateral interactions. Although the possibilities for encoding specificity may seem slight, the aliphatic-for-aliphatic substitutions in glycophorin A that disrupt dimerization[216,238] show that even side chains of the same volume and hydrophobicity (leucine and isoleucine) can have dramatically different abilities to support dimerization in the context of a GxxxG motif. Indeed, glycines or polar residues are not required for helix−helix interactions: sequences from the library selection of DiMaio and colleagues show that exclusively hydrophobic spans lacking small residues can mediate functional association of helices whose native sequence relies on a glutamine for interhelical hydrogen bonding.[315] However, upon examination of those selected hydrophobic sequences, it is not clear which of these residues participate in the dimerization interface.

Langosch and colleagues have explored the involvement of leucine residues in the self-assembly of both designed and biological hydrophobic sequences. Polyleucine gives a 4-fold stronger self-association signal than polyalanine in the ToxR assay,[322] although the polyleucine interaction is 3- or 4-fold weaker than glycophorin A in TOXCAT.[302,304] A membrane span with 12 leucines and 6 alanines that retains leucines at the *a*, *d*, *e*, and *g* positions of a heptad repeat (see Figure 10) gives a signal equivalent to polyleucine.[322] Although single replacements of leucines with alanine do not decrease the ToxR signal measurably, simultaneous replacement of four leucines with alanine has a substantial effect. Upon testing seven biological transmembrane domains with such leucine repeats, the authors found one sequence (from the erythropoietin receptor, see section 4.3.4) that associates more tightly than polyleucine, three others that associate somewhat less tightly than polyleucine, and three that are considerably weaker.[322] Further development of the ToxR assay to make a selectable and inducible version (termed POSSYCCAT) increased the discriminating power of the method and enabled several libraries of heptad repeat sequences to be analyzed.[262] While sequences exhibiting a range of association strengths were identified and prolines are underrepresented in the strongly associating sequences, no strong positional dependencies were observed among the bulky hydrophobic residues. Thus, much like the sequences identified by DiMaio and colleagues and described in section 4.2.2, it is not clear which residues participate in self-association.

As pointed out by Langosch and colleagues,[262] it is possible that residues at the *b*, *c*, or *f* positions of their heptad repeats may influence self-association. (Indeed, subsequent work with model peptides suggests that placing alanines at these positions in the middle of a span of 24 leucines may increase helix−helix association compared to polyleucine alone.[323]) The potential influence of supposedly "flanking" residues has also been seen by others. In the library selections of Russ and Engelman, randomized sites biased for right-handed helix crossing angles identified the GxxxG motif spacing in both a polyleucine and a polyalanine background, but the *positions* of the GxxxG sites in the two libraries were not the same even though the randomized codons and their relative positions were identical[275] (see Figure 9). This marked difference across the dozens of selected sequences shows, for the GxxxG motif, that the context provided by flanking residues (leucine versus alanine) of the host library sequence has a large impact on the randomized interface under selection. This presents a serious caveat for the interpretation of experiments where wholesale changes are made away from a putative interface, as well as challenging the idea of a "neutral" host sequence that does not affect the self-association properties of any motif sequence grafted into it.

How then are the potential contributions of such residues to be assessed? Alanine insertion scanning mutagenesis[218,324] could be used to determine whether the *spacing* of the alanines and leucines, rather than the *composition*, was modulating the ToxR signal; however, this method will not identify the interacting face of the helix. Asparagine scanning mutagenesis, which looks for reinforcement of a self-association signal upon scanning an asparagine along the membrane span, has been put forward as an approach to mapping such interfaces.[307] Support for this approach includes the mapping of the eyrthropoietin receptor interface[325] and the propensity in a selected library for a polar residue to align on the same face of the helix as in a wild-type sequence.[315] However, the relative strength of the hydrogen bond interaction and its dependence on flanking sequences[310] suggests that this approach may lack general applicability. The time-consuming approach of saturation mutagenesis of a given sequence seems the most reliable way to determine how each position contributes to self-association. Because the most dramatic effects of mutations seen with glycophorin A are for changes that introduce steric clashes,[216] using bulky residues (tryptophan, phenylalanine, or isoleucine) might help to identify hotspots quickly, as recommended in a review by Senes and DeGrado.[5]

Biophysical measurements of interaction energies for leucine zipper membrane spans provide a means of calibrating the relative propensities for dimerization of helix−helix association motifs, which is important for understanding the extent to which these interactions may influence biological phenomena. Although such data have not been reported for these leucine zippers, experiments with peptides and model membranes indicate that the free energy of association between polyleucine membrane spans bearing a single

tryptophan or dibromotyrosine residue is between 1.3 and 2.5 kcal mol^{-1}, so lipid/peptide ratios of 125:1 through 20:1 give significant fractional association.[130] While this is significant with respect to thermal energy, it is considerably lower than the values reported for other biological transmembrane helix−helix interactions such as glycophorin A or influenza M2 protein (see sections 4.1.3 and 4.3.2). Extensive studies with a polyleucine-based model peptide at a wide range of lipid-to-peptide ratios also suggest that these peptides are largely monomeric, although the formation of discrete low-order oligomers cannot be formally excluded (reviewed in ref 326). The application of robust methods to measure the sequence dependence of helix−helix interaction energies in bilayers of different lipid compositions, not just in detergents, may well be necessary to understand the potential contributions of leucine zipper type interactions to the function of membrane proteins.

4.3. Interacting Biological Single Spans

The transmembrane domains of many single-spanning proteins have been suggested to make functional protein−protein interactions. This section of the review considers six well-known systems, presents the many types of data used in these analyses, and attempts to evaluate the current tools used to explore the interaction propensities of these membrane spans. While tight transmembrane domain interactions are currently identified in a consistent and straightforward manner, experimental data are sometimes conflicting for weak potential interactions and the interpretation of the results from various approaches becomes complicated.

4.3.1. Phospholamban

Phospholamban is a 52 residue protein found in cardiac sarcoplasmic reticulum that binds to and regulates the activity of the calcium pump in native membranes and reconstituted bilayers.[327] The recently published solution NMR structure of the phospholamban pentamer[328] provides a structural framework within which to consider a wealth of mutagenesis data that have identified residues important to homo-oligomerization[329−331] and to interactions with the calcium pump.[331−333] Mutations along one face of the phospholamban transmembrane domain abolish pentamer formation,[329−331] and a structural model of a "leucine−isoleucine zipper" based on mutagenesis data[330] predicts the residues at the center of the pentameric bundle quite closely.[328] Leucine residues 37, 44, and 51 from the *a* position of a heptad repeat pack near the axis of symmetry and are supported by isoleucines at positions 33, 40, and 47, which lie at the *d* positions of the heptad repeat.[328] The availability of an atomic resolution structure in this system will undoubtedly further stimulate interest in the thermodynamics of pentamerization within the membrane, which may regulate the ability of phospholamban to inhibit the calcium pump.[327,331]

4.3.2. Influenza A Protein M2

The M2 protein of influenza virus forms a tetrameric helical bundle that acts as a proton-selective channel to facilitate acid-induced virus uncoating in the endosome, an important step in the viral life cycle.[334] Extensive solid-state NMR studies by Cross and colleagues on the transmembrane domain region of the influenza A M2 protein,[335−338] as well as data from the 92 residue full-length protein,[339,340] have provided a detailed view of the closed state of the channel, which is formed by straight α-helices that are strongly tilted by 38° with respect to the membrane normal.[338] These NMR data and structural model are in close agreement[337] with site-specific FTIR data,[341] with structures derived from these data by restrained molecular modeling,[341] and with structures derived from molecular dynamics[342] or site-directed mutagenesis data,[343] although the solid-state NMR orientational data along the whole transmembrane span indicates that the helices are quite straight, rather than forming a coiled-coil bundle.[338] The transmembrane domain of influenza A M2 protein has been a proving ground for site-specific labeling FTIR approaches to helix tilt and rotational information,[344,345] in part because the large helix tilt gives rise to large positional dependencies of the observable dichroic ratios. The strong variation seen in these dichroic ratios is analogous to the dispersion in oriented solid-state NMR PISA wheels.[346]

Sedimentation equilibrium studies revealed that tetramerization of the transmembrane domain of the M2 channel is specific and quite tight (16 kcal mol^{-1} in 15 mM dodecylphosphocholine detergent) but about 6 kcal mol^{-1} weaker than full length M2.[347] Binding of amantadine, an inhibitor of the proton-selective channel, stabilizes the tetrameric form of the wild-type M2 transmembrane domain.[348] Single point mutations in the transmembrane domain targeted at the residues lining the pore showed a range of effects:[349] four mutations stabilize the tetramer by about −1.2 kcal mol^{-1} (Val27Ala, Trp41Ala, Trp41Phe, Ala30Phe), one does not affect stability (Gly34Ala), and three destabilize by between +0.7 and +3.1 kcal mol^{-1}. However, of ten mutations targeted to the helix−helix interface, none are significantly disruptive, and most actually stabilize the tetramer, including one small-to-large mutation (Ser31Phe).[350] Amantadine binding to these mutants is attenuated or even abolished, and the effect of a mutation on tetramerization correlates negatively with the effect on amantadine binding.[350] Possible rationales for these observations include the idea that the function of the M2 protein may depend on switching between different conformations: as shown by spin−spin coupling EPR measurements, the lipid environment can modulate the structure of the M2 transmembrane domain, possibly affecting the tilt.[351] Mutations may perturb the balance between different conformations, or the energetics of lateral interactions between the transmembrane domains may be less than optimally stable to permit structural changes needed to accommodate function. It is noteworthy that stabilizing mutations for other membrane proteins appear to be common.[252,255,352]

4.3.3. Integrins

Integrins are heterodimeric cell surface receptors that mediate bidirectional signaling in the cell−cell and cell−matrix interactions that underlie development, differentiation, and cell death.[353] Large conformational changes occur in going from the low-affinity to the high-affinity state of the receptor,[354−357] but the detailed oligomeric interactions and clustering that accompany these conformational changes are controversial. Heterodimeric interactions between the single membrane spans of cognate α and β receptors have been implicated in both low- and high-affinity states,[358] although work with proteins containing the transmembrane and cytosolic portions of integrins αIIb and β3 suggests that these species form homodimers and homotrimers, respectively.[359] A designed transmembrane domain mutation, Gly708Asn in the β3 integrin, activates αIIbβ3 in vivo and also promotes

β3 homotrimer formation,[360] suggesting that the heterodimeric transmembrane domain interactions inferred in EM reconstructions of the αIIbβ3 low-affinity state[361] may give way to homooligomeric transmembrane interactions in the activated state. Designed disulfide cross-links that lock together an αIIbβ3 transmembrane domain heterodimer abolish activation[362] and negate the effects of juxtamembranous constitutively activating mutations in the cytoplasmic domain.[363] These findings further support the idea that tight interactions between αIIb and β3 membrane spans are linked to the low-affinity conformation.

Both heteromeric and homomeric interactions between the transmembrane domains of integrins have been measured using reporter assays. Schneider and Engelman developed the GALLEX assay, based on an asymmetric LexA operator, to measure heteromeric interactions of parallel helices in membranes.[265] Examining interactions of the transmembrane domains of a series of α and β subunits revealed that both β3 and β7 show some tendency for homodimer formation, while cognate pairs of α and β subunits show some heterooligomeric interactions that can be attenuated by mutations at GxxxG motifs within the transmembrane domains.[265,364]

TOXCAT analysis of αIIb transmembrane domain homodimerization identified interfacial residues and suggests that homodimer formation relies on the residues VGxxGGxxxL that map to one face of the helix.[365] A leucine scanning mutagenesis study of the transmembrane domains of αIIb and β3 showed that ligand binding of full-length integrins in mammalian cell culture becomes constitutive when Gly972 or Gly976 of αIIb or Gly708 of β3 is changed to leucine.[366] These mutations, as well as the previously described β3 Gly708Asn, seem to increase ligand affinity but not induce macroclustering of the receptors. The known disruptive effects of leucine substitutions at GxxxG motif positions argues that these mutations have their effect through disruption of heteromeric interactions but not through stabilization of homomeric interactions,[366] as had been suggested previously based on the mutation β3 Gly708Asn.[360] The identification of numerous activating mutations in the transmembrane domain and juxtamembranous region of β3, but not the distal cytoplasmic regions, confirms that the transmembrane domain is involved in activation.[367] While this process identified single point mutations on the same face of the helix of β3 implicated in association in other studies,[360,362,366] activation by clones with transmembrane domains exhibiting internal deletions and charged substitutions that reduce the effective length of the hydrophobic span indicate that disruption of intramembranous interactions leads to activation.[367]

4.3.4. Erythropoietin Receptor

The erythropoietin receptor transduces the signals that regulate the proliferation and differentiation of red blood cells in response to the cytokine erythropoietin.[368] The single transmembrane span of this receptor is important to its function,[369] and a juxtamembranous sequence motif is necessary for phosphorylation of the receptor.[370] The intact receptor forms dimers at the cell surface in the absence of ligand.[371] The erythropoietin receptor transmembrane domain self-associates in the ToxR assay,[322] and mutations that disrupt this interaction block signaling by the receptor even when combined with a constitutively activating mutation in the extracellular domain.[372] Replacement of the extracellular ligand-binding domain with designed soluble coiled-coils having different phases with respect to the transmembrane domain identified a preferred orientation of the transmembrane spans with respect to one another for maximal stimulation of downstream signal transduction pathways; modeling of the interface suggests that polar residues Ser231, Ser238, and Thr242 are involved in self-association.[373] A helical interface differing by only a slight rotation was identified by hydrophobic point mutations in the ToxR assay[325] and by asparagine scanning mutagenesis.[307,325] Transmembrane domain interactions also underlie heterooligomeric interactions between the erythropoietin receptor and gp55 of spleen focus forming virus that cause receptor activation.[369,374] The gp55 transmembrane domain self-associates in the TOXCAT assay,[375] and structural characterization of the gp55 interface identified a right-handed crossing angle between the helices by a ^{2}H solid-state NMR method that distinguishes between leucines at and away from the interface based on the mobility of their terminal methyls.[376] The differential ability of variants of gp55 to activate human and murine erythropoietin receptors results from differences in the transmembrane sequences of both receptor and gp55;[369,375] structural and energetic characterization of these interactions should provide new insights into the specificity and stability of lateral interactions between helices.

4.3.5. ErbB Receptors

Transmembrane domains have been implicated in the modulation of signaling by the ErbB family of receptor tyrosine kinases for decades. Mutation of a single valine to glutamic acid at position 664 in the transmembrane domain of the ErbB2 receptor constitutively activates the receptor, turning it into an oncogene.[377] The presence of the glutamic acid does not grossly affect the association of the sequence with the membrane,[378] suggesting that it alters lateral interactions between helices instead. Solid-state NMR chemical shifts indicate that the protonated glutamic acid forms strong hydrogen bonds, perhaps with another glutamic acid,[379] although rotational resonance distance measurements suggest that the adjacent Gly665 experiences close interhelical packing.[380] Dimerization of the oncogenic form of ErbB2 through its transmembrane domain is necessary, although not sufficient, for its transforming activity.[381] NMR studies in fluid bilayers indicate that both the normal and oncogenic form of ErbB2 exist as monomers and small oligomers at low ratios of peptide to lipid and that higher order aggregates occur at high peptide concentrations.[382]

Using the TOXCAT assay, Lemmon and colleagues showed that the transmembrane domains of the ErbB family self-associate in *E. coli* membranes, giving signals that range from 35% to 65% of wild-type glycophorin A, or about 4 to 8 times higher than the disruptive Gly83Ile glycophorin A mutant.[299] Exploring the sequence dependence of these associations with targeted mutagenesis revealed that mutations at GxxxG-like motifs in either the N-terminal or C-terminal part of the ErbB2 membrane span reduce homodimerization, while disruption of a TxxxG motif in ErbB3 does not affect self-association. Surprisingly, the oncogenic mutation Val662Glu in ErbB2 reduces dimerization in the context of the TOXCAT assay.[299] Interestingly, while both ErbB1 and ErbB4 contain multiple GxxxG-like motifs, the spatial distribution of the critical motifs is different: the C-terminal motif in ErbB1 contributes to homodimerization, while in ErbB4 a motif toward the N-terminal side of the membrane span is important. These findings may have

implications for homodimeric and heterodimeric interactions among this family of receptors, as further explored using the ToxR system by Shai and colleagues.[300] Consistent with these findings of interactions between the transmembrane domains of ErbB receptors, Hubert and colleagues have shown that expression of small designed proteins bearing the transmembrane domain of an ErbB1 or ErbB2 receptor can have a dominant negative effect on signaling by the receptor.[273]

These results contrast starkly with the demonstration by Stanley and Fleming that none of the ErbB transmembrane domains show strong homomeric or heteromeric interactions as chimeric proteins under conditions of sedimentation equilibrium in detergent.[298] A fusion construct bearing the membrane span of ErbB4 (which gave the strongest signal in TOXCAT[299]) shows some slight tendency to associate at low concentrations of the detergent C_8E_5. While there is considerable precedent for sequences that contain GxxxG motifs to fail to associate tightly[216,238,240] (see sections 4.1.1, 4.1.2, and 4.2.1) and membrane spans bearing polar residues may fail to associate in detergents while showing strong association in TOXCAT[261] (see section 4.1.4), the discrepancy between the TOXCAT data and the centrifugation data for four uniformly hydrophobic sequences is quite surprising. Aside from determining how transmembrane domains participate in receptor tyrosine kinase signaling, reconciling the differences between these measurements will be important for understanding how detergents influence helix−helix interactions and for determining what the results from TOXCAT and other biological assays really mean. It may be that certain classes of helix−helix interactions depend critically on the stabilizing influences of bulk bilayer properties (i.e., lateral pressure) and therefore do not associate in detergents.

Experimental approaches presented by Hristova and colleagues may be useful for resolving these issues. Working with synthetic peptides corresponding to the transmembrane domain of the fibroblast growth factor receptor 3, which contains one GxxxG motif and one SxxxG motif, these authors have shown that FRET measurements of bilayer-reconstituted peptides can yield thermodynamic parameters for lateral interactions between these helices (about −3 kcal mol^{-1}, giving significant self-association at protein/lipid ratios of 1000:1).[245,246] This is substantially weaker than the association of a tightly interacting system like glycophorin A, but it may be sufficient to influence the structure and mechanism of receptor tyrosine kinase signaling. Measurements of the sequence dependence of these interactions will be of great interest. The development of new approaches to measuring FRET in bilayers and to ensuring the homogeneity of the samples under study[244] will hopefully allow the exploration of these weaker interactions. The potential for using FRET approaches in both bilayers and detergent micelles, for comparison of the effects of the lipidic medium on association, is also attractive.

4.3.6. Synaptobrevin

Synaptobrevin is a member of the SNARE super-family[383] of proteins that mediate intracellular membrane fusion events.[384] Interactions between synaptobrevin (which is anchored in the vesicle membrane) and syntaxin and SNAP-25 (which are anchored in the plasma membrane) drive neuronal exocytosis in response to increases in calcium ions that enter the cytoplasm of the nerve terminal through voltage-gated calcium channels.[385]

A series of lines of evidence implicate the transmembrane domains of synaptobrevin, synaptophysin, and syntaxin in homomeric and heteromeric interactions. Synaptobrevin, syntaxin, and SNAP-25 form ternary complexes[386] that can be disassembled by components of the membrane fusion machinery, NSF and αSNAP.[387] The complex can assemble in nonionic detergents but not in SDS; however, preformed complexes are resistant to SDS unless boiled.[388] While only the 1:1:1 complex is seen upon assembly in vitro,[386] higher-order complexes form in vivo.[388] Co-reconstitution of synaptobrevin and syntaxin gives significant levels of SDS-resistant homodimeric and heterodimeric complexes only if the constructs include the transmembrane regions of these proteins, and proteolytic removal of the first 75 residues of synaptobrevin leaves the C-terminal membrane-spanning fragment complexed to syntaxin.[389] Binding of synaptophysin to synaptobrevin depends on the synaptophysin transmembrane domain and results in an SDS-resistant complex that prevents synaptopbrevin from binding syntaxin or SNAP-25,[390] and the transmembrane domain of synaptobrevin is sufficient to bind synaptophysin.[391] Cross-linking studies reveal synaptobrevin dimers and synaptobrevin/synaptophysin heterodimers in rat synaptic vesicles.[392] The higher molecular weight complexes detected under these conditions usually represent a small fraction of the total protein loaded onto the gel, but because the proteins probably cycle through different functional states the complexes may still be biologically relevant.

Experiments to probe the self-association of synaptobrevin transmembrane domains have been undertaken in two laboratories, with somewhat different results and quite different interpretations. Laage and Langosch showed that a fusion protein of staphylococcal nuclease and synaptobrevin that was overexpressed in *E. coli* and extracted in the detergent CHAPS at a protein concentration of ∼1 mg ml^{-1} could be cross-linked to dimers, while a fusion lacking the transmembrane domain did not cross-link.[393] These authors identified a mild urea/SDS−PAGE condition that supported dimerization without cross-linking and showed by alanine scanning mutagenesis that point mutations to alanine on one face of a helix could lower the dimer−monomer ratio, although only by as much as a factor of 2 (a triple mutant reduced the dimer−monomer ratio by a factor of 5).[393] Langosch and colleagues also showed that dimerization of the synaptobrevin fusion construct under conditions of mild SDS−PAGE was not affected by simultaneous conversion of eight noninterface residues to alanine.[394] Alignment of the synaptobrevin and syntaxin transmembrane domains suggested that these proteins shared a conserved interface, and a syntaxin fusion construct also formed dimers on mild SDS−PAGE. Surprisingly, mutating eight residues away from the inferred interface to alanine actually enhanced dimerization on mild SDS−PAGE severalfold, potentially indicating the formation of a new interface. Analysis of wild-type synaptobrevin and syntaxin transmembrane domains in the ToxR reporter system showed that both transmembrane domains gave self-association signals above background; while the variants with eight alanine replacements showed weak association in this assay, variants with only six alanine replacements show wild-type levels of ToxR signal. Mutants of either transmembrane domain that simultaneously replace three interfacial residues with alanines show minimal self-association by ToxR.[394]

Bowen, Engelman, and Brunger examined five synaptobrevin transmembrane domain constructs of different lengths and one syntaxin construct using the TOXCAT assay and compared the signals to a positive control (glycophorin A) and a negative control (the glycophorin A Gly83Ile disruptive point mutant). They found that while each of these constructs, including one harboring the disruptive triple alanine substitution of Langosch and colleagues, gave a stronger signal than the negative control, the signal was never more than twice the level of the negative control, and the positive control was 10 times higher than the negative.[395] These low levels of TOXCAT signals relative to glycophorin A indicated to these authors that the synaptobrevin self-association was extremely weak. (While these authors did test sequences of between 15 and at least 26 residues in length, they did not assay the precise sequence used by Langosch and colleagues, whose 15 residue construct is shifted to the C-terminal side by one residue.) Under standard SDS−PAGE conditions where a glycophorin A fusion construct forms strong dimers, the synaptobrevin fusion construct shows only trace amounts of dimer, again suggesting minimal dimer stability relative to glycophorin A.

Langosch and colleagues subsequently tested the 15 amino acid construct of Brunger and colleagues alongside their own 15 residue construct and the glycophorin A controls in the ToxR assay.[392] In the somewhat compressed scale of the ToxR assay relative to TOXCAT (see section 4.1.4), the wild-type glycophorin A transmembrane domain gives about five times the signal of the disruptive negative control, and the Langosch synaptobrevin 15-mer associates twice as strongly as the negative control while the Brunger 15-mer associates only slightly more strongly than the negative control.[392] These disparate results for very closely related sequences may be caused by sensitivity of the ToxR/TOXCAT dimerization signals to the relative orientations of the cytosolic and periplasmic domains and the dimer interface of the transmembrane domain.[392] However, Brunger and colleagues showed that in their 15 residue construct, converting a cysteine proposed by Langosch and colleagues to be in the dimer interface to an asparagine increased the TOXCAT signal 20-fold, to twice the level seen for wild-type glycophorin A, suggesting that the relative spacing of this construct can support strong self-association.[395] While the fusion constructs of Langosch and colleagues do associate under mild SDS−PAGE conditions, it should be pointed out that the rank order of the stabilities of sequence variants of synaptobrevin and syntaxin do not match between the ToxR assay and mild SDS−PAGE, suggesting that these assays may not be reporting on the same phenomenon. The indication that synaptobrevin is palmitoylated on a cysteine implicated in the Langosch interface further complicates matters.[396]

The experiments that most convincingly demonstrate a tight synaptobrevin−syntaxin protein−protein interaction that may underlie biological function are those that examine the native proteins in synaptic vesicle preparations. The semi-quantitative nature of both the gel assays and the in vivo association assays of heterologously expressed fusion proteins makes interpretation of the weak dimerization signals difficult for these systems, and the minimal effects of single point mutations on observed dimerization call into question the sequence specificity of the results. Using standardized positive and negative control sequences (such as glycophorin A) across various assays at least enables comparisons to be made between different bodies of work, but the significance of weak interactions in the ToxR and TOXCAT assays remains unclear. This situation should be contrasted with the case of the ErbB receptor transmembrane domains (section 4.3.5), where the TOXCAT and ToxR assays both indicate moderate to strong self-association that can be abolished by point mutations, while the sedimentation equilibrium data in detergents shows little evidence of significant interactions. In that case, the different conclusions drawn using in vivo and in vitro methods probably reflect differences in the lipidic environment that modulate the self-association properties of the transmembrane spans as measured by these diverse methods. In the case of synaptobrevin and syntaxin, small variations in the implementation of extremely similar methods lead to different conclusions, suggesting that the parameters surrounding the implementation and interpretation of these methods have yet to be completely established. While applying ToxR or TOXCAT to strongly associating systems allows the transmembrane domain interactions to be studied in isolation, thus clarifying the analysis by eliminating potentially confusing contributions from other parts of the molecules under study, in weakly associating systems this strategy seems to provide as many complications as clarifications.

4.4. Inhibition of Function by Interacting Membrane Spans

Specific helix−helix interactions that underlie functional assembly of helical bundle integral membrane proteins are potential targets for altering or disrupting function. Since helix−helix association is an equilibrium process, folded or assembled bundles of helices sample unfolded or dissociated states, in which one or more membrane spans transiently exist as independent helices. Introduction of a peptide bearing similar structural and sequence determinants for stable helix−helix interactions into the same membrane therefore establishes a thermodynamic competition: a sufficiently abundant peptide, or one that can make preferential interactions with the bundle, could effectively displace the native membrane span from participating in an oligomeric complex or even from making interactions within a folded polytopic mem-

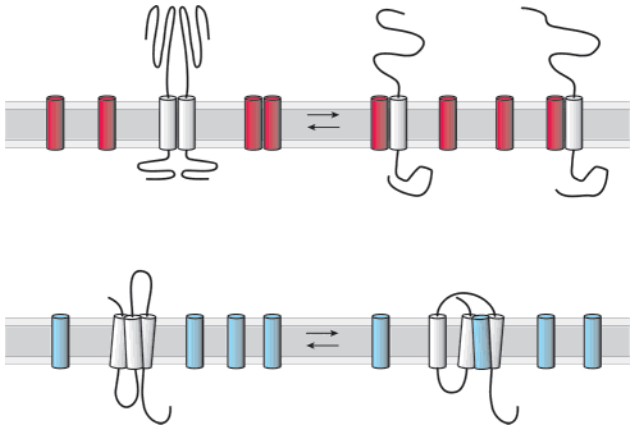

Figure 11. Schematics for how lateral interactions with transmembrane peptides might affect the structure and function of membrane proteins. Transbilayer helices compete reversibly with the lateral interactions that stabilize the native state; in cases where the peptide is in great excess or the sequence of the peptide supports a stronger than native interaction, the population of native protein may be significantly diminished.

brane protein (see Figure 11). Such peptides could have "dominant negative" effects on the function of the biological system. While small molecules may offer therapeutic advantages over peptides in terms of delivery or stability, peptides or small proteins are an excellent means of demonstrating that helix−helix interactions in a given system are a viable target for modulating function. This section presents examples from the literature in which lateral interactions of transmembrane helices within membranes affect the behavior of target proteins, either as part of a biological control mechanism or as tests of the importance of native interactions, supporting the view that manipulating the specificity of interactions within membranes provides a possible pathway to modifying function in biological systems and in medicine.

Lateral interactions with a single transmembrane helix can have subtle effects on the function of even quite complex membrane proteins in their native environment. Interactions between phospholamban (as a monomer or pentamer) and the calcium pump in the membranes of sarcoplasmic reticulum depend on residues that map to one face of the transmembrane domain of phospholamban,[333] while mutations on another face decrease pentamer formation and enhance interaction with the pump.[331] The interplay between these possible interactions and with contacts that occur between the aqueous domains of these proteins is now beginning to be understood in atomic detail with the recently published solution NMR structure of the phospholamban pentamer[328] and the crystal structure of the calcium pump[397] (see section 4.3.1).

The γ subunit of the sodium pump is a single span membrane protein that alters the affinity of the other two subunits of the Na,K-ATPase for substrates,[398,399] and the transmembrane domain of the γ subunit modulates sodium affinity.[400] The γ subunit transmembrane domain has been shown to form dimers in the mild detergent perfluorooctanoate but not in SDS, and this dimerization is disrupted by mutations Gly41Arg or Gly41Leu but not Gly35Arg.[401] Coexpression in cultured cells of full length γ subunit but not transmembrane domain mutants that disrupt dimerization is able to decrease the apparent affinity of the Na,K-ATPase for sodium. Interestingly, addition of the wild-type (but not disruptive mutant) γ subunit transmembrane peptide to membranes devoid of γ subunit recapitulates these results and demonstrates that an interaction between transmembrane domains modulates the activity of the pump.[271] These experiments provide a physical basis for the physiological function provided by the kidney-specific expression of the γ subunit.[402]

Transmembrane peptides from several G-protein coupled receptors (GPCRs) have been shown to specifically inhibit the parental GPCR in isolated membranes and in vivo. Incubation of a peptide corresponding to the sixth membrane span of the β2-adrenergic receptor with full-length receptor prevents both receptor dimerization and stimulation of adenylyl cyclase activity by β-adrenergic agonists.[268] The sixth membrane span from the D1 dopamine receptor reduces both binding of antagonists and receptor-mediated stimulation of adenylyl cyclase activity by agonists, although it does not apparently affect oligomerization.[269] Work with peptides from several GPCRs and a monoamine transporter indicates that injection of transmembrane peptides into live animals causes physiological effects that are consistent with specific antagonistic targeting of the polytopic protein from which the peptide is derived.[270] It is not clear how these peptides are delivered to their sites of action or how they insert into the target membranes.

The well-characterized protein diacylglycerol kinase, which contains three membrane spans, catalyzes the direct phosphorylation of diacylglycerol with high specificity.[403] Addition of a peptide corresponding to the second membrane span, but not a point mutant of that peptide, inhibits diacylglycerol kinase activity assayed in decylmaltoside by as much as 50%.[272] Mixing the disruptive peptide with the purified, full-length protein gives a complex that can be resolved on SDS−PAGE,[272] directly demonstrating the physical interaction between the peptide and its target protein.

Demonstration of the effect of competing transmembrane helices on biological signaling pathways has been presented for the ErbB family of receptor tyrosine kinases.[273] In human cell lines overexpressing either ErbB2 or the EGF receptor, cell surface coexpression of minigene constructs bearing transmembrane domains of the cognate receptor tyrosine kinase, but not control transmembrane domains, significantly inhibits the phosphorylation of the receptor and of the downstream kinase in the signaling pathway, ERK. Incubation with peptides corresponding to the transmembrane domains alone gives similar results.[273] Note that dimerization of ErbB family transmembrane domains has been detected in *E. coli* membranes using the TOXCAT assay,[299] but studies in detergent micelles reveal extremely weak interactions at best[298] (see section 4.3.5). Peptides have also been shown to disrupt ToxR signals for constructs bearing ErbB transmembrane domains.[300]

As discussed at the end of section 4.1.4, homodimerization of a ToxR−GpA construct is inhibited by addition of peptides carrying glycophorin A dimerization motifs but not by variants that partition into model membranes but lack the dimerization motif.[267] Unexpectedly, a glycophorin A transmembrane peptide with the two motif valine residues replaced with D-valine also inhibits the ToxR−GpA dimerization signal,[404] as does an all-D version of the peptide.[405] These findings are explained by the authors using models based on the wild-type glycophorin A solution NMR structure. Since the use of D-amino acids could improve peptide stability in vivo, it would be valuable to have biochemical and biophysical information about the stability and specificity of the interactions that underlie this biological result.

4.5. Polytopic Membrane Proteins and the Two-Stage Model

Bacteriorhodopsin is the polytopic protein for which lateral interactions within the membrane have been most extensively analyzed to determine their contributions to functional native structure. Intact bacteriorhodopsin can be refolded from a highly denatured state,[38,41] and two sets of fragments derived by proteolysis can also regain native structure when cofolded.[39,40,42] The demonstration that two of these fragments can refold independently to helical transmembrane conformations in separate liposomes and then assemble spontaneously into functional protein following liposome fusion[43] was an important experimental impetus in the formulation of the two-stage model of membrane protein folding. The nature and extent of helix−helix interactions within the independently reconstituted fragments is not known, but after

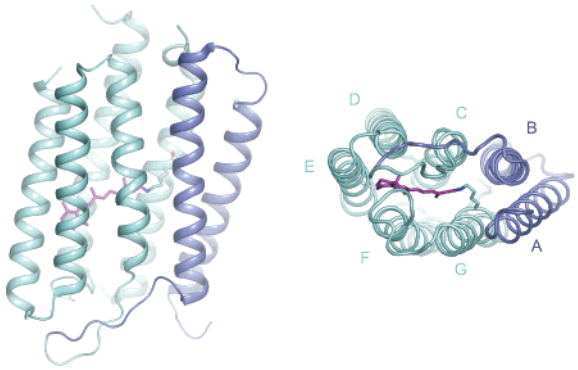

Figure 12. Cartoon representation of bacteriorhodopsin (PDB 1C3W) identifying one of the sets of fragments from which bacteriorhodopsin can be functionally reconsituted. Chymotrypsin cleaves bacteriorhodopsin after Phe71; the amino-terminal 71 residues are colored in slate, while the remainder of the protein is in cyan. The loop between helices B and C forms antiparallel β structure, but cleavage between these strands still allows proper folding of the combined fragments. The retinal chromophore is represented using purple sticks.

liposome fusion the native structure is acquired when the first and second spans (helices A and B in Figure 12) interact with the fragment corresponding to the last five helices. Independently reconstituted synthetic peptides corresponding to the first and second transmembrane domains of bacteriorhodopsin can also reassociate with the reconstituted five helix fragment following liposome fusion to yield functional bacteriorhodopsin and purple membrane lattice,[406,407] demonstrating that the first two loops are dispensable for functional assembly, although both these loops and the binding of retinal contribute to the thermal stability of the protein.[408] While the third, fourth, and fifth membrane spans of bacteriorhodopsin also reconstitute as transmembrane helices, the sixth span does not form stable secondary structure, and the seventh forms surface-associated β-structure in membranes.[409] Although these sequences might be successfully inserted as helices into membranes by the translocon in the appropriate sequence context, these biochemical reconstitutions (and the pH-dependent membrane insertion of the third membrane span[410]) demonstrate that the seven transmembrane domains of bacteriorhodopsin exhibit different behavior with respect to helical refolding. Synthesis and characterization of the individual transmembrane spans of G-protein coupled receptors, including the yeast α receptor[411] and the human adenosine A2A receptor,[412] have similarly shown that most but not all of these membrane spans can be readily incorporated into bilayers as independently stable helices. Interestingly, the fifth helix of the A2A receptor shows evidence of self-association that may be related to receptor dimerization.[413]

A role for topological links or lateral interactions between spans in stabilizing the helical conformation of membrane domains may be inferred from the failure of either span 6 or span 7 to form stable helices in isolation[409] despite the successful reconstitution of membrane spans 6 and 7 in the context of full-length bacteriorhodopsin[38,41] and when linked by the intervening loop as a helical hairpin.[40] Marti and colleagues explored the roles of the loops between bacteriorhodopsin membrane spans and showed that while fragments consisting of the last five transmembrane spans or the first two, three, four, or five spans insert stably into detergent micelles as helices at pH 6, shorter fragments containing the C-terminal four, three, or two spans would insert only at pH 4.[414] Pairs of complementary fragments that include all seven spans regenerate functional bacteriorhodopsin when combined, demonstrating that four of the loops in bacteriorhodopsin are individually dispensable for function.[414,415] However, stability and kinetic folding studies of bacteriorhodopsin with modified loops implicate not only the topological connection, but the sequence identity of loops as factors in folding and stability,[416,417] as discussed further in sections 5.1 and 6.2.1.

Work with bovine rhodopsin extends the view of polytopic proteins as being composed of domains that interact within the membrane. Fragments of bovine rhodopsin containing two, three, four, or five membrane spans can be successfully expressed in COS cells but do not bind the retinal chromophore; however, upon coexpression of two or three complementary fragments, retinal binding and wild-type-like absorption spectra are obtained.[418] The ability to express functional rhodopsin as fragments has led to the development of cysteine cross-linking strategies for mapping protein−protein contacts by detecting formation of disulfide bonds.[419] Cross-link formation differs for dark-adapted and photobleached rhodopsin,[420] and while certain cross-links abolish the ability to activate transducin without affecting the photocycle of rhodopsin,[421] up to four cross-links connecting cytoplasmic or extracellular ends of membrane spans can be incorporated while maintaining light-dependent rhodopsin activation of transducin.[422,423] Although the thermodynamic effects of splitting the receptor into pieces or of adding disulfide cross-links have not been determined, an engineered disulfide bond between residue 2 and residue 282 (in the extracellular loop between helices 6 and 7) significantly stabilizes the protein to detergent solubilization.[424] These experiments show that considerable insight into polytopic helical bundle membrane protein structure and function can be achieved by analyzing split variants under the basic assumptions of the two-stage model.

The generation of other functional polytopic proteins from coexpressed fragments further supports the dispensability of many[425−427] but not all[428] topological connections for functional folding. Generating a split variant of a membrane protein can be more complicated: in some instances, the fragments must be coexpressed to achieve wild-type levels of protein in membranes,[427] suggesting that the individual fragments may be unstable or degraded when expressed alone and that lateral interactions between the fragments help to stabilize them in vivo. Even more restrictive conditions can apply: coexpression of a six-span and a two-span fragment of the *E. coli* $IIBC^{Glc}$ subunit of the glucose transporter from two separate replicons does not give functional protein, but providing the same constructs as a dicistronic message results in proper targeting to membranes and functional activity.[426] This suggests that lateral interactions between helical domains at the stage of membrane insertion by the translocon machinery are necessary for proper in vivo folding and insertion of these fragments. Such interactions may occur even for proteins whose membrane spans can independently direct proper topological insertion.[429] Together, these examples demonstrate that the two-stage model provides useful insight into designing and analyzing a wide range of properties exhibited by fragments of polytopic membrane proteins.

5. Stability of Polytopic α-Helical Membrane Proteins

5.1. Sequence Dependence of Stability

Point mutations in membrane proteins are often made for purposes other than exploring the sequence dependence of protein stability or folding. Mutagenesis has been used to map function by exploring the functional consequences of alterations in side chain chemistry in proteins such as bacteriorhodopsin[430] or to incorporate cysteine residues for labeling or cross-linking studies. Extensive work in lac permease by Kaback and colleagues showed the viability of engineering a functional cysteine-less protein,[431] generating single cysteine mutants in this background (the overwhelming majority of which are active),[432] analyzing the local environment of these positions and distances between them with site-directed probes,[432−434] and using cysteine cross-linking to map interactions within a protein.[435−437] These studies have revealed structural, functional, and dynamic aspects of the permease, but they have also indicated that transport function shows a broad tolerance toward sequence changes. This tolerance of function to substitution has been seen in other polytopic membrane proteins. Random mutagenesis of a yeast G-protein coupled receptor followed by screening for function has revealed that every residue in the seven membrane spans accepts hydrophobic substitutions, and half of the sites that could mutate to ionizable residues by a single base change in fact do so while maintaining some level of function.[438] Do proteins that fold in a largely hydrophobic environment show small thermodynamic effects for sequence changes? Or are the destabilizing effects of substitutions readily accommodated in these proteins because of their high intrinsic stability? Can the functions of membrane proteins be supported by sequence variants that fold into rather different structures? Interesting answers to the first two of these questions have been provided from studies of diacylglycerol kinase and to all three of these questions by work with bacteriorhodopsin.

The enzyme diacylglycerol kinase contains three membrane spans, forms a homotrimeric complex of about 40 kDa, and was shown by Sanders and colleagues to insert into preformed bilayers with moderate efficiency upon dilution from a detergent solubilized state.[439] Although diacylglycerol kinase exhibits high specificity for substrates and its catalytic rate approaches the diffusion limit,[403] its activity shows a remarkable tolerance to single substitutions[440] and indeed to replacement of the first membrane span by polyalanine, although more hydrophobic variants showed better stability.[441] Lau and Bowie developed an approach in mixed micelles for measuring the energetics of unfolding of diacylglycerol kinase[249] and suggested that the stability of the transmembrane domain, estimated at 16 kcal mol^{-1}, might explain the tolerance of the protein to substitutions. Analysis of a set of mutant proteins identified sequence changes that improved resistance of the protein to thermal inactivation,[442] and combination of four such mutations resulted in a highly stabilized version of the protein.[255] The example of diacylglycerol kinase, in which sequence changes at any particular site have similar chances of being destabilizing, isoenergetic, or stabilizing, has demonstrated that the stability of a membrane protein in detergents may be readily optimized by making sequence changes.[352] The generality of this finding has yet to be demonstrated, but this possibility could find considerable utility in the study of proteins that are hard to express or purify. In the absence of a three-dimensional structure for diacylglycerol kinase, the detailed basis for the stabilizing effects of these mutations is not known, and the tolerance of the first membrane span to wholesale substitution may indicate that the property of optimizable stability is peculiar to this system. However, because diacylglycerol kinase activity can be inhibited by addition of a peptide corresponding to the second transmembrane span (but not by a mutant peptide),[272] it seems that lateral interactions among its membrane spans exhibit the same kind of specificity that is displayed by other model systems.

Bacteriorhodopsin is the polytopic helical protein that has been most extensively studied with respect to its stability, the sequence dependence of that stability, and the kinetics of its folding. Refolding of intact bacteriorhodopsin from a denatured state[38,41] can be monitored by circular dichroism, by fluorescence spectroscopy of intrinsic tryptophans, and by the absorption properties of the retinal chromophore, which change upon binding and upon formation of a Schiff base with Lys216 on the seventh membrane span. Identification of intermediates in the folding pathway[443] and of the formation of about 30 additional residues of α-helical structure during the folding process[444] have shown that the kinetic folding of this protein is more complex than might be anticipated from the tidy thermodynamic perspective of the two-stage model of integral membrane protein folding; the kinetic aspects of bacteriorhodopsin refolding are reviewed in section 6.1. The ability to refold bacteriorhodopsin has led to the development of expression systems that allow researchers to explore the sequence dependence of the stability of monomeric detergent-solubilized bacteriorhodopsin[445,446] or of the protein in the purple membrane lattice.[447,448] Thermodynamic measurements of the stability of monomeric bacteriorhodopsin in mixed micelle systems,[250,252] along with high-resolution crystal structures of trimeric[449] and monomeric bacteriorhodopsin,[450] have now enabled the investigation of the influence of sequence on the structure and the stability of this polytopic helical bundle protein.

Gouaux and colleagues showed that while bacteriorhodopsin can tolerate multiple polar substitutions at lipid-facing positions,[446] such mutants are destabilized by comparison to the wild-type protein,[250] as might be expected from considerations of hydrophobicity and from the two-stage model. Bowie and colleagues modified this approach using lessons from their studies of diacylglycerol kinase[249] and have since analyzed the stability of dozens of point mutants of bacteriorhodopsin; interestingly, many of these mutations are stabilizing,[252] as had been seen for diacylglycerol kinase.[255]

Bowie and colleagues performed alanine scanning mutagenesis on the second membrane span of bacteriorhodopsin (see Figure 13), replacing the wild-type residue with alanine at each of 24 sites to determine the change in the unfolding free energy of the protein ($\Delta\Delta G_u$) associated with truncation of each side chain on this helix.[252] Alanine substitutions at four large residues (Val49, Met56, Leu61, Leu62) stabilized the protein by between 0.5 and 1.4 kcal mol^{-1}. Eight substitutions had no significant effect on stability; interestingly, one of these mutations, Pro50Ala, occurs at the site where the helix is kinked in the wild-type crystal structure. Seven substitutions destabilized only moderately (by between 0.5 and 1.4 kcal mol^{-1}), while five strongly destabilized the protein (by between 1.6 and 3.7 kcal mol^{-1}); four of these

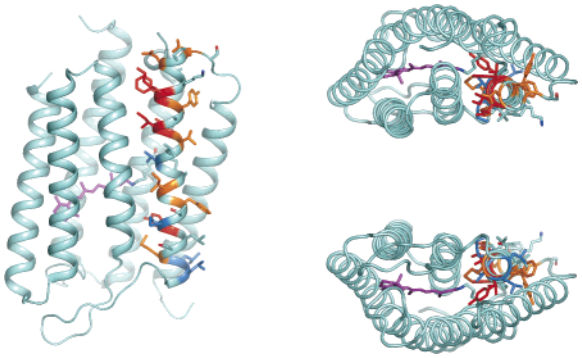

Figure 13. Cartoon representation of bacteriorhodopsin (PDB 1C3W) displaying the alanine scanning mutagenesis results of Bowie and colleagues[252] for the second membrane span. Side chains for residues 25 through 62 are shown as sticks. Those residues whose substitution by alanine strongly destabilizes the protein are shown in red, while those that slightly destabilize are shown in orange. Sites where alanine substitution improves stability are in blue, while sites where mutations do not significantly alter stability are in cyan. The retinal chromophore is in purple. Residues that destabilize when mutated to alanine tend to point toward other helices, and residues that do not change stability tend to point out from the helical bundle. Stabilizing mutations are in the C-terminal half of the span but do not cluster on one face of the helix. Adpated with permission from ref 252. Copyright 2003 Elsevier Ltd.

five sites were clustered in a space of six residues. Although the structural basis for the stabilizing mutants was not clear, the extent to which mutations *destabilized* the protein correlated strongly with the buried surface area of the native side chain. The empirical relationship of 38 Å^2 of buried surface area corresponding to 1 kcal mol^{-1} is in good agreement with alanine scanning mutagenesis thermodynamic data for glycophorin A helix−helix interaction[240] and with other substitutions in that system.[238] The close correspondence of the behavior of these systems argues that the thermodynamic contributions to helix−helix interactions in an ideal two-stage system, such as glycophorin A, can be very similar to the driving forces for polytopic membrane protein folding.

Bowie and colleagues have also performed a proline mutagenesis scan in the second membrane span of bacteriorhodopsin.[251] Six of the proline point mutants gave no active protein, five gave changes in the free energy of unfolding of between −1.1 and −2.4 kcal mol^{-1}, and three destabilized only slightly ($\Delta\Delta G_u$ about −0.5 kcal mol^{-1}).[251] The least destabilizing proline substitutions occur in the N-terminal end of the helix, where prolines are more frequently seen in membrane protein structures[195] and where prolines have the least destabilizing effect on the incorporation of a single transmembrane span in a membrane.[192] (Prolines are also less disruptive to glycophorin A dimerization when located at the N-terminal end of the membrane span.[221]) Unlike the alanine substitutions, the disruptive effects of proline replacements in bacteriorhodopsin do not correlate with the burial of the wild-type residue. Structure determination of the bacteriorhodopsin mutant Lys41Pro showed that the proline is accommodated by local changes in structure, while the structure of mutant Ala51Pro revealed conformational adjustments along the helix.[251] These findings suggest that the disruptive effects of introducing a proline substitution into interacting helices are mediated by effects on the stability of the transmembrane span and the complex response of the interacting helices to structural alterations at the proline.

Replacing proline residues might be expected to alleviate transmembrane kinks, but structure determinations for proline-to-alanine substitutions in three different membrane spans of bacteriorhodopsin show that these kinks are largely retained, although the distortion of the helix can be spread over more residues as a smooth bend instead of an abrupt kink.[252,451] This indicates that prolines can favor tertiary interactions between certain membrane spans by inducing kinks but that prolines are not absolutely necessary for such kinks to exist. The existence of a non-proline kink in the L subunit of the photosynthetic reaction center at the same position where a proline kink occurs in the structurally homologous M subunit[452] and other statistical correlations observed across structures and sequences led Bowie and colleagues to propose an evolutionary pathway to structural diversity and stability in which proline substitutions that introduce kinks are stabilized by subsequent mutations in other parts of the sequence that enhance lateral tertiary interactions with the proline-containing helix.[451] After such compensatory changes, the kink would be retained even if the proline were subsequently changed to another residue.

These findings have significance for the two-stage model of membrane protein folding. In the case of wild-type bacteriorhodopsin, it could be argued that the proline kinks seen in transmembrane helices in the high resolution crystal structures arise from predispositions to kinking due to proline or that the kinks might be preformed in isolated helical membrane spans. For each of the proline-to-alanine mutants, however, the structure of the isolated, independently stable helix in question should be essentially ideal[453] at that position, yet in the folded structures, each exhibits wild-type-like kinks. From the mutant proteins, then, it is clear that considerable deviations from helical ideality can be *induced* by tertiary interactions between membrane spans. Does this kinking of a helix in response to the lateral association of helices run counter to the two-stage view of membrane protein folding?

Since the second membrane span of bacteriorhodopsin containing wild-type Pro50 can be independently reconstituted into membranes and can associate with the first transmembrane span and a five span fragment to form functional bacteriorhodopsin,[406,407] this system is well described by the two-stage model: lateral interactions do specify the final structure from stable transmembrane spans. (It is interesting to speculate as to how readily a peptide of the second membrane span bearing the Pro50Ala mutation might assemble under similar conditions.) The kinetic effects of mutating this proline in the full length protein are complex,[454] but the two-stage model is a useful tool in sorting through these effects, as discussed in section 6.2. The two-stage model thus retains its tidy energetic perspective in outline, but the structural and energetic details become complicated: extending or expanding the definition of an "independently stable transmembrane helix" to an "independently stable span" constitutes a reworking that is more than merely semantic. It should be noted, however, that the possibility of the need for this detail was anticipated in the original presentation of the two-stage model.[37]

One naive structural interpretation of the two-stage model is that helical bundle membrane proteins would be formed by packing of ideal "preformed" helices. This seems to be essentially the case for the glycophorin A transmembrane helix dimer,[229,235] and this favorable example provides a tempting avenue to membrane protein structure prediction.

Membrane protein structures that exhibit highly noncanonical transmembrane spans prove that such approaches to membrane protein structure prediction cannot be entirely general.[94] The demonstration that even in the canonical α-helical polytopic protein bacteriorhodopsin, membrane spans can be kinked or bent at particular points because of tertiary interactions and not simply because of their primary sequence may seem like a further indictment of the practice of assuming that membrane spans could be usefully modeled as preformed helices. However, combining the prediction of the positions of kinks based on sequence alignments within families of proteins[451] with an understanding of the biases for the geometry of proline-induced kinks[195] might allow expectations about possible site-specific kinking into prediction schemes that otherwise treat membrane spans as ideal helices. Such approaches would still be a long way from providing insights into how sequence specifies the highly noncanonical structures of helical membrane proteins such as the ClC chloride channel,[95] but extending the prediction of membrane protein structure by rational modification of approaches that are successful in simple systems seems to be a worthwhile area of endeavor.

5.2. Lipid Dependence of Stability

The lipids of the bilayer in which integral membrane proteins reside are expected to play a major role in determining protein structure, stability, and function by binding directly to specific sites and by modulating the overall physical properties of the bilayer, which depend on lipid composition. High-resolution structures of membrane proteins have revealed the structural details of tightly bound lipids (reviewed in refs 455 and 456), and the use of spin-labeled or deuterated lipid probes have revealed the stoichiometry and dynamic aspects of lipid−protein interactions over a range of interaction strengths (reviewed in ref 456). Nonbilayer lipids in biological membranes have been shown to influence membrane proteins through the lateral pressure profile (reviewed in ref 457), and approaches to modifying the lipid contents of various biological membranes have identified the in vivo folding dependencies of proteins on specific lipids (reviewed in ref 53). Studies of model lipid systems have shown that cholesterol can enhance fluid−fluid phase separation in bilayers, and work in these systems is providing insights into the properties of the lipid rafts that are proposed to provide specialized functional microdomains within cell membranes (reviewed in refs 458−462). In addition to the implications for biological membrane protein function, understanding how the lipid composition of membranes influences protein expression and stability[463] and how the interplay between proteins, lipids, and detergents influences in vitro membrane protein stability[6] has practical implications for the study of membrane proteins.

Delipidation during purification can destabilize membrane proteins, and addition of lipids has been used to overcome stability problems in the isolation of proteins for biophysical studies.[6,455] Lipids can also be crucial for crystallization. Although at least two native lipid species copurify with the cyanobacterial cytochrome b6f complex,[464] adding approximately 10 DOPC lipids per b6f monomer (0.1% DOPC in the presence of 0.05% β-undecylmaltoside) dramatically improved crystal diffraction limit, in part by protecting the complex from proteolysis.[465] The structures of the algal[466] and cyanobacterial[467] cytochrome b6f complexes both contain ordered lipid; interestingly, the position of an ordered sulfolipid in the algal complex is occupied by a (non-native) DOPC molecule in the cyanobacterial complex. It seems likely that a native lipid is bound at this position in cyanobacterial membranes; the reasons for the displacement of this lipid and the retention of the algal lipid probably include both the purification methods and the details of the protein−lipid interactions.

Ordered lipids interact with membrane proteins through acyl chains, glycerol backbones, and polar headgroups. Jones and colleagues used mutagenesis to explore the roles of specific protein−lipid polar contacts between an arginine side chain in the M subunit of the bacterial reaction center and the headgroup of an ordered cardiolipin.[468] Differential scanning calorimetry in 0.1% LDAO revealed that the melting point of the reaction center containing the Arg267Leu mutant is about 5 °C lower than wild-type.[468] Mutating arginine to leucine had no measurable effects on reaction center expression, function, or absorption spectra, and except for the lack of an ordered cardiolipin, the mutant structure is identical to wild-type,[469] suggesting that the binding of the ordered lipid contributes to the stability of the reaction center but does not significantly perturb its structure.

Lipid composition has been shown to influence the assembly and stability of the tetrameric form of the KcsA potassium channel. KcsA made by transcription/translation in the presence of liposomes can associate with membranes and assemble into tetramers.[470] High-efficiency tetramer assembly is supported by liposomes with high mole fractions of phosphatidylethanolamine (40%) and phosphatidylglycerol (20%) but not pure phosphatidylcholine. These observations correlate with the measured thermal stability of the channel: detergent solubilized KcsA tetramer is dissociated to monomers after incubation at moderate temperatures, but KcsA reconstituted into bilayers of 70:30 PE/PG shows high thermal stability.[470] Perturbation of KcsA tetramerization in membranes by a series of alcohols suggests that the partitioning of the alcohols to the bilayer interface reduces lateral pressure at the middle of the bilayer, reducing the driving force for tetramerization.[471,472] The potential for the nonbilayer lipid phosphatidylethanolamine to increase lateral pressure[473] is consistent with its importance in KcsA tetramer stability. While it is possible that KcsA has binding sites for particular lipids, the correlation between the effects on tetramerization and on bilayer properties suggests that lipids influence KcsA oligomerization by altering the physical properties of the bilayer as a whole. Lateral pressure is also implicated in decreasing the folding efficiency of bacteriorhodopsin from the dependence of membrane insertion on phosphatidylethanolamine content and the opposing effect of 1-palmitoyl-2-hydroxy-*sn*-glycero-3phosphocholine.[474] Membrane insertion of the multidrug transporter EmrE, but not reconstitution of EmrE by dialysis, is inhibited by phosphatidylethanolamine,[475] suggesting that there may be both kinetic effects[476,477] and thermodynamic effects of lipid composition in some experiments.

The direct influence of lipid composition on the free energy of helical integral membrane protein folding has not been systematically examined. However, Hong and Tamm have explored in considerable detail the urea-induced reversible folding of the β-barrel membrane protein OmpA in the presence of bilayers composed of POPC/POPG and an additional guest lipid.[478] The data show a strong dependence of folding free energy on the headgroup and the acyl chain

length of the third lipid species: shorter acyl chain phosphatidylcholines decrease the folding free energy, while phosphatidylethanolamine increases it. The negative spontaneous curvature of the lipid mixtures (or lateral pressure of the membranes) seems to explain much of the variation in folding free energies; hydrophobic mismatch also plays a role. This pioneering work was made possible by the identification of urea unfolding conditions that are reversible in the presence of membranes. The mixed micelle systems in which the stabilities of several helical proteins have been studied also exhibit lipid dependencies, but because folding in these systems is in micelles and not membranes, linking lipid effects to bulk bilayer properties such as lateral pressure will be tenuous.

Exploring the effects of particular lipids on the properties of proteins in vivo requires genetic manipulation of the lipid biosynthetic pathways of interest and appropriate tools for probing protein targeting, structure, and function;[53] in some instances, biochemical and biophysical data provide a context for the biological experiment. Cardiolipin binding to the respiratory bc_1 complex has been shown to be important for function,[479,480] and the structure of the bc_1 complex contains ordered cardiolipin.[481] As might be anticipated, yeast altered to have repressible phosphatidylglycerol phosphate synthase expression are defective in respiration and are unable to incorporate at least five respiratory chain transmembrane proteins into their inner mitochondrial membranes when phosphatidylglycerol and cardiolipin are not present.[482] Blocking only the pathway that converts phosphatidylglycerol to cardiolipin allows functional expression and proper targeting of all proteins needed for respiration, but destabilizes supercomplex formation among these proteins.[483−485]

Lipid composition also influences membrane protein topology in vivo (reviewed in ref 486). Anionic phospholipids help enforce the positive-inside rule for proteins being inserted into the *E. coli* inner membrane, implicating a general electrostatic effect in the kinetics or thermodynamics of translocon-mediated folding,[186] but protein-specific effects are also seen. Dowhan and colleagues have shown that the proper topology of lac permease in *E. coli* membranes depends on the presence of phosphatidylethanolamine.[487] The misfolded protein exhibits an inverted topology for the six N-terminal membrane spans and their connecting loops, with the large loop between the sixth and seventh helices exposed to the periplasm instead of the cytosol.[488] The first six membrane spans of LacY do not fully integrate into the membrane while the ribosome is synthesizing the cytosolic loop between the N and C domains,[489] suggesting that these regions may not make a topological decision until late in the translation of the protein. It is noteworthy that another *E. coli* membrane protein, YidC, is important for the proper folding but not membrane integration of LacY.[490] Analogous dual topology results are obtained for PheP, an amino acid transporter, although only the two N-terminal membrane spans are inverted in the absence of phosphatidylethanolamine.[491] GapP, another member of the same transporter family, also exhibits topological inversion of its first two membrane spans in phosphatidylethanolamine deficient cells.[492] Phosphatidylethanolamine is essential for targeting several amino acid transporters to the plasma membrane of yeast[493,494] although the effects on topology are not known.

Multiple topologies have been observed for other proteins under conditions of normal lipid metabolism,[495−500] but the in vitro and in vivo data for the lac permease system combine to yield particularly interesting implications for membrane protein folding. Reconstitution of permease into bilayers in the absence of phosphatidylethanolamine gives the partially inverted topology and in the presence of phosphatidylethanolamine gives the normal topology, independent of the lipid content of the cells from which the protein is isolated.[501] This finding indicates that bilayer lipid composition, and not the in vivo folding history of the protein, controls the topology of the reconstituted protein. Pulse−chase experiments show that permease topologically misfolded in vivo can be posttranslationally converted to the correct topology following induction of phosphatidylethanolamine synthesis;[488] PheP exhibits a similar posttranslational reorganization.[491] Such reorganizations may be able to occur spontaneously, but it seems more likely that the translocon is involved in this process, possibly with the assistance of other proteins. The LacY reconstitution data suggest that the thermodynamically favored topology varies with lipid composition, and the in vivo data suggest that the translocon allows the permease to sample topologies and to choose the more favorable orientation, even after translation has been completed and the protein has been released to the membrane. This complements and extends the "lipid partitioning hypothesis" for translocon-mediated integration of membrane spans[190] and the thermodynamic insights into this process.[192] Lipid-dependent topology preferences may be properties of the sequences of individual membrane spans, but because helix−helix interactions also affect integration,[502] lipids could influence topology by modulating helix−helix interactions. Because phosphatidylethanolamine, a nonbilayer lipid, enhances the negative spontaneous curvature of membranes, PE-dependent topologies described above may be mediated by lateral pressure effects on interactions between membrane spans of the transporters in question. While the current data do not yet provide a clear picture of the extent to which the processes that determine the topology of membrane spans are normally coupled to the energetics of helix−helix interactions, the potential influences hinted at by the lipid-dependence of topology of certain proteins in prokaryotes can readily be incorporated into a thermodynamic picture of membrane protein folding in vivo.

It appears that *E. coli* inner membrane proteins, since they coexist in the same bilayer with the translocon, can be subject to "proofreading" of topology even after their translation has ended.[488,491] For a model protein membrane span being inserted by the eukaryotic translocon, the window of opportunity to change topology appears to close after synthesis of the remainder of the protein is complete.[99] This difference may be a reflection of the membrane protein substrate used in this particular study, or it may reflect real differences in the prokaryotic and eukaryotic translocons and their associated machinery; the latter alternative would mean that a kinetic model for topology determination would be more appropriate for eukaryotic systems. It is noteworthy, however, that the eukaryotic translocation machinery is inhibited by sterols:[503] translocons that inadvertently leave the endoplasmic reticulum for the Golgi and sterol-rich membranes would therefore be inactivated. This is important to ensure that translocation of nascent chains is localized to a single compartment (the endoplasmic reticulum), but it may also play a role in preventing membrane proteins in other cellular membranes from having their topology "proofread" by a runaway translocon in the context of their specific lipid composition.

6. Kinetic Folding of α-Helical Transmembrane Proteins

The thermodynamic cycle in Figure 3 suggests that the energetics of a protein folding from an aqueous state to a transmembrane helical protein may be analyzed productively by considering the energies of the (folded or unfolded) aqueous and interfacial states of the protein, as well as by examining the energies of helix insertion into and association within the membrane. Kinetic analysis of the folding of helical membrane proteins can begin with whatever experimentally accessible conformation of the protein can be successfully refolded: for polytopic proteins such as bacteriorhodopsin, this may correspond to a detergent-denatured polypeptide, while for small peptides or self-inserting polytopic toxins, the process may start with a water-soluble species, making the kinetics of membrane insertion accessible in some systems. Manipulation of the lipid and detergent composition of refolding conditions is critical for successful reconstitution of proteins for biochemical studies and also for identification of conditions that permit study of the kinetics and thermodynamics of membrane protein folding.[6] As progress in the field advances our understanding of the factors at work in refolding membrane proteins, the rational exploration of experimental conditions is extending the applicability of these methods to more proteins and is broadening and deepening our understanding of membrane protein folding, including the detection of intermediates and the nature of the activation barriers in these processes.

6.1. Spontaneous Insertion

Insertion of a protein from a soluble state to a transbilayer conformation necessarily involves traversing the membrane interfacial region and contacting the hydrophobic core of the membrane. Thinking about the energetics of a polypeptide interacting with these regions is facilitated by the whole-residue interfacial[65] and octanol[64] scales, but the importance of partitioning−folding coupling at the membrane interface and the partitioning of the peptide bond[2,55] (see section 2.2) strongly suggest that secondary structure formation will be important in this process. How will the sequence adopt different secondary structures or tertiary folds as the insertion process takes a particular part of the protein from an aqueous environment to the membrane interface to the membrane core, and perhaps out the other side? The difference in the chemical environment presented by the interfacial region and the core of a bilayer can be partly appreciated by examining the different folds adopted by proteins in those environments. Structures of five monotopic proteins, which probably insert across only one leaflet of the bilayer, reveal interactions with the membrane using loops and turns as well as helices and sheets that run parallel to the membrane surface.[504] For proteins that are classified as α-helical bundles, the canonical helical structure dominates the central 30 Å region of the bilayer, but the membrane interfacial region contains both helices lying on the membrane and irregular secondary structure; β-strands make a significant contribution only 25 Å from the middle of the membrane.[505] Some contribution to the effects of environment on secondary structure must derive from the strong tendency for tryptophan and tyrosine to be found in the interfacial region of the bilayer and for positively charged residues to be interacting with phosphate groups of lipids.[506] These data come from well-ordered regions of proteins in a thermodynamic minimum; folding intermediates may exhibit dynamic interconversion between these or other states.

While most integral membrane proteins are constitutively folded into bilayers by an insertion machinery,[52,100,102,177−179] certain soluble proteins have the unusual property of being able to associate with target membranes and insert themselves as polytopic integral membrane proteins. Examples of secreted bacterial pore-forming toxins with this property include species that form α-helical bundles (e.g., colicins[507]) or β-barrel structures (α-hemolysin[508] and others[509]) in membranes. These systems provide a means of studying the membrane insertion process of isolated polypeptides (reviewed elsewhere[510]).

Colicin E1 inserts into membranes at low pH[511] but can exchange between the surfaces of different liposomes upon return to neutral pH,[512] suggesting that the binding and insertion processes are reversible under some conditions. The destabilization of the native state under acid conditions is consistent with the formation of a "molten globule" in solution,[513] but when associated with the membrane surface, the toxin appears to form an extended, loose array of interfacial helices.[514] The lipid dependence of pore activity implicates a role for nonbilayer lipids in stabilizing or forming the pore.[515]

The pore-forming toxins of *Staphyloccocus aureus* are soluble proteins that form homomeric or heteromeric β-barrels in target membranes.[516] Crystal structures of the soluble, monomeric form of LukF toxin[517,518] and the homoheptameric α-hemolysin pore[519] provide snapshots of the initial and final states for these homologous systems;[520] the comparison identifies large conformational changes that accompany insertion and suggests possible pathways for the insertion process. Pore assembly of α-hemolysin is cooperative and involves distinct intermediates,[521] with heptamerization occurring at the membrane interface followed by full insertion and concomitant pore formation. Engineered disulfide bonds that restrict conformational rearrangements can halt this process, trapping an oligomeric prepore species that is located in the membrane interfacial region.[522,523] The structure of the α-hemolysin pore with bound phosphocholine headgroups[524] suggests how interactions with lipids favor pore insertion.

Diphtheria toxin is a modular soluble protein consisting of catalytic, transmembrane (T), and receptor-binding domains that can insert into target membranes at low pH and form a channel, with translocation of the catalytic domain across the membrane resulting in inhibition of protein synthesis.[525−528] Crystal structures of the monomeric and dimeric forms of the toxin[529−531] reveal the fold of the protein, which includes two hydrophobic helices in the T-domain. Proteolysis of bilayer-inserted toxin[532] and EPR studies of spin labels incorporated at engineered cysteines[533] map the eighth and ninth helices of the T domain (TH8 and TH9) as membrane-spanning. The T-domain alone is capable of self-inserting into membranes, and designed disulfide bridges that lock TH8 or TH9 into the soluble conformation inhibit this insertion, while reducing agents restore insertion.[534] Fluorescent probes conjugated to the cysteines that support cross-linking self-quench in the soluble conformation, but this is alleviated upon insertion, indicating a large conformational change.[534] The loop between TH8 and TH9 that is inserted across the membrane[535] contains three acidic residues that facilitate insertion[536,537] but can be replaced by amides[538] or even lysines with retention of pH-dependent insertion;[539] the hairpin-forming propensity of a proline in this loop is also

important for insertion.[540] Fluorescent probes attached at engineered cysteine residues in TH8 and TH9 identify a shallow insertion state that can convert to a deep transmembrane state in thinner bilayers or at high protein concentration.[541] Helices 5−7 of the T-domain also insert into membranes[542] but do not actually span the bilayer.[543]

Ladokhin and colleagues have used an environment-sensitive fluorescent probe attached to cysteines engineered at three different positions within the T-domain to show that the isolated domain can bind to either zwitterionic or negatively charged large unilamellar vesicles at neutral pH.[544] In POPG/POPC (but not pure POPC) bilayers, the T-domain can also undergo a reversible, pH-dependent conformational change that corresponds to transmembrane insertion.[544] The reversibility of the insertion process indicates that this system may be amenable to thermodynamic analysis; measuring the partitioning constants of T-domain for vesicles at pH 7.0 and pH 4.3 yielded estimates of the free energy changes for binding of T-domain to the membrane surface (about −7.5 kcal mol^{-1}) and for insertion of the T-domain across the bilayer (about −8.3 kcal mol^{-1}). The modest differences between these free energies suggest that the system is energetically poised for acid-triggered insertion, as would be experienced in a slowly acidifying endosomal vesicle. The observed binding to neutral membranes, even at neutral pH, shows that the isolated T-domain readily converts to a surface-associated species with minimal involvement of electrostatics. Differences between the behavior of isolated T-domain and intact toxin suggest that interactions between domains may modulate binding in a manner that is relevant to biological fuction,[533] but the important demonstration of the reversible insertion of isolated T-domain provides the possibility of thermodynamic analysis of the sequence and lipid dependence of hairpin insertion across membranes.

The annexins are Ca^{2+}-binding proteins present in higher eukaryotes that respond to intracellular calcium signals to modulate events at biological membranes.[545] Soluble annexin monomers bind to the surfaces of negatively charged membranes utilizing a conserved core domain, with some species forming trimers[546] but others remaining monomeric.[547] Crystal structures of annexin V suggest that the monomer or trimer can interact with membranes with a concave face that also binds calcium ions.[548−551]

Annexin B12 can insert across the membrane as well as interact with its surface.[552] EPR studies of nitroxide labels on engineered cysteines have shown that residues 134−163, which form a helix−loop−helix in the crystal structure of the annexin B12 soluble form,[553] adopt a similar conformation in the aqueous monomer or calcium-dependent membrane surface-associated form[554,555] but are inserted in a calcium-independent fashion into the membrane at low pH as a single continuous helix.[556] Another pair of helices in the crystal structure of the soluble form of the protein, extending from residues 251−273, also insert across the membrane, with one face of this helix apparently exposed to an aqueous pore.[557] FRET measurements detect a transient intermediate and demonstrate that the fully inserted species exist as monomers, not as oligomers, suggesting that pores made by annexin B12 must contain several transmembrane spans contributed by a single protein molecule.[558] Ladokhin and colleagues have shown that annexin B12 converts between the surface-associated and transmembrane conformation reversibly in a pH-dependent fashion,[559] opening up the possibility of using this system to study the thermodynamics of helix insertion into membranes. The strong lipid dependence of calcium-independent insertion[555] suggests that this system may yield information on how lipid composition can affect the kinetics and thermodynamics of the insertion of helices into membranes.

Other eukaryotic proteins that undergo conformational changes to insert into membranes, including Bax[560] and other members of the Bcl-2 super-family,[561] are being intensively studied to understand how these insertion processes underlie various biological processes (such as mitochondria-mediated apoptosis). These and other tail-anchored proteins can insert into various subcellular membranes posttranslationally and apparently without the assistance of a translocation machinery.[562,563] Interestingly, spontaneous insertion of the hydrophobic C-terminal tail of cytochrome b5 into liposomes or microsomes can be blocked by incorporation of cholesterol into the bilayers,[564] which suggests a role for lipid composition in directing the targeting of tail-anchored proteins.

Short peptides that insert spontaneously into or across membranes have been the subject of biochemical and biophysical investigations for decades because of their experimental accessibility. The classic example is the bee venom lytic peptide melittin, a 26 amino acid amphipathic peptide that is largely unstructured in aqueous solution at low salt but can partition onto or insert across bilayers in a manner that is coupled to helix formation;[71] this system has been extensively reviewed.[565,566] The interfacial positioning of melittin was determined by an X-ray diffraction method[567] to be near the glycerol backbone of the lipids in a fluid bilayer.[568] Oriented circular dichroism measurements indicate that melittin can assume a transbilayer orientation in POPC but not when POPG is present,[569] indicating a lipid dependence to the transition between a surface-associated state and the transmembrane conformation[570] that parallels the effect of lipid composition on melittin-mediated lysis.[571−573] Kinetic studies of the association of melittin with model bilayers by both fluorescence and CD indicate the presence of intermediates in the insertion process and demonstrate that lipid composition influences the process.[476,574]

Direct measurement of the kinetics and thermodynamics of the spontaneous insertion of a single transmembrane helix into a bilayer is an important goal in the study of membrane protein folding. Availability of such a system would allow the sequence dependence and lipid dependence of the process to be explored in detail. However, many potentially complicating factors present fundamental problems for these studies. Hydrophobicity is a major driving force for transmembrane insertion of apolar sequences, but strongly hydrophobic sequences are likely to aggregate in solution, rendering thermodynamic interpretations difficult or impossible. For instance, the pH-dependent, reversible interconversion of the surface-associated and transbilayer states of a peptide corresponding to the third membrane span of bacteriorhodopsin is complicated by oligomerization and aggregation of the peptide in the aqueous phase.[410] Even if such aggregation can be avoided, the partitioning of many model or biological sequences could be driven by more than 20 kcal mol^{-1}, which would be experimentally challenging to quantify. Inclusion of polar residues might help prevent aggregation in the aqueous phase and should lower the total free energy of partitioning (as discussed in sections 3.3 and 3.4), but polar residues could also drive association within the membrane (as discussed in section 4.2.2), complicating thermodynamic analysis. However, with current knowledge

of the terms that influence partitioning of peptides into the interfacial regions of membranes, several model systems have been developed to address this process.

White and colleagues have designed two peptides that insert spontaneously across bilayers.[575,576] The initial study characterized the properties of a 31 amino acid peptide containing a 21 residue apolar core that includes 15 alanines, thus keeping the overall hydrophobicity of the peptide low and enabling it to be water soluble. This positively charged peptide partitions strongly (apparent free energy of −7.1 kcal mol^{-1}) and reversibly into POPC bilayers, and most of the peptide acquires a transbilayer orientation.[575] The tendency of this peptide to aggregate in solution was reduced in a later design by including two histidines and a proline in the membrane span and removing an unintentional alanine heptad repeat; this peptide partitions reversibly into the membrane interface in a pH-dependent manner that involves protonation of the histidines and can achieve 30−40% transbilayer insertion, although this last process is not entirely reversible.[576]

Meijberg and Booth performed a detailed analysis of the insertion kinetics of a polyalanine-based peptide flanked by a total of six lysines into bilayers of different lipid compositions and identified kinetic intermediates and lipid dependences for the observed rates.[477] The model hydrophobic peptide[577−579] is soluble in water and partitions to the interface of POPC membranes at pH 10.5 on ice but can insert across the bilayer at pH 10.5 and 30 °C with biphasic kinetics exhibiting time constants of 30 and 430 s.[477] The temperature threshold and the time constants for the insertion process are strongly affected by the inclusion of DOPE in the membranes, which opposes peptide insertion;[477] the Arrhenius activation barrier for insertion goes from 21 ± 2 kcal mol^{-1} in pure DOPC to about 31 kcal mol^{-1} in 60:40 DOPE/DOPC. These data suggest that insertion of helices into membranes may be inhibited by lateral pressure.

Matsuzaki and colleagues have investigated the membrane partitioning behavior of a model peptide without polar or ionizable side chains[117] by observing its exchange from one set of vesicles into another.[118] Although this alanine-based peptide lacks formal charges that make the other peptides discussed here readily soluble in water, transfer between POPC vesicles occurs through an aqueous monomer, and the activation barrier for the process is 17.7 ± 1.3 kcal mol^{-1}. This barrier is attributed to the dissociation process, indicating that the barrier to insertion is probably much lower.[118] This system appears to be amenable to the study of peptide partitioning between bilayers of different compositions and to kinetic characterization of aspects of transmembrane insertion, although the observed tendency of the peptide to form weak antiparallel dimers[117] will complicate thermodynamic measurements and interpretations.

6.2. Refolding from Denaturants

6.2.1. Bacteriorhodopsin

The ability to fold the SDS-denatured apoprotein bacterioopsin to functional bacteriorhodopsin[38,41] provided the basis for the first kinetic refolding studies of a helical membrane protein, which have been extensively reviewed elsewhere.[4,580,581] Booth, Khorana, and colleagues described the fluorescence changes on the millisecond time scale that follow rapid mixing of SDS-denatured bacterioopsin with SDS/DMPC/CHAPS mixed micelles in the presence or absence of the chromophore retinal.[443] Five kinetic phases with time constants ranging from 4 ms to hundreds of seconds are observed in the processes that lead to folded bacteriorhodopsin (see Figure 14); only the three fast phases are observed in the absence of retinal, while addition of retinal to prefolded bacterioopsin gives the slower phases. These fluorescence data suggest a minimal linear kinetic scheme for bacteriorhodopsin folding that includes an intermediate on the pathway to a partially folded apoprotein, followed by the noncovalent binding of retinal to apoprotein, and finally covalent Schiff base formation with acquisition of native structure.[443] The rate-limiting step is independent of retinal concentration, but one second-order step results in formation of a noncovalent complex between retinal and protein.[582] Prefolded bacterioopsin appears to bind retinal to form two different noncovalent intermediates that decay to folded bacteriorhodopsin; while the two species form at a rate of about 1 s^{-1} and decay about 100-fold more slowly, the pH of the sample modulates the fraction of protein that passes through each of these parallel pathways.[583]

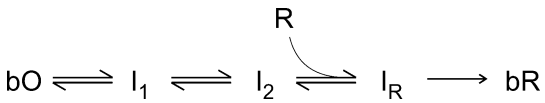

Figure 14. Kinetic scheme for the folding of bacteriorhodopsin in mixed micelles. Unfolded apoprotein bacterioopsin (bO) proceeds through an intermediate I_1 to form I_2, the folded apoprotein, which can be isolated in the absence of retinal. In the presence of retinal (R), the folded apoprotein binds retinal noncovalently to form intermediate I_R, followed by a rearrangement that leads to covalent bonding to the retinal and folded bacteriorhodopsin, bR. This simplified scheme does not depict the existence of multiple observable states for I_R or other intermediates. Adapted with permission from ref 581. Copyright 2000 Elsevier Ltd.

Manipulating the conditions under which refolding occurs has yielded additional structural details for these kinetic phases. Substituting the short-chain lipid dihexanoylphosphatidylcholine (DHPC) for the detergent CHAPS in the mixed micelles of the refolding buffer facilitates CD spectroscopy measurements, and changing the mole ratio of DMPC/DHPC and the pH can slow the rate-limiting step by an order of magnitude.[584] With the time resolution afforded by manipulating the conditions to favor slower folding, Booth and colleagues showed that about 30 residues of bacteriorhodopsin acquire helical structure with a time constant of 80−120 s in the step that results in formation of folded apoprotein. This kinetic complexity demonstrates that SDS-denatured bacterioopsin, which has ∼60% of native α-helical structure, gains helicity in distinct kinetic phases; the authors suggest that these steps may correspond to extension of shorter, preexisting helices that are stable in SDS. Slow acquisition of α-helical secondary structure that leads to formation of folded apoprotein either is rate-limiting or occurs rapidly after some other rate-limiting process. The physical basis for the slow rates of folding is not clear. Modulation of the folding rate by the mole fraction of DHPC may be mediated through the effect of lipid composition on the bending rigidity or lateral pressure of the mixed micelles.[4,584,585] Native lipids can also alter the rates of the slow steps in bacteriorhodopsin folding, although this may be due to tight, specific interactions between lipids and protein.[586]

Refolding a membrane protein in a lipid bilayer (as opposed to detergent/lipid mixed micelles) provides the opportunity to fully explore the lipid dependence of various folding steps, but it is technically challenging because of

contributions of scattered light in the optical methods used to probe the folding process. Pioneering work in high efficiency refolding of SDS-denatured bacterioopsin directly into phosphatidylcholine bilayers showed a decrease in overall efficiency as the mole fraction of phosphatidylethanolamine is increased, adding to the evidence that bilayer lateral pressure inhibits folding.[587] Kinetic studies of this refolding process reveal an additional intermediate compared to refolding in mixed micelles, and the influence of the mole fraction of phosphatidylethanolamine indicates that the equilibrium between two forms of the apoprotein is influenced by lateral pressure within the bilayer.[588] In experiments where saturated phosphatidylcholine or lyso-phosphatidylcholine are used to relieve the spontaneous negative curvature of an unsaturated phosphatidylcholine membrane or phosphatidylethanolamine is used to enhance the lateral lipid pressure, bacteriorhodopsin refolding efficiency correlates with conditions of decreasing lateral lipid pressure.[474] These studies of bacteriorhodopsin and analogous studies with the multidrug transporter EmrE[475] strongly implicate bulk bilayer properties in the kinetics of integral membrane protein folding and suggest ways in which the folding, stability, and function of helical membrane proteins can be modulated by lipid composition.

Changes to the loop sequences of bacteriorhodopsin have been shown to alter the kinetics of folding of either the apoprotein or the holoprotein, depending on the loop. Replacement of each of the loops between membrane spans with (presumably flexible) repeats of glycine and serine has varied effects on refolding, with the loops between the third and fourth span and between the fifth and sixth span being implicated in the rate-limiting step for folding and the stability of the apoprotein intermediate, while the loop between the sixth and seventh span seems to affect the final folding and covalent bond formation with the retinal cofactor.[416,417] These results show that the *sequences* of loops, and not merely the existence of a topological connection between membrane spans, contribute to bacteriorhodopsin folding kinetics. Intact loops are not absolutely required for formation of functional protein but do contribute to thermodynamic stability[406−408,415] as discussed in section 4.5.

Alanine or glycine point mutations at Pro50 or Pro91, which lie in transmembrane helices of folded bacteriorhodopsin, slowed the rate of formation of folded apoprotein by a factor of 5−7, while the same mutations at Pro186, the other membrane-embedded proline, have no effect on this kinetic step.[454] These findings eliminate cis−trans proline isomerization as an important factor in folding of the apoprotein. The substitutions at these prolines have minimal effects on the noncovalent binding of retinal to the folded apoprotein but exhibit a wide array of effects in the final step that involves Schiff base formation and folding to the native state. Biexponential kinetics for this last phase are observed for Pro50Ala and Pro50Gly, which fold more than five times faster than wild-type, while Pro91Ala and Pro91Gly fold about 12 times more slowly than wild-type with a single rate constant. Pro186Ala exhibits slow biexponential decay to the folded state, while Pro186Gly folds to a state with a blue-shifted chromophore with approximately wild-type kinetics.[454]

These data point to transmembrane span proline residues as important factors in determining the rates of folding of the apoprotein and the subsequent binding and bonding to the retinal chromophore. Perhaps the most interesting mutant

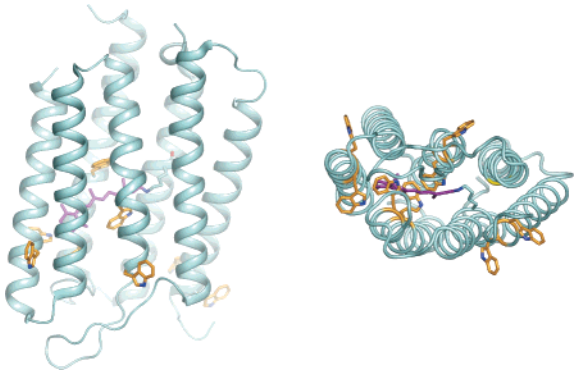

Figure 15. Cartoon representation of bacteriorhodopsin (PDB 1C3W) with the retinal chromophore (purple) and the eight native tryptophan side chains (orange) drawn as sticks. Although the tryptophans cluster to one side of the membrane, they make many contacts with other helices and some help to form the retinal binding pocket, which is composed of atoms from each of the seven membrane spans.

is Pro50Ala, in the second membrane span of bacteriorhodopsin, which has recently been shown by Bowie and colleagues to have the same stability and nearly the same helical kink in the final folded mutant structure as wild-type.[252] The kinetic data show that the mutation Pro50Ala *slows* the formation of the folded apoprotein about 4-fold, has essentially no effect on the noncovalent binding of retinal, but *increases* the rate of the final folding step (3−9-fold, depending on the component in this biexponential decay).[454] These data allow inferences to be drawn about the nature of the apoprotein intermediate in the bacteriorhodopsin folding pathway. In the SDS-denatured state, the wild-type protein may have a tendency to kink at Pro50, but the mutant should have no such tendency at Ala50. The slower rate at which unfolded Pro50Ala converts to folded apoprotein compared to wild-type suggests that formation of the folded apoprotein requires a kink or bend similar to that seen in the wild-type and Pro50Ala folded structures: kinking the wild-type sequence at Pro50 would be easy, but introducing a bend at Ala50 should be more difficult. This implies that folded apoprotein makes helix−helix interactions that are near-native. Since the mutant Pro50Ala speeds the subsequent rearrangement that leads to Schiff base formation and the final folded state, the noncovalent retinal/protein complex may undergo a partial unfolding of the retinal binding pocket to accommodate the final folding events; this unfolding would be opposed by native Pro50 but assisted by Ala50. Although the presence of such kinks is a subtlety not often considered in the two-stage view of membrane protein folding, the potential effects of a proline on lateral interactions between helices seems to rationalize these kinetic observations quite well. It should be noted that the experimental separation of the refolding of bacteriorhodopsin into retinal-dependent and retinal-independent steps[443] is critical to the observation of these various effects since the slow rate of formation of the folded apoprotein could be masked at high retinal concentrations.

Bacteriorhodopsin provides an excellent system in which to explore both the thermodynamics and the kinetic refolding of a helical membrane protein: it is stable, folds reversibly in detergents, can be expressed in high yield and purity, gives crystals that diffract to high resolution, and contains intrinsic tryptophans and a bound retinal chromophore (see Figure 15)to that are sensitive optical probes of the conformation of the protein. Although few membrane proteins are likely

to be as amenable on all these fronts, methods for probing the kinetics of folding using other techniques such as fluorescence resonance energy transfer[589] are being tested in bacteriorhodopsin and may be transferable to other systems that lack the spectroscopic probes intrinsic to bacteriorhodopsin.

6.2.2. Diacylglycerol Kinase

Kinetic studies of folding and misfolding of the trimeric integral membrane protein diacylglycerol kinase complement the stability studies in mixed micelles reviewed in section 5.1. Spontaneous but low efficiency insertion refolding of diacylglycerol kinase into bilayers from micellar solutions (30% efficiency) or from a urea-denatured state (4% efficiency) was first observed using an assay for the kinase activity of the protein.[439] Detergent extraction of diacylglycerol kinase and point mutant variants expressed at high levels in *E. coli* yields protein with low and poorly reproducible specific activity, but these proteins can typically be refolded to yield improved activity.[590] However, properly folded and functional diacylglycerol kinase in detergent solution becomes inactivated over time, not through covalent modification but by a conformational change that can be overcome by unfolding and refolding.[591] The slowing of inactivation at high protein concentrations and a strong correlation between the SDS denaturation midpoint and the thermal inactivation half-life for cysteine substitution variants of the protein suggests that inactivation occurs through the monomeric unfolded state, perhaps by a misfolding event.[591]

Activity assays and solution NMR spectra reveal that a highly stable triple mutant variant of diacylglycerol kinase is sometimes purified as a mixture of folded, active protein and alternately folded, inactive protein.[592] Surprisingly, both species are homotrimers and the "misfolded" species gives rise to a distinct set of NMR resonances with high chemical shift dispersion, indicating that a specific, near-native, but catalytically inactive conformation may be kinetically trapped during the expression, extraction, and reconstitution process. This alternately folded species is resistant to refolding protocols, suggesting that it is separated from the folded state by significant energy barriers.[592] (By contrast, NMR evidence for alternate conformations of the β-barrel membrane protein PagP indicates that these species do interconvert, with low temperature favoring the folded conformation of a loop that unfolds at high temperature.[593,594]) While membrane protein misfolding in vivo that is related to disease states often involves mistargeting or degradation of misfolded proteins (reviewed in refs 595−597), it is not clear what the precise determinants of "misfolding" are or whether species such as the triple mutant would misfold during normal biogenesis or be recognized by quality control machinery.

Sanders and colleagues used the appearance of enzyme activity to explore the refolding of diacylglycerol kinase from detergent micelles, urea, or guanidinium hydrochloride solutions into mixed micelles or lipid bilayers on the time scale of tens of seconds.[598] Under these conditions, diacylglycerol kinase refolds most rapidly and efficiently when diluted from detergents, where the protein retains its trimeric structure, into detergent/lipid mixed micelles. However, functional enzyme can be refolded from acidic 6.5 M urea, where it is α-helical and retains tertiary structure, or from 8 M guanidinium hydrochloride, where the protein is largely unfolded. The refolding of helical bundle membrane proteins from chaotropic denaturants such as urea or guanidinium hydrochloride makes it possible to eliminate the potential membrane-perturbing influences of detergent molecules.

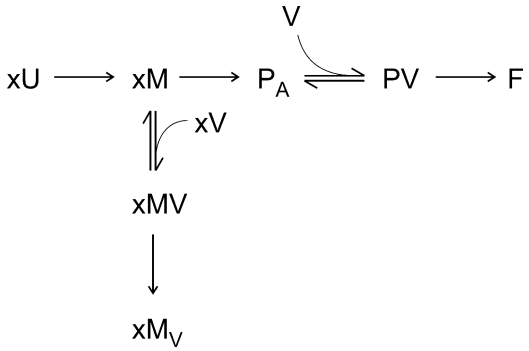

Figure 16. Kinetic scheme for the folding and misfolding of acid-urea denatured diacylglycerol kinase by dilution into vesicles. Unfolded monomers, U, in 7 M urea are converted to a monomeric state M upon dilution; this state can form a low order aggregate P_A in water, which then associates reversibly with vesicles (V) as a PV complex before inserting and folding to the trimeric folded state F. At high lipid concentrations, M associates directly with vesicles to form MV complexes that become vesicle-inserted monomers (M_V) but cannot fold to the trimer since each vesicle contains fewer than three proteins. This simplified scheme does not include off-pathway fates for U or P_A that are discussed in ref 599. Adapted with permission from ref 599. Copyright 2004 Elsevier Ltd.

Lorch and Booth used fluorescence spectroscopy to probe the early stages of refolding of diacylglycerol kinase from an acidic urea-denatured state into vesicles.[599] Data at the millisecond time scale showed that while the unfolded protein associates with lipid bilayers rapidly, the urea-unfolded monomer also tends to aggregate after dilution to low urea levels. Competition between monomer insertion and aggregation/oligomerization is modulated by the concentration of protein and of lipid, with maximal folding efficiency (as measured by protein activity) proceeding through the formation of aggregates (see Figure 16). The authors point out that if dilute monomers insert into abundant vesicles, there may be fewer than three proteins per vesicle, causing a topological barrier to functional trimerization.[599]

Nagy and Sanders showed that a single point mutation (Tyr16Cys) that has little effect on the activity or thermal stability of diacylglycerol kinase dramatically reduces the efficiency of refolding from urea into bilayers but not into detergent micelles. The rate of functional folding into bilayers is slowed 10-fold, suggesting that the substitution affects how the protein inserts into and crosses the membrane.[600] It is tempting to speculate that this alteration is due to a kinetic effect from the loss of the preferential interfacial membrane partitioning of tyrosine in some intermediate or transient state (as indicated by the WW scale[55,65]) that does not affect the overall stability of the folded protein. However, for most other sequence variants surveyed, the resistance of the mutant protein to thermal inactivation or denaturation by SDS correlates with the ability of that variant to insert into membranes from an unfolded state.[601]

6.2.3. Other Systems

The success of these pioneering studies and the ability to refold other polytopic helical proteins by rapid dilution suggests that detailed folding kinetics studies of this class of proteins will be more common in the future. Otzen has used stopped-flow fluorescence to monitor both rapid folding and rapid unfolding of disulfide-bond reducing protein B

(DsbB, which spans the membrane four times and whose function is reviewed elsewhere[602]) and has characterized the protein at different mole fractions of the denaturant SDS in SDS−dodecylmaltoside detergent mixed micelles.[603] The kinetic data are well approximated by single exponentials, and where folding and unfolding rates can be measured for the same mole fraction of SDS, the rates correspond closely, indicating microscopic reversibility. Folding and unfolding rates, as well as thermodynamic stability, are perturbed by reducing the active site disulfide bonds and by a point mutation (Ala57Gly).

The light-harvesting chlorophyll a/b complex has been refolded from SDS[604] and from guanidinium hydrochloride,[605] and the binding of carotenoids and chlorophylls provides a number of spectroscopic signatures well into the visible spectrum that may enable characterization of folding in samples that scatter light strongly in the ultraviolet. The refolding of this complex has been used to screen random mutagenesis libraries on 96-well plates for protein sequence variants with altered pigment binding abilities;[606,607] mutations in the stromal and lumenal loops as well as transmembrane domains affect reconstitution and the stability of the complex.[608] The three-dimensional structure of the complex[609,610] provides a basis for making structure-based inferences about stability.

6.3. Cotranslational and Posttranslational Folding

Most eukaryotic helical integral membrane proteins arrive at membranes after the ribosome that has translated their N-terminal signal peptide[611] interacts with the signal recognition particle[178] and is brought to the Sec61 translocon[176] to allow secretion or membrane integration of the translated and translocated polypeptide chain.[102] Section 3.4 of this review outlines how the sequences and flanking charges of potential membrane spans help determine their orientation and integration propensity. The mechanistic details of these cotranslational processes are the subject of intense investigation and some controversy, as reviewed in refs 100, 101, and 177−179. However, it seems clear that the portion of a protein passing through the translocon can integrate laterally into the bilayer if that stretch is sufficiently hydrophobic to partition favorably into the membrane.[190−193]

Because it takes many minutes for a large polytopic membrane protein to be translated, one plausible scheme for determining the topology of such a protein would be for the orientation of the first hydrophobic span to be decided based on flanking charges (the positive-inside rule) and for any subsequent membrane spans to simply recross the membrane, following the topological decision of the span that preceded them.[184] While it has long been known that simple models of this sort cannot account for all experimentally observed topologies,[183,185] the versatility of the translocon machinery and the impact of kinetics on topogenesis are still being explored (reviewed in ref 99). Even for a protein such as aquaporin-4, in which each transmembrane domain independently directs its own sequential insertion and topology,[612] contacts between the nascent polypeptide chain and the translocon change progressively as chain elongation occurs, with membrane spans contacting the translocon for different lengths of time and at different locations within the translocon.[429] Considerable complexity underlies the biogenesis of even apparently straightforward integral membrane proteins.

Signal anchor sequences are internal hydrophobic stretches within a protein that can be incorporated into a membrane in either an N_{exo}/C_{cyt} orientation (type II) or an N_{cyt}/C_{exo} orientation (type III). Both the flanking charges and the hydrophobicity of the sequence influence topology, with more hydrophobic spans favoring the N_{exo}/C_{cyt} topology.[197,613,614] N_{cyt}/C_{exo} topology requires that the protein N-terminal to the signal anchor, which has been translated in the cytoplasm, be translocated to the lumen of the endoplasmic reticulum; although hundreds of amino acids can be translocated in this manner, if this part of the protein has folded into a tight structure then translocation is blocked.[615] This shows that folding in the aqueous domains can be coupled to topogenesis and that the energetics of the protein substrate can affect kinetic processes being implemented by the translocation machinery. A signal anchor span with a strong tendency for N_{exo}/C_{cyt} orientation can "pull" a hydrophilic sequence to its immediate N-terminal side into the membrane even if the topology of a preceding span means that this hydrophilic sequence will be topologically trapped in a transmembrane configuration.[616]

The success of the base biological hydrophobicity scale at predicting the fractional integration of model and biological membrane spans into bilayers[192,193] described in section 3.4 reinforces the view that translocon-mediated integration is controlled at least in part by thermodynamics and suggests that other aspects of translocon function might also be explained by apparent free energy scales. Spiess and colleagues have shown that the propensity for N-terminal versus C-terminal translocation of a single stop-anchor sequence in COS cells is explained largely by amino acid composition, although both glycine and proline exhibit positional effects.[197] Despite the potential presence of kinetic effects in such systems, as reviewed elsewhere[99] and described below, it is tempting to think that such approaches might enable construction of scales for topology decisions as well as integration into the membrane.

Topology of membrane proteins can apparently be dynamic, and sequences that are transiently exposed to the lumen of the ER may become trapped there by glycosylation.[617] Recent results suggest that the N_{cyt}/C_{exo} orientation of a lone signal-anchor sequence is acquired after the span initially inserts as N_{exo}/C_{cyt}.[618] Reorientation from N_{exo}/C_{cyt} to N_{cyt}/C_{exo} can occur while a soluble loop is being translocated; the rate of reorientation is enhanced by flanking charges as per the positive-inside rule and is slowed by strong hydrophobicity of the first span[618] (see Figure 17). Aromatic residues in different positions within the hydrophobic sequence can strongly modulate the rate of reorientation,[619] and translocation of the next membrane span of a polytopic membrane protein can halt reorientation.[620]

Membrane integration and topology depend on the sequence of the span in question but can also be determined by context and by interactions between different potential membrane spans.[621] The topology and length of the first span, as well as the length of the intervening loop, help determine whether the second span of erythrocyte band 3 is integrated or secreted,[622] although it seems that the precise sequence of the first membrane span is not critical. Analysis of translocation intermediates by proteolysis and cross-linking showed that the first transmembrane domain of bacterial leader peptidase integrates into the ER membrane on its own and is able to diffuse away from the translocon when the second span is still within the ribosome, but the first span is once again associated with the translocon in intermediates where the second membrane span is becoming incorpo-

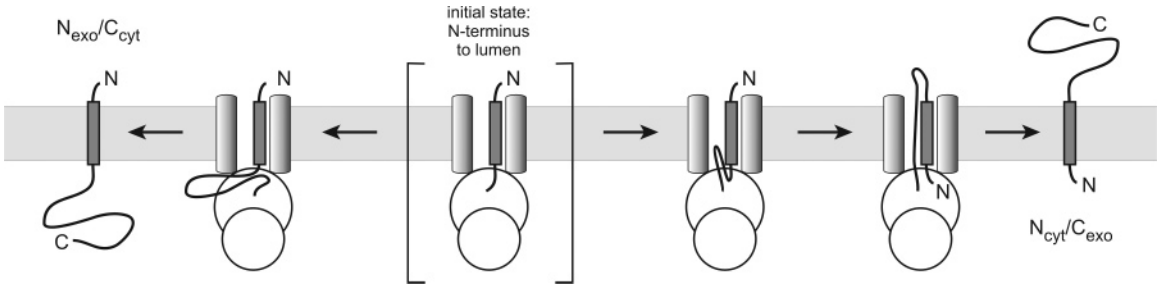

Figure 17. Mechanism by which a model bitopic protein can achieve either N_{exo}/C_{cyt} or N_{cyt}/C_{exo} topology. A protein that is initially oriented N_{exo}/C_{cyt} retains this orientation if the signal anchor is strongly hydrophobic (arrows to left) but can reorient to follow the positive-inside rule (arrows to right) if the charge difference across the span is large and if the span is weakly hydrophobic.[618] Alternative mechanisms, such as variable insertion orientation depending on flanking charges, may be used for different constructs. Adapted with permission from ref 618. Copyright 2003 by the European Molecular Biology Organization (Nature Press Group).

rated.[502] Mutation of charged residues in one membrane span affects integration efficiencies of other spans of a potassium channel, indicating that sequence-specific charge−charge interactions between helices help stabilize membrane insertion of helices[623] and that folding within the membrane is thus coupled to topogenesis.

Cotranslational and posttranslational processes including the folding of soluble domains, interdomain association, and helix−helix interactions within membranes all contribute to the folding of the cystic fibrosis transmembrane regulator (CFTR) chloride channel, the gene underlying the childhood disease cystic fibrosis (reviewed elsewhere, refs 101 and 624). CFTR is composed of six membrane spans followed by a large cytoplasmic nucleotide binding domain and a regulatory domain, followed by six more membrane spans and another nucleotide binding domain. Misfolding of the protein is implicated in disease states resulting from mutations in the coding region.[625−627] The sixth membrane span of CFTR, which contains three positively charged residues, fails to act as a stop-transfer sequence on its own or with the preceding five spans but is able to integrate into membranes when followed by the first cytoplasmic domain.[628] Another manifestation of how CFTR topology depends on cooperation of different parts of the protein is given by the eighth membrane span of wild-type CFTR, which is unable to act as a stop-transfer sequence when inserted into a secreted protein due to the presence of an aspartic acid in the middle of the span, but does incorporate successfully into membranes when preceded by the seventh CFTR membrane span.[629]

Initial studies had shown that the most common CFTR mutation, deletion of Phe508 within the first nucleotide binding domain, affects the folding and nucleotide binding activity of a synthetic peptide spanning the mutation site.[630] However, this mutation has only minor local effects on the structure (and no effect on the stability) of the isolated, full-length domain;[631,632] substitutions at Phe508 also have minimal effects on structure and stability of the domain.[631] These findings suggest that the deletion affects the interaction of the folded domain with another portion of the protein rather than the stability of the domain itself.[631,632] Deletion or replacement of Phe508 has severe consequences on the proper folding and maturation of full-length protein in vivo,[631,632] with the *second* nucleotide binding domain becoming highly susceptible to proteolysis upon mutation of the first,[633] further supporting the idea that interdomain interactions dependent on Phe508 underlie the maturation and stability of CFTR. Hydrophobic substitutions at position 508 reduce the folding efficiency of the protein without greatly influencing the half-life of the mature protein, while all charged substitutions, most polar substitutions, and the deletion mutation severely reduce both the fraction of properly folded protein and its half-life in vivo,[633] consistent with a role for protein degradation in the CFTR phenotype.[634,635] Both wild-type and mutant CFTR protein undergo ER-associated degradation, but different pathways are implicated in this process for the different species.[636]

Studies with model membrane proteins have shown that a 40 residue polyleucine sequence that inserts in a N_{cyt}/C_{exo} orientation can be induced to form a hairpin by placing a strongly polar residue two or three turns of helix from each end of the hydrophobic stretch.[637] This suggests that as the translated polypeptide chain is threaded through the translocon to push the C-terminal end of the membrane span into the lumen, the chain samples the hairpin conformation and can "choose" to integrate as a hairpin rather than continue translocating to the lumen. Substitutions in the middle of a long hydrophobic span can also induce hairpin formation.[187−189] Thus, it appears that proteins being passed through the translocon have the option not only to partition into the membrane, depending on their hydrophobicity,[190−193] but also to sample,[638] at least for some limited time period, the *orientation* in which such partitioning occurs.

This possibility may explain how changing the lipid composition of the *E. coli* inner membrane results in altered topology of several polytopic integral membrane proteins[491,492] including LacY,[487,488] as discussed in section 5.2. Since the presence or absence of phosphatidylethanolamine determines whether lac permease is reconstituted into artificial bilayers with proper or altered topology,[501] lipid composition seems to influence which topology is energetically favored. Studies of LacY translocation in *E. coli* membrane vesicles with normal lipid composition have shown that none of the first six membrane spans of LacY integrate into the membrane until late in the translation of the protein.[489] Thus, one explanation is that the permease tests various topologies for its N-terminal half with the help of the translocon machinery and, when the phosphatidylethanolamine content of the cell membrane is lowered, the permease acquires the altered topology because it is energetically preferred. There might be a direct influence of lipid composition on orientation or integration of the seventh membrane span, which would normally be oriented N_{cyt}/C_{exo}, or lipids might affect helix−helix interactions in a way that favored one final topology over another. The observed posttranslational correction of topology following restoration of normal lipid composition for both LacY[488] and the transporter PheP[491] suggests that, at least in *E. coli*, proteins

can have their topology "proofread" posttranslationally. This process seems likely to involve the translocon machinery, but could also involve additional proteins such as chaperones that might recognize "misfolded" integral membrane proteins or assist in the topological rearrangement. This posttranslational reorganization strongly favors thermodynamic, rather than kinetic, control in the lipid dependence of the topology of these two particular proteins. But how does this occur? Does a ribosome have to be recruited to the membrane? Because reorientation of membrane spans in the eukaryotic translocon appears to be halted with the end of translation and often before, this potential mechanism is probably not broadly generalizable; there appear to be significant kinetic components to the regulation of eukaryotic polytopic integral membrane protein topogenesis. This potential mechanistic difference between the translocons suggests a possible explanation for why eukaryotic integral membrane proteins often express poorly in prokaryotes: eukaryotic membrane protein sequences may have evolved kinetic control elements for folding (including glycosylation), but prokaryotes cannot use these inputs and instead "proofread" the topology of proteins in their membranes continuously.

6.4. Chemical and Pharmacological Chaperones

Mutations in integral membrane proteins underlie various human disease states (reviewed elsewhere[597]), and many such mutations alter the normal trafficking or assembly of the protein, rather than perturbing function directly. A growing body of evidence that proper targeting and folding of these mutant proteins can be restored by the addition of nonspecific stabilizing agents or of ligands that target the particular protein of interest[639] suggests therapeutic approaches for many genetic diseases and provides insights into the mechanisms at work in the folding of membrane proteins in vivo.

The cystic fibrosis transmembrane regulator (CFTR) chloride channel is the gene underlying the childhood disease cystic fibrosis (reviewed elsewhere[624]). Many CFTR mutants are expressed but fail to be transported to the plasma membrane in cultured cells[627] and in patients;[625] these trafficking defects are temperature sensitive in many cases,[626] and the role of the translocon machinery in the assembly and function of this protein is under intense investigation (reviewed elsewhere[101]). The demonstration that glycerol (~1 M) and trimethylamine oxide (~100 mM) rescue the cell-surface expression of the most common CFTR mutant, deletion of amino acid 508, suggests that certain osmolytes (or osmophobes[640]) could help stabilize the mutant protein at a critical stage of its folding.[641,642] Indeed, the polyhydric alcohol myoinositol, alone or with taurine and betaine, functionally rescues this CFTR mutation in bronchial airway cells.[643] Glycerol also improves cell-surface expression of aquaporin-2 mutants[644] and functional overexpression of human P-glycoprotein in *S. cerevisiae*.[645] The implication from these findings is that the mutations block the folding pathway (or enhance misfolding) in a way that can be corrected by stabilizing agents: once the proteins fold correctly, they are active and reasonably stable, so the mutations have not altered residues that are critical to structure, stability, or catalysis.

Many integral membrane proteins that are misprocessed due to mutations can be rescued by ligands that bind specifically to the folded protein, so-called pharmacological chaperones. Mutations in the transmembrane domains, loops, and nucleotide binding domain of P-glycoprotein that cause retention of synthesized protein in the endoplasmic reticulum and degradation can be rescued by substrates or modulators of this energy-dependent drug effluxer.[646] Proper folding and expression of a temperature sensitive mutant of the HERG potassium channel can be restored by treating the cells with glycerol or with three different channel blockers, which can even rescue the protein after translation is complete.[647,648] Cell surface expression of the V2 vasopressin receptor can be increased by antagonists that readily permeate through membranes,[649] and these agents are also effective when applied posttranslationally.[650] Both agonists and antagonists of the δ opioid receptor reduce the half-life of a precursor form of the receptor and increase cell surface expression of mature protein when applied at sub-micromolar concentrations.[651] The effectiveness of many pharmacological chaperones correlates with their binding affinities,[648,650,651] suggesting that the free energy of ligand binding contributes to stabilizing either a folding intermediate or the final folded form of the proteins. Because these chemically diverse ligands induce protein-specific rescue of folding, the ligands are probably binding to a native or near-native state of the protein that is accessible to the mutant but that otherwise would fail to proceed to the native folded state. Binding of the ligand may stabilize a nativelike folding intermediate long enough to allow the correct folding pathway to proceed.

These studies highlight the possibility of using ligands therapeutically to induce proper folding of otherwise misprocessed mutant membrane proteins; indeed, the drug diazoxide (which is used to treat patients having mutations in an ATP-sensitive potassium channel) is not only a channel opener, but also a ligand that helps restore proper folding and trafficking to the mutant channel.[652] The ability of the pharmacological chaperone 11-*cis*-7-ring retinal to rescue the folding and cell-surface expression of the Pro23His mutant of rhodopsin, the most common point mutation for autosomal dominant retinitis pigmentosa, suggests that this or other similar agents may have therapeutic value. An initial report that the compound curcumin suppresses the phenotype of mice bearing mutant CFTR channels by rescuing functional expression[653] has been contradicted by other studies,[654−656] but curcumin may stimulate opening of defective but cell-surface associated channels in a way that helps restore function.[657,658] High-throughput screening has identified not only CFTR activators[659] and agents that correct channel gating defects,[660] but also pharmacological chaperones that enhance cell-surface expression and function of CFTR mutant proteins.[661] The ability to screen not only for function but also for proper subcellular localization adds another method to the approaches that can be employed in seeking therapeutic agents to correct the disease-related effects of misfolding mutations on membrane proteins.

7. Summary

Although the current data have not yet yielded a complete picture of the energetics of interfacial association, transbilayer insertion, and lateral assembly of helical membrane spans, the advances made thus far indicate strongly that the thermodynamic frameworks of Popot and Engelman and of Wimley and White can incorporate a multiplicity of experimental inputs to explain the behavior of helical membrane proteins. Experiments on the kinetics and thermodynamics of membrane protein folding are providing insight into the dependence of folding on protein sequence and lipid composition, and advances in the biophysical approaches

used to study these processes, as well as the availability of more structures of membrane proteins, are increasing the number of systems in which fundamental questions about protein folding and stability can be asked and answered. The close correspondence between the concept of hydrophobicity, the peptide-based free energy scales for partitioning, and the biological hydrophobicity scale derived from measurements with the eukaryotic translocon shows that the general principles that underlie the physicochemical basis for membrane protein behavior can inform both membrane biophysicists and membrane biologists. The methods being employed in the study of membrane protein folding are growing more diverse, with important contributions from in vivo, ex vivo, and in vitro experiments. The field of membrane protein folding will benefit greatly as findings from these different approaches continue to complement one another.

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9. References

(1) Wiener, M. C.; White, S. H. *Biophys. J.* **1992**, *61*, 437.
(2) White, S. H.; Wimley, W. C. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 319.
(3) Popot, J. L.; Engelman, D. M. *Annu. Rev. Biochem.* **2000**, *69*, 881.
(4) Booth, P. J.; Templer, R. H.; Meijberg, W.; Allen, S. J.; Curran, A. R.; Lorch, M. *Crit. Rev. Biochem. Mol. Biol.* **2001**, *36*, 501.
(5) Senes, A.; Engel, D. E.; DeGrado, W. F. *Curr. Opin. Struct. Biol.* **2004**, *14*, 465.
(6) Seddon, A. M.; Curnow, P.; Booth, P. J. *Biochim. Biophys. Acta* **2004**, *1666*, 105.
(7) Booth, P. J.; High, S. *Mol. Membr. Biol.* **2004**, *21*, 163.
(8) Jelinek, R.; Kolusheva, S. *Curr. Protein Pept. Sci.* **2005**, *6*, 103.
(9) Magzoub, M.; Graslund, A. *Q. Rev. Biophys.* **2004**, *37*, 147.
(10) Powers, J. P.; Hancock, R. E. *Peptides* **2003**, *24*, 1681.
(11) Tamm, L. K.; Crane, J.; Kiessling, V. *Curr. Opin. Struct. Biol.* **2003**, *13*, 453.
(12) Blumenthal, R.; Clague, M. J.; Durell, S. R.; Epand, R. M. *Chem. Rev.* **2003**, *103*, 53.
(13) Peisajovich, S. G.; Shai, Y. *Biochim. Biophys. Acta* **2003**, *1614*, 122.
(14) Epand, R. M. *Biochim. Biophys. Acta* **2003**, *1614*, 116.
(15) Cohen, F. S.; Melikyan, G. B. *J. Membr. Biol.* **2004**, *199*, 1.
(16) Sollner, T. H. *Curr. Opin. Cell Biol.* **2004**, *16*, 429.
(17) Schibli, D. J.; Weissenhorn, W. *Mol. Membr. Biol.* **2004**, *21*, 361.
(18) Tamm, L. K.; Hong, H.; Liang, B. *Biochim. Biophys. Acta* **2004**, *1666*, 250.
(19) Wimley, W. C. *Curr. Opin. Struct. Biol.* **2003**, *13*, 404.
(20) Ash, W. L.; Zlomislic, M. R.; Oloo, E. O.; Tieleman, D. P. *Biochim. Biophys. Acta* **2004**, *1666*, 158.
(21) Roux, B.; Schulten, K. *Structure (Cambridge)* **2004**, *12*, 1343.
(22) Lehnert, U.; Xia, Y.; Royce, T. E.; Goh, C. S.; Liu, Y.; Senes, A.; Yu, H.; Zhang, Z. L.; Engelman, D. M.; Gerstein, M. *Q. Rev. Biophys.* **2004**, *37*, 121.
(23) White, S. H. *Protein Sci.* **2004**, *13*, 1948.
(24) Capaldi, R. A.; Vanderkooi, G. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 930.
(25) Vanderkooi, G.; Capaldi, R. A. *Ann. N. Y. Acad. Sci.* **1972**, *195*, 135.
(26) Tanford, C. *Science* **1978**, *200*, 1012.
(27) Tanford, C. *The Hydrophobic Effect*; Wiley: New York, 1980.
(28) Rees, D. C.; DeAntonio, L.; Eisenberg, D. *Science* **1989**, *245*, 510.
(29) Deisenhofer, J.; Epp, O.; Miki, K.; Huber, R.; Michel, H. *J. Mol. Biol.* **1984**, *180*, 385.
(30) Deisenhofer, J.; Michel, H. *EMBO J.* **1989**, *8*, 2149.
(31) Yeates, T. O.; Komiya, H.; Rees, D. C.; Allen, J. P.; Feher, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6438.
(32) Allen, J. P.; Feher, G.; Yeates, T. O.; Komiya, H.; Rees, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5730.
(33) Allen, J. P.; Feher, G.; Yeates, T. O.; Komiya, H.; Rees, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6162.
(34) Henderson, R.; Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. *J. Mol. Biol.* **1990**, *213*, 899.
(35) Nagle, J. F.; Wiener, M. C. *Biophys. J.* **1989**, *55*, 309.
(36) Wiener, M. C.; Suter, R. M.; Nagle, J. F. *Biophys. J.* **1989**, *55*, 315.
(37) Popot, J. L.; Engelman, D. M. *Biochemistry* **1990**, *29*, 4031.
(38) London, E.; Khorana, H. G. *J. Biol. Chem.* **1982**, *257*, 7003.
(39) Liao, M. J.; London, E.; Khorana, H. G. *J. Biol. Chem.* **1983**, *258*, 9949.
(40) Liao, M. J.; Huang, K. S.; Khorana, H. G. *J. Biol. Chem.* **1984**, *259*, 4200.
(41) Huang, K. S.; Bayley, H.; Liao, M. J.; London, E.; Khorana, H. G. *J. Biol. Chem.* **1981**, *256*, 3802.
(42) Popot, J. L.; Trewhella, J.; Engelman, D. M. *EMBO J.* **1986**, *5*, 3039.
(43) Popot, J. L.; Gerchman, S. E.; Engelman, D. M. *J. Mol. Biol.* **1987**, *198*, 655.
(44) von Heijne, G. *Eur. J. Biochem.* **1981**, *120*, 275.
(45) Argos, P.; Rao, J. K.; Hargrave, P. A. *Eur. J. Biochem.* **1982**, *128*, 565.
(46) Kyte, J.; Doolittle, R. F. *J. Mol. Biol.* **1982**, *157*, 105.
(47) Eisenberg, D.; Schwarz, E.; Komaromy, M.; Wall, R. *J. Mol. Biol.* **1984**, *179*, 125.
(48) Engelman, D. M.; Steitz, T. A.; Goldman, A. *Annu. Rev. Biophys. Biophys. Chem.* **1986**, *15*, 321.
(49) Edelman, J.; White, S. H. *J. Mol. Biol.* **1989**, *210*, 195.
(50) DeGrado, W. F.; Gratkowski, H.; Lear, J. D. *Protein Sci.* **2003**, *12*, 647.
(51) Engelman, D. M.; Chen, Y.; Chin, C. N.; Curran, A. R.; Dixon, A. M.; Dupuy, A. D.; Lee, A. S.; Lehnert, U.; Matthews, E. E.; Reshetnyak, Y. K.; Senes, A.; Popot, J. L. *FEBS Lett.* **2003**, *555*, 122.
(52) Von Heijne, G. *Adv. Protein Chem.* **2003**, *63*, 1.
(53) Dowhan, W.; Mileykovskaya, E.; Bogdanov, M. *Biochim. Biophys. Acta* **2004**, *1666*, 19.
(54) Sanderson, J. M. *Org. Biomol. Chem.* **2005**, *3*, 201.
(55) White, S. H.; Wimley, W. C. *Biochim. Biophys. Acta* **1998**, *1376*, 339.
(56) Nozaki, Y.; Tanford, C. *J. Biol. Chem.* **1971**, *246*, 2211.
(57) Jacobs, R. E.; White, S. H. *Biochemistry* **1986**, *25*, 2605.
(58) Jacobs, R. E.; White, S. H. *Biochemistry* **1987**, *26*, 6127.
(59) Jacobs, R. E.; White, S. H. *Biochemistry* **1989**, *28*, 3421.
(60) Li, S. C.; Deber, C. M. *FEBS Lett.* **1992**, *311*, 217.
(61) Li, S. C.; Deber, C. M. *J. Biol. Chem.* **1993**, *268*, 22975.
(62) Li, S. C.; Deber, C. M. *Nat. Struct. Biol.* **1994**, *1*, 558.
(63) O'Neil, K. T.; DeGrado, W. F. *Science* **1990**, *250*, 646.
(64) Wimley, W. C.; Creamer, T. P.; White, S. H. *Biochemistry* **1996**, *35*, 5109.
(65) Wimley, W. C.; White, S. H. *Nat. Struct. Biol.* **1996**, *3*, 842.
(66) Ben-Tal, N.; Ben-Shaul, A.; Nicholls, A.; Honig, B. *Biophys. J.* **1996**, *70*, 1803.
(67) Jayasinghe, S.; Hristova, K.; White, S. H. *J. Mol. Biol.* **2001**, *312*, 927.
(68) Ladokhin, A. S.; Selsted, M. E.; White, S. H. *Biophys. J.* **1997**, *72*, 794.
(69) Thorgeirsson, T. E.; Russell, C. J.; King, D. S.; Shin, Y. K. *Biochemistry* **1996**, *35*, 1803.
(70) Tamm, L. K.; Bartoldus, I. *FEBS Lett.* **1990**, *272*, 29.
(71) Ladokhin, A. S.; White, S. H. *J. Mol. Biol.* **1999**, *285*, 1363.
(72) Wimley, W. C.; Hristova, K.; Ladokhin, A. S.; Silvestro, L.; Axelsen, P. H.; White, S. H. *J. Mol. Biol.* **1998**, *277*, 1091.
(73) Wieprecht, T.; Apostolov, O.; Beyermann, M.; Seelig, J. *J. Mol. Biol.* **1999**, *294*, 785.
(74) Li, Y.; Han, X.; Tamm, L. K. *Biochemistry* **2003**, *42*, 7245.
(75) Hristova, K.; White, S. H. *Biochemistry* **2005**, *44*, 12614.
(76) White, S. H.; Wimley, W. C.; Ladokhin, A. S.; Hristova, K. *Methods Enzymol.* **1998**, *295*, 62.
(77) Wimley, W. C.; Gawrisch, K.; Creamer, T. P.; White, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2985.
(78) Tossi, A.; Sandri, L.; Giangaspero, A. *Biopolymers* **2000**, *55*, 4.
(79) Shai, Y. *Biochim. Biophys. Acta* **1999**, *1462*, 55.
(80) Shai, Y. *Biopolymers* **2002**, *66*, 236.
(81) Shai, Y.; Oren, Z. *Peptides* **2001**, *22*, 1629.
(82) Lear, J. D.; Gratkowski, H.; DeGrado, W. F. *Biochem. Soc. Trans.* **2001**, *29*, 559.
(83) Skehel, J. J.; Cross, K.; Steinhauer, D.; Wiley, D. C. *Biochem. Soc. Trans.* **2001**, *29*, 623.
(84) Tamm, L. K.; Han, X.; Li, Y.; Lai, A. L. *Biopolymers* **2002**, *66*, 249.
(85) Nieva, J. L.; Agirre, A. *Biochim. Biophys. Acta* **2003**, *1614*, 104.
(86) Landolt-Marticorena, C.; Williams, K. A.; Deber, C. M.; Reithmeier, R. A. *J. Mol. Biol.* **1993**, *229*, 602.
(87) Samatey, F. A.; Xu, C.; Popot, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4577.
(88) Arkin, I. T.; Brunger, A. T. *Biochim. Biophys. Acta* **1998**, *1429*, 113.

(89) Senes, A.; Gerstein, M.; Engelman, D. M. *J. Mol. Biol.* **2000**, *296*, 921.
(90) Kim, H.; Melen, K.; von Heijne, G. *J. Biol. Chem.* **2003**, *278*, 10208.
(91) Daley, D. O.; Rapp, M.; Granseth, E.; Melen, K.; Drew, D.; von Heijne, G. *Science* **2005**, *308*, 1321.
(92) Jayasinghe, S.; Hristova, K.; White, S. H. *Protein Sci.* **2001**, *10*, 455.
(93) Cuthbertson, J. M.; Doyle, D. A.; Sansom, M. S. *Protein Eng. Des. Sel.* **2005**, *18*, 295.
(94) White, S. H. *FEBS Lett.* **2003**, *555*, 116.
(95) Dutzler, R.; Campbell, E. B.; Cadene, M.; Chait, B. T.; MacKinnon, R. *Nature* **2002**, *415*, 287.
(96) Jiang, Y.; Lee, A.; Chen, J.; Cadene, M.; Chait, B. T.; MacKinnon, R. *Nature* **2002**, *417*, 523.
(97) Yost, C. S.; Hedgpeth, J.; Lingappa, V. R. *Cell* **1983**, *34*, 759.
(98) Davis, N. G.; Model, P. *Cell* **1985**, *41*, 607.
(99) Higy, M.; Junne, T.; Spiess, M. *Biochemistry* **2004**, *43*, 12716.
(100) White, S. H.; von Heijne, G. *Curr. Opin. Struct. Biol.* **2004**, *14*, 397.
(101) Sadlish, H.; Skach, W. R. *J. Membr. Biol.* **2004**, *202*, 115.
(102) Rapoport, T. A.; Goder, V.; Heinrich, S. U.; Matlack, K. E. *Trends Cell Biol.* **2004**, *14*, 568.
(103) Zhang, Y. P.; Lewis, R. N.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **1992**, *31*, 11579.
(104) Zhang, Y. P.; Lewis, R. N.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **1992**, *31*, 11572.
(105) Lewis, R. N.; Zhang, Y. P.; Hodges, R. S.; Subczynski, W. K.; Kusumi, A.; Flach, C. R.; Mendelsohn, R.; McElhaney, R. N. *Biochemistry* **2001**, *40*, 12103.
(106) Bechinger, B. *Biophys. J.* **2001**, *81*, 2251.
(107) Chen, H.; Kendall, D. A. *J. Biol. Chem.* **1995**, *270*, 14115.
(108) Zhang, Y. P.; Lewis, R. N.; Henry, G. D.; Sykes, B. D.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **1995**, *34*, 2348.
(109) Zhang, Y. P.; Lewis, R. N.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **1995**, *34*, 2362.
(110) Weiss, T. M.; van der Wel, P. C.; Killian, J. A.; Koeppe, R. E., 2nd; Huang, H. W. *Biophys. J.* **2003**, *84*, 379.
(111) Killian, J. A.; Salemink, I.; de Planque, M. R.; Lindblom, G.; Koeppe, R. E., 2nd; Greathouse, D. V. *Biochemistry* **1996**, *35*, 1037.
(112) Lew, S.; Caputo, G. A.; London, E. *Biochemistry* **2003**, *42*, 10833.
(113) Yau, W. M.; Wimley, W. C.; Gawrisch, K.; White, S. H. *Biochemistry* **1998**, *37*, 14713.
(114) Braun, P.; von Heijne, G. *Biochemistry* **1999**, *38*, 9778.
(115) Yuen, C. T.; Davidson, A. R.; Deber, C. M. *Biochemistry* **2000**, *39*, 16155.
(116) von Heijne, G. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 167.
(117) Yano, Y.; Takemoto, T.; Kobayashi, S.; Yasui, H.; Sakurai, H.; Ohashi, W.; Niwa, M.; Futaki, S.; Sugiura, Y.; Matsuzaki, K. *Biochemistry* **2002**, *41*, 3073.
(118) Yano, Y.; Matsuzaki, K. *Biochemistry* **2002**, *41*, 12407.
(119) Kuroiwa, T.; Sakaguchi, M.; Mihara, K.; Omura, T. *J. Biol. Chem.* **1991**, *266*, 9251.
(120) Whitley, P.; Grahn, E.; Kutay, U.; Rapoport, T. A.; von Heijne, G. *J. Biol. Chem.* **1996**, *271*, 7583.
(121) Nilsson, I.; von Heijne, G. *J. Mol. Biol.* **1998**, *284*, 1185.
(122) Monne, M.; von Heijne, G. *FEBS Lett.* **2001**, *496*, 96.
(123) Mouritsen, O. G.; Bloom, M. *Biophys. J.* **1984**, *46*, 141.
(124) Harroun, T. A.; Heller, W. T.; Weiss, T. M.; Yang, L.; Huang, H. W. *Biophys. J.* **1999**, *76*, 3176.
(125) Killian, J. A. *Biochim. Biophys. Acta* **1998**, *1376*, 401.
(126) Killian, J. A. *FEBS Lett.* **2003**, *555*, 134.
(127) de Planque, M. R.; Bonev, B. B.; Demmers, J. A.; Greathouse, D. V.; Koeppe, R. E., 2nd; Separovic, F.; Watts, A.; Killian, J. A. *Biochemistry* **2003**, *42*, 5341.
(128) Ren, J.; Lew, S.; Wang, Z.; London, E. *Biochemistry* **1997**, *36*, 10213.
(129) Ren, J.; Lew, S.; Wang, J.; London, E. *Biochemistry* **1999**, *38*, 5905.
(130) Mall, S.; Broadbridge, R.; Sharma, R. P.; East, J. M.; Lee, A. G. *Biochemistry* **2001**, *40*, 12379.
(131) de Planque, M. R.; Goormaghtigh, E.; Greathouse, D. V.; Koeppe, R. E., 2nd; Kruijtzer, J. A.; Liskamp, R. M.; de Kruijff, B.; Killian, J. A. *Biochemistry* **2001**, *40*, 5000.
(132) Rinia, H. A.; Kik, R. A.; Demel, R. A.; Snel, M. M.; Killian, J. A.; van Der Eerden, J. P.; de Kruijff, B. *Biochemistry* **2000**, *39*, 5852.
(133) Morein, S.; Strandberg, E.; Killian, J. A.; Persson, S.; Arvidson, G.; Koeppe, R. E., 2nd; Lindblom, G. *Biophys. J.* **1997**, *73*, 3078.
(134) Morein, S.; Koeppe, I. R.; Lindblom, G.; de Kruijff, B.; Killian, J. A. *Biophys. J.* **2000**, *78*, 2475.
(135) Strandberg, E.; Morein, S.; Rijkers, D. T.; Liskamp, R. M.; van der Wel, P. C.; Killian, J. A. *Biochemistry* **2002**, *41*, 7190.
(136) van Duyl, B. Y.; Meeldijk, H.; Verkleij, A. J.; Rijkers, D. T.; Chupin, V.; de Kruijff, B.; Killian, J. A. *Biochemistry* **2005**, *44*, 4526.
(137) Zhang, Y. P.; Lewis, R. N.; Hodges, R. S.; McElhaney, R. N. *Biophys. J.* **1995**, *68*, 847.
(138) Liu, F.; Lewis, R. N.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **2001**, *40*, 760.
(139) Harroun, T. A.; Heller, W. T.; Weiss, T. M.; Yang, L.; Huang, H. W. *Biophys. J.* **1999**, *76*, 937.
(140) Mitra, K.; Ubarretxena-Belandia, I.; Taguchi, T.; Warren, G.; Engelman, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4083.
(141) Nezil, F. A.; Bloom, M. *Biophys. J.* **1992**, *61*, 1176.
(142) Pare, C.; Lafleur, M.; Liu, F.; Lewis, R. N.; McElhaney, R. N. *Biochim. Biophys. Acta* **2001**, *1511*, 60.
(143) de Planque, M. R.; Greathouse, D. V.; Koeppe, R. E., 2nd; Schafer, H.; Marsh, D.; Killian, J. A. *Biochemistry* **1998**, *37*, 9333.
(144) Harzer, U.; Bechinger, B. *Biochemistry* **2000**, *39*, 13106.
(145) van der Wel, P. C.; Strandberg, E.; Killian, J. A.; Koeppe, R. E., 2nd *Biophys. J.* **2002**, *83*, 1479.
(146) Strandberg, E.; Ozdirekcan, S.; Rijkers, D. T.; van der Wel, P. C.; Koeppe, R. E., 2nd; Liskamp, R. M.; Killian, J. A. *Biophys. J.* **2004**, *86*, 3709.
(147) Ozdirekcan, S.; Rijkers, D. T.; Liskamp, R. M.; Killian, J. A. *Biochemistry* **2005**, *44*, 1004.
(148) Aisenbrey, C.; Bechinger, B. *J. Am. Chem. Soc.* **2004**, *126*, 16676.
(149) Aisenbrey, C.; Bechinger, B. *Biochemistry* **2004**, *43*, 10502.
(150) Park, S. H.; Opella, S. J. *J. Mol. Biol.* **2005**, *350*, 310.
(151) Liu, F.; Lewis, R. N.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **2002**, *41*, 9197.
(152) Demmers, J. A.; van Duijn, E.; Haverkamp, J.; Greathouse, D. V.; Koeppe, R. E., 2nd; Heck, A. J.; Killian, J. A. *J. Biol. Chem.* **2001**, *276*, 34501.
(153) Jensen, M. O.; Park, S.; Tajkhorshid, E.; Schulten, K. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 6731.
(154) Jensen, M. O.; Mouritsen, O. G. *Biochim. Biophys. Acta* **2004**, *1666*, 205.
(155) Lew, S.; Ren, J.; London, E. *Biochemistry* **2000**, *39*, 9632.
(156) Caputo, G. A.; London, E. *Biochemistry* **2003**, *42*, 3265.
(157) Caputo, G. A.; London, E. *Biochemistry* **2003**, *42*, 3275.
(158) Caputo, G. A.; London, E. *Biochemistry* **2004**, *43*, 8794.
(159) Monne, M.; Nilsson, I.; Johansson, M.; Elmhed, N.; von Heijne, G. *J. Mol. Biol.* **1998**, *284*, 1177.
(160) Chamberlain, A. K.; Lee, Y.; Kim, S.; Bowie, J. U. *J. Mol. Biol.* **2004**, *339*, 471.
(161) Deol, S. S.; Bond, P. J.; Domene, C.; Sansom, M. S. *Biophys. J.* **2004**, *87*, 3737.
(162) Strandberg, E.; Killian, J. A. *FEBS Lett.* **2003**, *544*, 69.
(163) Richardson, J. S.; Richardson, D. C. *Science* **1988**, *240*, 1648.
(164) Presta, L. G.; Rose, G. D. *Science* **1988**, *240*, 1632.
(165) Li, S. C.; Deber, C. M.; Shoelson, S. E. *Biochemistry* **1994**, *33*, 14333.
(166) Williams, K. A.; Deber, C. M. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 1.
(167) Williams, K. A.; Deber, C. M. *Biochemistry* **1996**, *35*, 10472.
(168) Williams, K. A.; Farrow, N. A.; Deber, C. M.; Kay, L. E. *Biochemistry* **1996**, *35*, 5145.
(169) Li, S. C.; Goto, N. K.; Williams, K. A.; Deber, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6676.
(170) de Planque, M. R.; Rijkers, D. T.; Fletcher, J. I.; Liskamp, R. M.; Separovic, F. *Biochim. Biophys. Acta* **2004**, *1665*, 40.
(171) Nilsson, I. M.; von Heijne, G. *J. Biol. Chem.* **1993**, *268*, 5798.
(172) Nilsson, I.; Saaf, A.; Whitley, P.; Gafvelin, G.; Waller, C.; von Heijne, G. *J. Mol. Biol.* **1998**, *284*, 1165.
(173) Chin, C. N.; von Heijne, G. *J. Mol. Biol.* **2000**, *303*, 1.
(174) Wigley, W. C.; Corboy, M. J.; Cutler, T. D.; Thibodeau, P. H.; Oldan, J.; Lee, M. G.; Rizo, J.; Hunt, J. F.; Thomas, P. J. *Nat. Struct. Biol.* **2002**, *9*, 381.
(175) Hill, R. B.; Raleigh, D. P.; Lombardi, A.; DeGrado, W. F. *Acc. Chem. Res.* **2000**, *33*, 745.
(176) Simon, S. M.; Blobel, G. *Cell* **1991**, *65*, 371.
(177) Johnson, A. E. *FEBS Lett.* **2005**, *579*, 916.
(178) Shan, S. O.; Walter, P. *FEBS Lett.* **2005**, *579*, 921.
(179) Osborne, A. R.; Rapoport, T. A.; van den Berg, B. *Annu. Rev. Cell Dev. Biol.* **2005**, *21*, 529.
(180) von Heijne, G.; Gavel, Y. *Eur. J. Biochem.* **1988**, *174*, 671.
(181) Sipos, L.; von Heijne, G. *Eur. J. Biochem.* **1993**, *213*, 1333.
(182) Whitley, P.; Nilsson, I.; von Heijne, G. *Nat. Struct. Biol.* **1994**, *1*, 858.
(183) Gafvelin, G.; von Heijne, G. *Cell* **1994**, *77*, 401.
(184) Blobel, G. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1496.
(185) Gafvelin, G.; Sakaguchi, M.; Andersson, H.; von Heijne, G. *J. Biol. Chem.* **1997**, *272*, 6119.
(186) van Klompenburg, W.; Nilsson, I.; von Heijne, G.; de Kruijff, B. *EMBO J.* **1997**, *16*, 4261.
(187) Monne, M.; Nilsson, I.; Elofsson, A.; von Heijne, G. *J. Mol. Biol.* **1999**, *293*, 807.
(188) Monne, M.; Hermansson, M.; von Heijne, G. *J. Mol. Biol.* **1999**, *288*, 141.
(189) Saaf, A.; Hermansson, M.; von Heijne, G. *J. Mol. Biol.* **2000**, *301*, 191.

(190) Heinrich, S. U.; Mothes, W.; Brunner, J.; Rapoport, T. A. *Cell* **2000**, *102*, 233.
(191) Van den Berg, B.; Clemons, W. M., Jr.; Collinson, I.; Modis, Y.; Hartmann, E.; Harrison, S. C.; Rapoport, T. A. *Nature* **2004**, *427*, 36.
(192) Hessa, T.; Kim, H.; Bihlmaier, K.; Lundin, C.; Boekel, J.; Andersson, H.; Nilsson, I.; White, S. H.; von Heijne, G. *Nature* **2005**, *433*, 377.
(193) Hessa, T.; White, S. H.; von Heijne, G. *Science* **2005**, *307*, 1427.
(194) White, S. H.; von Heijne, G. *Curr. Opin. Struct. Biol.* **2005**, *15*, 378.
(195) Cordes, F. S.; Bright, J. N.; Sansom, M. S. *J. Mol. Biol.* **2002**, *323*, 951.
(196) Schiffer, M.; Chang, C. H.; Stevens, F. J. *Protein Eng.* **1992**, *5*, 213.
(197) Rosch, K.; Naeher, D.; Laird, V.; Goder, V.; Spiess, M. *J. Biol. Chem.* **2000**, *275*, 14916.
(198) Meier, S.; Neupert, W.; Herrmann, J. M. *J. Cell Biol.* **2005**, *170*, 881.
(199) Marchesi, V. T.; Andrews, E. P. *Science* **1971**, *174*, 1247.
(200) Segrest, J. P.; Jackson, R. L.; Marchesi, V. T.; Guyer, R. B.; Terry, W. *Biochem. Biophys. Res. Commun.* **1972**, *49*, 964.
(201) Tosteson, M. T.; Lau, F.; Tosteson, D. C. *Nat. New Biol.* **1973**, *243*, 112.
(202) Grant, C. W.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4653.
(203) Marchesi, V. T.; Tillack, T. W.; Jackson, R. L.; Segrest, J. P.; Scott, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1445.
(204) Tomita, M.; Furthmayr, H.; Marchesi, V. T. *Biochemistry* **1978**, *17*, 4756.
(205) Armitage, I. M.; Shapiro, D. L.; Furthmayr, H.; Marchesi, V. T. *Biochemistry* **1977**, *16*, 1317.
(206) van Zoelen, E. J.; van Dijck, P. W.; de Kruijff, B.; Verkleij, A. J.; van Deenen, L. L. *Biochim. Biophys. Acta* **1978**, *514*, 9.
(207) Mendelsohn, R.; Dluhy, R.; Taraschi, T.; Cameron, D. G.; Mantsch, H. H. *Biochemistry* **1981**, *20*, 6699.
(208) Dluhy, R. A.; Mendelsohn, R.; Casal, H. L.; Mantsch, H. H. *Biochemistry* **1983**, *22*, 1170.
(209) Mendelsohn, R.; Dluhy, R. A.; Crawford, T.; Mantsch, H. H. *Biochemistry* **1984**, *23*, 1498.
(210) Ong, R. L. *J. Membr. Biol.* **1984**, *78*, 1.
(211) Sami, M.; Dempsey, C. *FEBS Lett.* **1988**, *240*, 211.
(212) Furthmayr, H.; Marchesi, V. T. *Biochemistry* **1976**, *15*, 1137.
(213) Silverberg, M.; Furthmayr, H.; Marchesi, V. T. *Biochemistry* **1976**, *15*, 1448.
(214) Bormann, B. J.; Knowles, W. J.; Marchesi, V. T. *J. Biol. Chem.* **1989**, *264*, 4033.
(215) Lemmon, M. A.; Flanagan, J. M.; Hunt, J. F.; Adair, B. D.; Bormann, B. J.; Dempsey, C. E.; Engelman, D. M. *J. Biol. Chem.* **1992**, *267*, 7683.
(216) Lemmon, M. A.; Flanagan, J. M.; Treutlein, H. R.; Zhang, J.; Engelman, D. M. *Biochemistry* **1992**, *31*, 12719.
(217) Lemmon, M. A.; Treutlein, H. R.; Adams, P. D.; Brunger, A. T.; Engelman, D. M. *Nat. Struct. Biol.* **1994**, *1*, 157.
(218) Mingarro, I.; Whitley, P.; Lemmon, M. A.; von Heijne, G. *Protein Sci.* **1996**, *5*, 1339.
(219) Orzaez, M.; Perez-Paya, E.; Mingarro, I. *Protein Sci.* **2000**, *9*, 1246.
(220) Orzaez, M.; Lukovic, D.; Abad, C.; Perez-Paya, E.; Mingarro, I. *FEBS Lett.* **2005**, *579*, 1633.
(221) Orzaez, M.; Salgado, J.; Gimenez-Giner, A.; Perez-Paya, E.; Mingarro, I. *J. Mol. Biol.* **2004**, *335*, 631.
(222) Challou, N.; Goormaghtigh, E.; Cabiaux, V.; Conrath, K.; Ruysschaert, J. M. *Biochemistry* **1994**, *33*, 6902.
(223) Smith, S. O.; Jonas, R.; Braiman, M.; Bormann, B. J. *Biochemistry* **1994**, *33*, 6334.
(224) Adair, B. D.; Engelman, D. M. *Biochemistry* **1994**, *33*, 5539.
(225) Smith, S. O.; Bormann, B. J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 488.
(226) Smith, S. O.; Song, D.; Shekar, S.; Groesbeek, M.; Ziliox, M.; Aimoto, S. *Biochemistry* **2001**, *40*, 6553.
(227) Smith, S. O.; Eilers, M.; Song, D.; Crocker, E.; Ying, W.; Groesbeek, M.; Metz, G.; Ziliox, M.; Aimoto, S. *Biophys. J.* **2002**, *82*, 2476.
(228) Liu, W.; Crocker, E.; Siminovitch, D. J.; Smith, S. O. *Biophys. J.* **2003**, *84*, 1263.
(229) MacKenzie, K. R.; Prestegard, J. H.; Engelman, D. M. *Science* **1997**, *276*, 131.
(230) Chothia, C.; Levitt, M.; Richardson, D. *J. Mol. Biol.* **1981**, *145*, 215.
(231) Adams, P. D.; Engelman, D. M.; Brunger, A. T. *Proteins* **1996**, *26*, 257.
(232) Treutlein, H. R.; Lemmon, M. A.; Engelman, D. M.; Brunger, A. T. *Biochemistry* **1992**, *31*, 12726.
(233) Mottamal, M.; Lazaridis, T. *Biochemistry* **2005**, *44*, 1607.
(234) Senes, A.; Ubarretxena-Belandia, I.; Engelman, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9056.
(235) MacKenzie, K. R.; Engelman, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3583.
(236) Fleming, K. G. *Methods Enzymol.* **2000**, *323*, 63.
(237) Fleming, K. G.; Ren, C. C.; Doura, A. K.; Eisley, M. E.; Kobus, F. J.; Stanley, A. M. *Biophys. Chem.* **2004**, *108*, 43.
(238) Doura, A. K.; Kobus, F. J.; Dubrovsky, L.; Hibbard, E.; Fleming, K. G. *J. Mol. Biol.* **2004**, *341*, 991.
(239) Doura, A. K.; Fleming, K. G. *J. Mol. Biol.* **2004**, *343*, 1487.
(240) Fleming, K. G.; Engelman, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14340.
(241) Fleming, K. G.; Ackerman, A. L.; Engelman, D. M. *J. Mol. Biol.* **1997**, *272*, 266.
(242) Fisher, L. E.; Engelman, D. M.; Sturgis, J. N. *J. Mol. Biol.* **1999**, *293*, 639.
(243) Fisher, L. E.; Engelman, D. M.; Sturgis, J. N. *Biophys. J.* **2003**, *85*, 3097.
(244) Li, E.; Hristova, K. *Langmuir* **2004**, *20*, 9053.
(245) You, M.; Li, E.; Wimley, W. C.; Hristova, K. *Anal. Biochem.* **2005**, *340*, 154.
(246) Li, E.; You, M.; Hristova, K. *Biochemistry* **2005**, *44*, 352.
(247) Cristian, L.; Lear, J. D.; DeGrado, W. F. *Protein Sci.* **2003**, *12*, 1732.
(248) Cristian, L.; Lear, J. D.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 14772.
(249) Lau, F. W.; Bowie, J. U. *Biochemistry* **1997**, *36*, 5884.
(250) Chen, G. Q.; Gouaux, E. *Biochemistry* **1999**, *38*, 15380.
(251) Yohannan, S.; Yang, D.; Faham, S.; Boulting, G.; Whitelegge, J.; Bowie, J. U. *J. Mol. Biol.* **2004**, *341*, 1.
(252) Faham, S.; Yang, D.; Bare, E.; Yohannan, S.; Whitelegge, J. P.; Bowie, J. U. *J. Mol. Biol.* **2004**, *335*, 297.
(253) Fleming, K. G. *J. Mol. Biol.* **2002**, *323*, 563.
(254) Sehgal, P.; Mogensen, J. E.; Otzen, D. E. *Biochim. Biophys. Acta* **2005**, *1716*, 59.
(255) Zhou, Y.; Bowie, J. U. *J. Biol. Chem.* **2000**, *275*, 6975.
(256) Melnyk, R. A.; Kim, S.; Curran, A. R.; Engelman, D. M.; Bowie, J. U.; Deber, C. M. *J. Biol. Chem.* **2004**, *279*, 16591.
(257) Langosch, D.; Brosig, B.; Kolmar, H.; Fritz, H. J. *J. Mol. Biol.* **1996**, *263*, 525.
(258) Kolmar, H.; Hennecke, F.; Gotze, K.; Janzer, B.; Vogt, B.; Mayer, F.; Fritz, H. J. *EMBO J.* **1995**, *14*, 3895.
(259) Miller, V. L.; Taylor, R. K.; Mekalanos, J. J. *Cell* **1987**, *48*, 271.
(260) Miller, V. L.; Mekalanos, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3471.
(261) Russ, W. P.; Engelman, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 863.
(262) Gurezka, R.; Langosch, D. *J. Biol. Chem.* **2001**, *276*, 45580.
(263) Leeds, J. A.; Beckwith, J. *J. Mol. Biol.* **1998**, *280*, 799.
(264) Leeds, J. A.; Boyd, D.; Huber, D. R.; Sonoda, G. K.; Luu, H. T.; Engelman, D. M.; Beckwith, J. *J. Mol. Biol.* **2001**, *313*, 181.
(265) Schneider, D.; Engelman, D. M. *J. Biol. Chem.* **2003**, *278*, 3105.
(266) Brosig, B.; Langosch, D. *Protein Sci.* **1998**, *7*, 1052.
(267) Gerber, D.; Shai, Y. *J. Biol. Chem.* **2001**, *276*, 31229.
(268) Hebert, T. E.; Moffett, S.; Morello, J. P.; Loisel, T. P.; Bichet, D. G.; Barret, C.; Bouvier, M. *J. Biol. Chem.* **1996**, *271*, 16384.
(269) George, S. R.; Lee, S. P.; Varghese, G.; Zeman, P. R.; Seeman, P.; Ng, G. Y.; O'Dowd, B. F. *J. Biol. Chem.* **1998**, *273*, 30244.
(270) George, S. R.; Ng, G. Y.; Lee, S. P.; Fan, T.; Varghese, G.; Wang, C.; Deber, C. M.; Seeman, P.; O'Dowd, B. F. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 481.
(271) Zouzoulas, A.; Therien, A. G.; Scanzano, R.; Deber, C. M.; Blostein, R. *J. Biol. Chem.* **2003**, *278*, 40437.
(272) Partridge, A. W.; Melnyk, R. A.; Yang, D.; Bowie, J. U.; Deber, C. M. *J. Biol. Chem.* **2003**, *278*, 22056.
(273) Bennasroune, A.; Fickova, M.; Gardin, A.; Dirrig-Grosch, S.; Aunis, D.; Cremel, G.; Hubert, P. *Mol. Biol. Cell* **2004**, *15*, 3464.
(274) Kim, S.; Chamberlain, A. K.; Bowie, J. U. *J. Mol. Biol.* **2003**, *329*, 831.
(275) Russ, W. P.; Engelman, D. M. *J. Mol. Biol.* **2000**, *296*, 911.
(276) Curran, A. R.; Engelman, D. M. *Curr. Opin. Struct. Biol.* **2003**, *13*, 412.
(277) Vargas, R.; Garza, J.; Dixon, D. A.; Hay, B. P. *J. Am. Chem. Soc.* **2000**, *122*, 4750.
(278) Scheiner, S.; Kar, T.; Gu, Y. *J. Biol. Chem.* **2001**, *276*, 9832.
(279) Arbely, E.; Arkin, I. T. *J. Am. Chem. Soc.* **2004**, *126*, 5362.
(280) Yohannan, S.; Faham, S.; Yang, D.; Grosfeld, D.; Chamberlain, A. K.; Bowie, J. U. *J. Am. Chem. Soc.* **2004**, *126*, 2284.
(281) Lazaridis, T. *Proteins* **2003**, *52*, 176.
(282) Op De Beeck, A.; Montserret, R.; Duvet, S.; Cocquerel, L.; Cacan, R.; Barberot, B.; Le Maire, M.; Penin, F.; Dubuisson, J. *J. Biol. Chem.* **2000**, *275*, 31428.
(283) Arselin, G.; Giraud, M. F.; Dautant, A.; Vaillier, J.; Brethes, D.; Coulary-Salin, B.; Schaeffer, J.; Velours, J. *Eur. J. Biochem.* **2003**, *270*, 1875.
(284) Saddar, S.; Stuart, R. A. *J. Biol. Chem.* **2005**, *280*, 24435.
(285) Bustos, D. M.; Velours, J. *J. Biol. Chem.* **2005**, *280*, 29004.
(286) McClain, M. S.; Cao, P.; Cover, T. L. *Infect. Immun.* **2001**, *69*, 1181.

(287) McClain, M. S.; Iwamoto, H.; Cao, P.; Vinion-Dubiel, A. D.; Li, Y.; Szabo, G.; Shao, Z.; Cover, T. L. *J. Biol. Chem.* **2003**, *278*, 12101.
(288) Kim, S.; Chamberlain, A. K.; Bowie, J. U. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 5988.
(289) Sulistijo, E. S.; Jaszewski, T. M.; MacKenzie, K. R. *J. Biol. Chem.* **2003**, *278*, 51950.
(290) Overton, M. C.; Chinault, S. L.; Blumer, K. J. *J. Biol. Chem.* **2003**, *278*, 49369.
(291) Morais, V. A.; Crystal, A. S.; Pijak, D. S.; Carlin, D.; Costa, J.; Lee, V. M.; Doms, R. W. *J. Biol. Chem.* **2003**, *278*, 43284.
(292) Takasugi, N.; Tomita, T.; Hayashi, I.; Tsuruoka, M.; Niimura, M.; Takahashi, Y.; Thinakaran, G.; Iwatsubo, T. *Nature* **2003**, *422*, 438.
(293) Lee, S. F.; Shah, S.; Yu, C.; Wigley, W. C.; Li, H.; Lim, M.; Pedersen, K.; Han, W.; Thomas, P.; Lundkvist, J.; Hao, Y. H.; Yu, G. *J. Biol. Chem.* **2004**, *279*, 4144.
(294) Edbauer, D.; Kaether, C.; Steiner, H.; Haass, C. *J. Biol. Chem.* **2004**, *279*, 37311.
(295) Murata, K.; Mitsuoka, K.; Hirai, T.; Walz, T.; Agre, P.; Heymann, J. B.; Engel, A.; Fujiyoshi, Y. *Nature* **2000**, *407*, 599.
(296) Fu, D.; Libson, A.; Miercke, L. J.; Weitzman, C.; Nollert, P.; Krucinski, J.; Stroud, R. M. *Science* **2000**, *290*, 481.
(297) Kobus, F. J.; Fleming, K. G. *Biochemistry* **2005**, *44*, 1464.
(298) Stanley, A. M.; Fleming, K. G. *J. Mol. Biol.* **2005**, *347*, 759.
(299) Mendrola, J. M.; Berger, M. B.; King, M. C.; Lemmon, M. A. *J. Biol. Chem.* **2002**, *277*, 4704.
(300) Gerber, D.; Sal-Man, N.; Shai, Y. *J. Biol. Chem.* **2004**, *279*, 21177.
(301) Choma, C.; Gratkowski, H.; Lear, J. D.; DeGrado, W. F. *Nat. Struct. Biol.* **2000**, *7*, 161.
(302) Zhou, F. X.; Cocco, M. J.; Russ, W. P.; Brunger, A. T.; Engelman, D. M. *Nat. Struct. Biol.* **2000**, *7*, 154.
(303) Gratkowski, H.; Lear, J. D.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 880.
(304) Zhou, F. X.; Merianos, H. J.; Brunger, A. T.; Engelman, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2250.
(305) Gratkowski, H.; Dai, Q. H.; Wand, A. J.; DeGrado, W. F.; Lear, J. D. *Biophys. J.* **2002**, *83*, 1613.
(306) Lear, J. D.; Gratkowski, H.; Adamian, L.; Liang, J.; DeGrado, W. F. *Biochemistry* **2003**, *42*, 6400.
(307) Ruan, W.; Lindner, E.; Langosch, D. *Protein Sci.* **2004**, *13*, 555.
(308) Cristian, L.; Nanda, V.; Lear, J. D.; DeGrado, W. F. *J. Mol. Biol.* **2005**, *348*, 1225.
(309) Howard, K. P.; Liu, W.; Crocker, E.; Nanda, V.; Lear, J.; Degrado, W. F.; Smith, S. O. *Protein Sci.* **2005**, *14*, 1019.
(310) Dawson, J. P.; Melnyk, R. A.; Deber, C. M.; Engelman, D. M. *J. Mol. Biol.* **2003**, *331*, 255.
(311) Freeman-Cook, L. L.; Dixon, A. M.; Frank, J. B.; Xia, Y.; Ely, L.; Gerstein, M.; Engelman, D. M.; DiMaio, D. *J. Mol. Biol.* **2004**, *338*, 907.
(312) Petti, L. M.; Reddy, V.; Smith, S. O.; DiMaio, D. *J. Virol.* **1997**, *71*, 7318.
(313) Klein, O.; Polack, G. W.; Surti, T.; Kegler-Ebo, D.; Smith, S. O.; DiMaio, D. *J. Virol.* **1998**, *72*, 8921.
(314) Mattoon, D.; Gupta, K.; Doyon, J.; Loll, P. J.; DiMaio, D. *Oncogene* **2001**, *20*, 3824.
(315) Freeman-Cook, L. L.; Edwards, A. P.; Dixon, A. M.; Yates, K. E.; Ely, L.; Engelman, D. M.; Dimaio, D. *J. Mol. Biol.* **2005**, *345*, 907.
(316) Dawson, J. P.; Weinger, J. S.; Engelman, D. M. *J. Mol. Biol.* **2002**, *316*, 799.
(317) Adamian, L.; Liang, J. *Proteins* **2002**, *47*, 209.
(318) Partridge, A. W.; Therien, A. G.; Deber, C. M. *Proteins* **2004**, *54*, 648.
(319) Peng, S.; Liu, L. P.; Emili, A. Q.; Deber, C. M. *FEBS Lett.* **1998**, *431*, 29.
(320) Therien, A. G.; Grant, F. E.; Deber, C. M. *Nat. Struct. Biol.* **2001**, *8*, 597.
(321) Partridge, A. W.; Melnyk, R. A.; Deber, C. M. *Biochemistry* **2002**, *41*, 3647.
(322) Gurezka, R.; Laage, R.; Brosig, B.; Langosch, D. *J. Biol. Chem.* **1999**, *274*, 9265.
(323) Renthal, R.; Velasquez, D. *J. Protein Chem.* **2002**, *21*, 255.
(324) Braun, P.; Persson, B.; Kaback, H. R.; von Heijne, G. *J. Biol. Chem.* **1997**, *272*, 29566.
(325) Ruan, W.; Becker, V.; Klingmuller, U.; Langosch, D. *J. Biol. Chem.* **2004**, *279*, 3273.
(326) Lewis, R. N.; Zhang, Y. P.; Liu, F.; McElhaney, R. N. *Bioelectrochemistry* **2002**, *56*, 135.
(327) Thomas, D. D.; Reddy, L. G.; Karim, C. B.; Li, M.; Cornea, R.; Autry, J. M.; Jones, L. R.; Stamm, J. *Ann. N. Y. Acad. Sci.* **1998**, *853*, 186.
(328) Oxenoid, K.; Chou, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10870.
(329) Arkin, I. T.; Adams, P. D.; MacKenzie, K. R.; Lemmon, M. A.; Brunger, A. T.; Engelman, D. M. *Embo. J.* **1994**, *13*, 4757.
(330) Simmerman, H. K.; Kobayashi, Y. M.; Autry, J. M.; Jones, L. R. *J. Biol. Chem.* **1996**, *271*, 5941.
(331) Kimura, Y.; Kurzydlowski, K.; Tada, M.; MacLennan, D. H. *J. Biol. Chem.* **1997**, *272*, 15061.
(332) Toyofuku, T.; Kurzydlowski, K.; Tada, M.; MacLennan, D. H. *J. Biol. Chem.* **1994**, *269*, 3088.
(333) Kimura, Y.; Kurzydlowski, K.; Tada, M.; MacLennan, D. H. *J. Biol. Chem.* **1996**, *271*, 21726.
(334) Kelly, M. L.; Cook, J. A.; Brown-Augsburger, P.; Heinz, B. A.; Smith, M. C.; Pinto, L. H. *FEBS Lett.* **2003**, *552*, 61.
(335) Kovacs, F. A.; Cross, T. A. *Biophys. J.* **1997**, *73*, 2511.
(336) Song, Z.; Kovacs, F. A.; Wang, J.; Denny, J. K.; Shekar, S. C.; Quine, J. R.; Cross, T. A. *Biophys. J.* **2000**, *79*, 767.
(337) Wang, J.; Kim, S.; Kovacs, F.; Cross, T. A. *Protein Sci.* **2001**, *10*, 2241.
(338) Nishimura, K.; Kim, S.; Zhang, L.; Cross, T. A. *Biochemistry* **2002**, *41*, 13170.
(339) Tian, C.; Tobler, K.; Lamb, R. A.; Pinto, L. H.; Cross, T. A. *Biochemistry* **2002**, *41*, 11294.
(340) Tian, C.; Gao, P. F.; Pinto, L. H.; Lamb, R. A.; Cross, T. A. *Protein Sci.* **2003**, *12*, 2597.
(341) Kukol, A.; Adams, P. D.; Rice, L. M.; Brunger, A. T.; Arkin, T. I. *J. Mol. Biol.* **1999**, *286*, 951.
(342) Zhong, Q.; Husslein, T.; Moore, P. B.; Newns, D. M.; Pattnaik, P.; Klein, M. L. *FEBS Lett.* **1998**, *434*, 265.
(343) Pinto, L. H.; Dieckmann, G. R.; Gandhi, C. S.; Papworth, C. G.; Braman, J.; Shaughnessy, M. A.; Lear, J. D.; Lamb, R. A.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11301.
(344) Torres, J.; Kukol, A.; Arkin, I. T. *Biophys. J.* **2000**, *79*, 3139.
(345) Torres, J.; Arkin, I. T. *Biophys. J.* **2002**, *82*, 1068.
(346) Marassi, F. M.; Opella, S. J. *J. Magn. Reson.* **2000**, *144*, 150.
(347) Kochendoerfer, G. G.; Salom, D.; Lear, J. D.; Wilk-Orescan, R.; Kent, S. B.; DeGrado, W. F. *Biochemistry* **1999**, *38*, 11905.
(348) Salom, D.; Hill, B. R.; Lear, J. D.; DeGrado, W. F. *Biochemistry* **2000**, *39*, 14160.
(349) Howard, K. P.; Lear, J. D.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 8568.
(350) Stouffer, A. L.; Nanda, V.; Lear, J. D.; DeGrado, W. F. *J. Mol. Biol.* **2005**, *347*, 169.
(351) Duong-Ly, K. C.; Nanda, V.; Degrado, W. F.; Howard, K. P. *Protein Sci.* **2005**, *14*, 856.
(352) Bowie, J. U. *Curr. Opin. Struct. Biol.* **2001**, *11*, 397.
(353) Geiger, B.; Bershadsky, A.; Pankov, R.; Yamada, K. M. *Nat. Rev. Mol. Cell. Biol.* **2001**, *2*, 793.
(354) Takagi, J.; Petre, B. M.; Walz, T.; Springer, T. A. *Cell* **2002**, *110*, 599.
(355) Kim, M.; Carman, C. V.; Springer, T. A. *Science* **2003**, *301*, 1720.
(356) Springer, T. A.; Wang, J. H. *Adv. Protein Chem.* **2004**, *68*, 29.
(357) Calvete, J. J. *Exp. Biol. Med. (Maywood)* **2004**, *229*, 732.
(358) Gottschalk, K. E.; Adams, P. D.; Brunger, A. T.; Kessler, H. *Protein Sci.* **2002**, *11*, 1800.
(359) Li, R.; Babu, C. R.; Lear, J. D.; Wand, A. J.; Bennett, J. S.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12462.
(360) Li, R.; Mitra, N.; Gratkowski, H.; Vilaire, G.; Litvinov, R.; Nagasami, C.; Weisel, J. W.; Lear, J. D.; DeGrado, W. F.; Bennett, J. S. *Science* **2003**, *300*, 795.
(361) Adair, B. D.; Yeager, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14059.
(362) Luo, B. H.; Springer, T. A.; Takagi, J. *PLoS Biol.* **2004**, *2*, 0776.
(363) Hughes, P. E.; Diaz-Gonzalez, F.; Leong, L.; Wu, C.; McDonald, J. A.; Shattil, S. J.; Ginsberg, M. H. *J. Biol. Chem.* **1996**, *271*, 6571.
(364) Schneider, D.; Engelman, D. M. *J. Biol. Chem.* **2004**, *279*, 9840.
(365) Li, R.; Gorelik, R.; Nanda, V.; Law, P. B.; Lear, J. D.; DeGrado, W. F.; Bennett, J. S. *J. Biol. Chem.* **2004**, *279*, 26666.
(366) Luo, B. H.; Carman, C. V.; Takagi, J.; Springer, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3679.
(367) Partridge, A. W.; Liu, S.; Kim, S.; Bowie, J. U.; Ginsberg, M. H. *J. Biol. Chem.* **2005**, *280*, 7294.
(368) Richmond, T. D.; Chohan, M.; Barber, D. L. *Trends Cell Biol.* **2005**, *15*, 146.
(369) Constantinescu, S. N.; Liu, X.; Beyer, W.; Fallon, A.; Shekar, S.; Henis, Y. I.; Smith, S. O.; Lodish, H. F. *EMBO J.* **1999**, *18*, 3334.
(370) Constantinescu, S. N.; Huang, L. J.; Nam, H.; Lodish, H. F. *Mol. Cell* **2001**, *7*, 377.
(371) Constantinescu, S. N.; Keren, T.; Socolovsky, M.; Nam, H.; Henis, Y. I.; Lodish, H. F. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4379.
(372) Kubatzky, K. F.; Ruan, W.; Gurezka, R.; Cohen, J.; Ketteler, R.; Watowich, S. S.; Neumann, D.; Langosch, D.; Klingmuller, U. *Curr. Biol.* **2001**, *11*, 110.
(373) Seubert, N.; Royer, Y.; Staerk, J.; Kubatzky, K. F.; Moucadel, V.; Krishnakumar, S.; Smith, S. O.; Constantinescu, S. N. *Mol. Cell* **2003**, *12*, 1239.
(374) Fang, C.; Choi, E.; Nie, L.; Li, J. P. *Virology* **1998**, *252*, 46.

(375) Constantinescu, S. N.; Keren, T.; Russ, W. P.; Ubarretxena-Belandia, I.; Malka, Y.; Kubatzky, K. F.; Engelman, D. M.; Lodish, H. F.; Henis, Y. I. *J. Biol. Chem.* **2003**, *278*, 43755.
(376) Liu, W.; Crocker, E.; Constantinescu, S. N.; Smith, S. O. *Biophys. J.* **2005**, *89*, 1194.
(377) Bargmann, C. I.; Hung, M. C.; Weinberg, R. A. *Cell* **1986**, *45*, 649.
(378) Gullick, W. J.; Bottomley, A. C.; Lofts, F. J.; Doak, D. G.; Mulvey, D.; Newman, R.; Crumpton, M. J.; Sternberg, M. J.; Campbell, I. D. *EMBO J.* **1992**, *11*, 43.
(379) Smith, S. O.; Smith, C. S.; Bormann, B. J. *Nat. Struct. Biol.* **1996**, *3*, 252.
(380) Smith, S. O.; Smith, C.; Shekar, S.; Peersen, O.; Ziliox, M.; Aimoto, S. *Biochemistry* **2002**, *41*, 9321.
(381) Burke, C. L.; Lemmon, M. A.; Coren, B. A.; Engelman, D. M.; Stern, D. F. *Oncogene* **1997**, *14*, 687.
(382) Sharpe, S.; Barber, K. R.; Grant, C. W. *Biochemistry* **2002**, *41*, 2341.
(383) Ungar, D.; Hughson, F. M. *Annu. Rev. Cell Dev. Biol.* **2003**, *19*, 493.
(384) Sollner, T. H. *Mol. Membr. Biol.* **2003**, *20*, 209.
(385) Mochida, S. *Neurosci. Res.* **2000**, *36*, 175.
(386) Hayashi, T.; McMahon, H.; Yamasaki, S.; Binz, T.; Hata, Y.; Sudhof, T. C.; Niemann, H. *EMBO J.* **1994**, *13*, 5051.
(387) Hayashi, T.; Yamasaki, S.; Nauenburg, S.; Binz, T.; Niemann, H. *EMBO J.* **1995**, *14*, 2317.
(388) Otto, H.; Hanson, P. I.; Jahn, R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6197.
(389) Margittai, M.; Otto, H.; Jahn, R. *FEBS Lett.* **1999**, *446*, 40.
(390) Edelmann, L.; Hanson, P. I.; Chapman, E. R.; Jahn, R. *EMBO J.* **1995**, *14*, 224.
(391) Yelamanchili, S. V.; Reisinger, C.; Becher, A.; Sikorra, S.; Bigalke, H.; Binz, T.; Ahnert-Hilger, G. *Eur. J. Cell Biol.* **2005**, *84*, 467.
(392) Roy, R.; Laage, R.; Langosch, D. *Biochemistry* **2004**, *43*, 4964.
(393) Laage, R.; Langosch, D. *Eur. J. Biochem.* **1997**, *249*, 540.
(394) Laage, R.; Rohde, J.; Brosig, B.; Langosch, D. *J. Biol. Chem.* **2000**, *275*, 17481.
(395) Bowen, M. E.; Engelman, D. M.; Brunger, A. T. *Biochemistry* **2002**, *41*, 15861.
(396) Veit, M.; Becher, A.; Ahnert-Hilger, G. *Mol. Cell. Neurosci.* **2000**, *15*, 408.
(397) Toyoshima, C.; Nakasako, M.; Nomura, H.; Ogawa, H. *Nature* **2000**, *405*, 647.
(398) Cornelius, F.; Mahmmoud, Y. A. *News Physiol. Sci.* **2003**, *18*, 119.
(399) Geering, K.; Beguin, P.; Garty, H.; Karlish, S.; Fuzesi, M.; Horisberger, J. D.; Crambert, G. *Ann. N. Y. Acad. Sci.* **2003**, *986*, 388.
(400) Lindzen, M.; Aizman, R.; Lifshitz, Y.; Lubarski, I.; Karlish, S. J.; Garty, H. *J. Biol. Chem.* **2003**, *278*, 18738.
(401) Therien, A. G.; Deber, C. M. *J. Mol. Biol.* **2002**, *322*, 583.
(402) Farman, N.; Fay, M.; Cluzeaud, F. *Ann. N. Y. Acad. Sci.* **2003**, *986*, 428.
(403) Badola, P.; Sanders, C. R., 2nd *J. Biol. Chem.* **1997**, *272*, 24176.
(404) Gerber, D.; Sal-Man, N.; Shai, Y. *J. Mol. Biol.* **2004**, *339*, 243.
(405) Gerber, D.; Shai, Y. *J. Mol. Biol.* **2002**, *322*, 491.
(406) Kahn, T. W.; Engelman, D. M. *Biochemistry* **1992**, *31*, 6144.
(407) Kataoka, M.; Kahn, T. W.; Tsujiuchi, Y.; Engelman, D. M.; Tokunaga, F. *Photochem. Photobiol.* **1992**, *56*, 895.
(408) Kahn, T. W.; Sturtevant, J. M.; Engelman, D. M. *Biochemistry* **1992**, *31*, 8829.
(409) Hunt, J. F.; Earnest, T. N.; Bousche, O.; Kalghatgi, K.; Reilly, K.; Horvath, C.; Rothschild, K. J.; Engelman, D. M. *Biochemistry* **1997**, *36*, 15156.
(410) Hunt, J. F.; Rath, P.; Rothschild, K. J.; Engelman, D. M. *Biochemistry* **1997**, *36*, 15177.
(411) Xie, H.; Ding, F. X.; Schreiber, D.; Eng, G.; Liu, S. F.; Arshava, B.; Arevalo, E.; Becker, J. M.; Naider, F. *Biochemistry* **2000**, *39*, 15462.
(412) Lazarova, T.; Brewin, K. A.; Stoeber, K.; Robinson, C. R. *Biochemistry* **2004**, *43*, 12945.
(413) Thevenin, D.; Lazarova, T.; Roberts, M. F.; Robinson, C. R. *Protein Sci.* **2005**, *14*, 2177.
(414) Luneberg, J.; Widmann, M.; Dathe, M.; Marti, T. *J. Biol. Chem.* **1998**, *273*, 28822.
(415) Marti, T. *J. Biol. Chem.* **1998**, *273*, 9312.
(416) Kim, J. M.; Booth, P. J.; Allen, S. J.; Khorana, H. G. *J. Mol. Biol.* **2001**, *308*, 409.
(417) Allen, S. J.; Kim, J. M.; Khorana, H. G.; Lu, H.; Booth, P. J. *J. Mol. Biol.* **2001**, *308*, 423.
(418) Ridge, K. D.; Lee, S. S.; Yao, L. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3204.
(419) Yu, H.; Kono, M.; McKee, T. D.; Oprian, D. D. *Biochemistry* **1995**, *34*, 14963.
(420) Yu, H.; Kono, M.; Oprian, D. D. *Biochemistry* **1999**, *38*, 12028.
(421) Yu, H.; Oprian, D. D. *Biochemistry* **1999**, *38*, 12033.
(422) Struthers, M.; Yu, H.; Kono, M.; Oprian, D. D. *Biochemistry* **1999**, *38*, 6597.
(423) Struthers, M.; Yu, H.; Oprian, D. D. *Biochemistry* **2000**, *39*, 7938.
(424) Xie, G.; Gross, A. K.; Oprian, D. D. *Biochemistry* **2003**, *42*, 1995.
(425) Martin, N. P.; Leavitt, L. M.; Sommers, C. M.; Dumont, M. E. *Biochemistry* **1999**, *38*, 682.
(426) Beutler, R.; Kaufmann, M.; Ruggiero, F.; Erni, B. *Biochemistry* **2000**, *39*, 3745.
(427) Ottolia, M.; John, S.; Qiu, Z.; Philipson, K. D. *J. Biol. Chem.* **2001**, *276*, 19603.
(428) Beutler, R.; Ruggiero, F.; Erni, B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1477.
(429) Sadlish, H.; Pitonzo, D.; Johnson, A. E.; Skach, W. R. *Nat. Struct. Mol. Biol.* **2005**, *12*, 870.
(430) Krebs, M. P.; Khorana, H. G. *J. Bacteriol.* **1993**, *175*, 1555.
(431) van Iwaarden, P. R.; Pastore, J. C.; Konings, W. N.; Kaback, H. R. *Biochemistry* **1991**, *30*, 9595.
(432) Frillingos, S.; Sahin-Toth, M.; Wu, J.; Kaback, H. R. *FASEB J.* **1998**, *12*, 1281.
(433) Voss, J.; Hubbell, W. L.; Kaback, H. R. *Biochemistry* **1998**, *37*, 211.
(434) Voss, J.; Wu, J.; Hubbell, W. L.; Jacques, V.; Meares, C. F.; Kaback, H. R. *Biochemistry* **2001**, *40*, 3184.
(435) Wu, J.; Kaback, H. R. *J. Mol. Biol.* **1997**, *270*, 285.
(436) Wu, J.; Hardy, D.; Kaback, H. R. *Biochemistry* **1999**, *38*, 1715.
(437) Wolin, C. D.; Kaback, H. R. *Biochemistry* **2000**, *39*, 6130.
(438) Martin, N. P.; Celic, A.; Dumont, M. E. *J. Mol. Biol.* **2002**, *317*, 765.
(439) Sanders, C. R., 2nd; Czerski, L.; Vinogradova, O.; Badola, P.; Song, D.; Smith, S. O. *Biochemistry* **1996**, *35*, 8610.
(440) Wen, J.; Chen, X.; Bowie, J. U. *Nat. Struct. Biol.* **1996**, *3*, 141.
(441) Zhou, Y.; Wen, J.; Bowie, J. U. *Nat. Struct. Biol.* **1997**, *4*, 986.
(442) Lau, F. W.; Nauli, S.; Zhou, Y.; Bowie, J. U. *J. Mol. Biol.* **1999**, *290*, 559.
(443) Booth, P. J.; Flitsch, S. L.; Stern, L. J.; Greenhalgh, D. A.; Kim, P. S.; Khorana, H. G. *Nat. Struct. Biol.* **1995**, *2*, 139.
(444) Riley, M. L.; Wallace, B. A.; Flitsch, S. L.; Booth, P. J. *Biochemistry* **1997**, *36*, 192.
(445) Chen, G. Q.; Gouaux, J. E. *Protein Sci.* **1996**, *5*, 456.
(446) Chen, G. Q.; Gouaux, E. *Protein Eng.* **1997**, *10*, 1061.
(447) Krebs, M. P.; Li, W.; Halambeck, T. P. *J. Mol. Biol.* **1997**, *267*, 172.
(448) Isenbarger, T. A.; Krebs, M. P. *Biochemistry* **1999**, *38*, 9023.
(449) Luecke, H.; Schobert, B.; Richter, H. T.; Cartailler, J. P.; Lanyi, J. K. *J. Mol. Biol.* **1999**, *291*, 899.
(450) Faham, S.; Bowie, J. U. *J. Mol. Biol.* **2002**, *316*, 1.
(451) Yohannan, S.; Faham, S.; Yang, D.; Whitelegge, J. P.; Bowie, J. U. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 959.
(452) von Heijne, G. *J. Mol. Biol.* **1991**, *218*, 499.
(453) Kim, S.; Cross, T. A. *Biophys. J.* **2002**, *83*, 2084.
(454) Lu, H.; Marti, T.; Booth, P. J. *J. Mol. Biol.* **2001**, *308*, 437.
(455) Palsdottir, H.; Hunte, C. *Biochim. Biophys. Acta* **2004**, *1666*, 2.
(456) Marsh, D.; Pali, T. *Biochim. Biophys. Acta* **2004**, *1666*, 118.
(457) van den Brink-van der Laan, E.; Killian, J. A.; de Kruijff, B. *Biochim. Biophys. Acta* **2004**, *1666*, 275.
(458) Silvius, J. R. *Biochim. Biophys. Acta* **2003**, *1610*, 174.
(459) Edidin, M. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 257.
(460) Munro, S. *Cell* **2003**, *115*, 377.
(461) Pike, L. J. *Biochem. J.* **2004**, *378*, 281.
(462) Simons, K.; Vaz, W. L. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 269.
(463) Opekarova, M.; Tanner, W. *Biochim. Biophys. Acta* **2003**, *1610*, 11.
(464) Zhang, H.; Cramer, W. A. *Methods Mol. Biol.* **2004**, *274*, 67.
(465) Zhang, H.; Kurisu, G.; Smith, J. L.; Cramer, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5160.
(466) Stroebel, D.; Choquet, Y.; Popot, J. L.; Picot, D. *Nature* **2003**, *426*, 413.
(467) Kurisu, G.; Zhang, H.; Smith, J. L.; Cramer, W. A. *Science* **2003**, *302*, 1009.
(468) Jones, M. R.; Fyfe, P. K.; Roszak, A. W.; Isaacs, N. W.; Cogdell, R. J. *Biochim. Biophys. Acta* **2002**, *1565*, 206.
(469) Fyfe, P. K.; Isaacs, N. W.; Cogdell, R. J.; Jones, M. R. *Biochim. Biophys. Acta* **2004**, *1608*, 11.
(470) van Dalen, A.; Hegger, S.; Killian, J. A.; de Kruijff, B. *FEBS Lett.* **2002**, *525*, 33.
(471) van den Brink-van der Laan, E.; Chupin, V.; Killian, J. A.; de Kruijff, B. *Biochemistry* **2004**, *43*, 4240.
(472) van den Brink-van der Laan, E.; Chupin, V.; Killian, J. A.; de Kruijff, B. *Biochemistry* **2004**, *43*, 5937.
(473) Gruner, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3665.
(474) Allen, S. J.; Curran, A. R.; Templer, R. H.; Meijberg, W.; Booth, P. J. *J. Mol. Biol.* **2004**, *342*, 1293.
(475) Curnow, P.; Lorch, M.; Charalambous, K.; Booth, P. J. *J. Mol. Biol.* **2004**, *343*, 213.
(476) Constantinescu, I.; Lafleur, M. *Biochim. Biophys. Acta* **2004**, *1667*, 26.

(477) Meijberg, W.; Booth, P. J. *J. Mol. Biol.* **2002**, *319*, 839.
(478) Hong, H.; Tamm, L. K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4065.
(479) Gomez, B., Jr.; Robinson, N. C. *Anal. Biochem* **1999**, *267*, 212.
(480) Gomez, B., Jr.; Robinson, N. C. *Biochemistry* **1999**, *38*, 9031.
(481) Lange, C.; Nett, J. H.; Trumpower, B. L.; Hunte, C. *EMBO J.* **2001**, *20*, 6591.
(482) Ostrander, D. B.; Zhang, M.; Mileykovskaya, E.; Rho, M.; Dowhan, W. *J. Biol. Chem.* **2001**, *276*, 25262.
(483) Zhang, M.; Mileykovskaya, E.; Dowhan, W. *J. Biol. Chem.* **2002**, *277*, 43553.
(484) Zhang, M.; Su, X.; Mileykovskaya, E.; Amoscato, A. A.; Dowhan, W. *J. Biol. Chem.* **2003**, *278*, 35204.
(485) Pfeiffer, K.; Gohil, V.; Stuart, R. A.; Hunte, C.; Brandt, U.; Greenberg, M. L.; Schagger, H. *J. Biol. Chem.* **2003**, *278*, 52873.
(486) van Dalen, A.; de Kruijff, B. *Biochim. Biophys. Acta* **2004**, *1694*, 97.
(487) Bogdanov, M.; Sun, J.; Kaback, H. R.; Dowhan, W. *J. Biol. Chem.* **1996**, *271*, 11615.
(488) Bogdanov, M.; Heacock, P. N.; Dowhan, W. *EMBO J.* **2002**, *21*, 2107.
(489) Nagamori, S.; Vazquez-Ibar, J. L.; Weinglass, A. B.; Kaback, H. R. *J. Biol. Chem.* **2003**, *278*, 14820.
(490) Nagamori, S.; Smirnova, I. N.; Kaback, H. R. *J. Cell Biol.* **2004**, *165*, 53.
(491) Zhang, W.; Bogdanov, M.; Pi, J.; Pittard, A. J.; Dowhan, W. *J. Biol. Chem.* **2003**, *278*, 50128.
(492) Zhang, W.; Campbell, H. A.; King, S. C.; Dowhan, W. *J. Biol. Chem.* **2005**, *280*, 26032.
(493) Robl, I.; Grassl, R.; Tanner, W.; Opekarova, M. *Yeast* **2001**, *18*, 251.
(494) Opekarova, M.; Robl, I.; Tanner, W. *Biochim. Biophys. Acta* **2002**, *1564*, 9.
(495) Bruss, V.; Lu, X.; Thomssen, R.; Gerlich, W. H. *EMBO J.* **1994**, *13*, 2273.
(496) Lu, Y.; Turnbull, I. R.; Bragin, A.; Carveth, K.; Verkman, A. S.; Skach, W. R. *Mol. Biol. Cell* **2000**, *11*, 2973.
(497) Kanki, T.; Sakaguchi, M.; Kitamura, A.; Sato, T.; Mihara, K.; Hamasaki, N. *Biochemistry* **2002**, *41*, 13973.
(498) Lambert, C.; Prange, R. *J. Biol. Chem.* **2001**, *276*, 22265.
(499) Lambert, C.; Prange, R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5199.
(500) Buck, T. M.; Skach, W. R. *J. Biol. Chem.* **2005**, *280*, 261.
(501) Wang, X.; Bogdanov, M.; Dowhan, W. *EMBO J.* **2002**, *21*, 5673.
(502) Heinrich, S. U.; Rapoport, T. A. *EMBO J.* **2003**, *22*, 3654.
(503) Nilsson, I.; Ohvo-Rekila, H.; Slotte, J. P.; Johnson, A. E.; von Heijne, G. *J. Biol. Chem.* **2001**, *276*, 41748.
(504) Bracey, M. H.; Cravatt, B. F.; Stevens, R. C. *FEBS Lett.* **2004**, *567*, 159.
(505) Granseth, E.; von Heijne, G.; Elofsson, A. *J. Mol. Biol.* **2005**, *346*, 377.
(506) Killian, J. A.; von Heijne, G. *Trends Biochem. Sci.* **2000**, *25*, 429.
(507) Zakharov, S. D.; Cramer, W. A. *Biochim. Biophys. Acta* **2002**, *1565*, 333.
(508) Montoya, M.; Gouaux, E. *Biochim. Biophys. Acta* **2003**, *1609*, 19.
(509) Heuck, A. P.; Tweten, R. K.; Johnson, A. E. *Biochemistry* **2001**, *40*, 9065.
(510) Parker, M. W.; Feil, S. C. *Prog. Biophys. Mol. Biol.* **2005**, *88*, 91.
(511) Davidson, V. L.; Brunden, K. R.; Cramer, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1386.
(512) Xu, S.; Cramer, W. A.; Peterson, A. A.; Hermodson, M.; Montecucco, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7531.
(513) van der Goot, F. G.; Gonzalez-Manas, J. M.; Lakey, J. H.; Pattus, F. *Nature* **1991**, *354*, 408.
(514) Zakharov, S. D.; Lindeberg, M.; Cramer, W. A. *Biochemistry* **1999**, *38*, 11325.
(515) Sobko, A. A.; Kotova, E. A.; Antonenko, Y. N.; Zakharov, S. D.; Cramer, W. A. *FEBS Lett.* **2004**, *576*, 205.
(516) Menestrina, G.; Dalla Serra, M.; Comai, M.; Coraiola, M.; Viero, G.; Werner, S.; Colin, D. A.; Monteil, H.; Prevost, G. *FEBS Lett.* **2003**, *552*, 54.
(517) Olson, R.; Nariya, H.; Yokota, K.; Kamio, Y.; Gouaux, E. *Nat. Struct. Biol.* **1999**, *6*, 134.
(518) Pedelacq, J. D.; Maveyraud, L.; Prevost, G.; Baba-Moussa, L.; Gonzalez, A.; Courcelle, E.; Shepard, W.; Monteil, H.; Samama, J. P.; Mourey, L. *Struct. Folding Des.* **1999**, *7*, 277.
(519) Song, L.; Hobaugh, M. R.; Shustak, C.; Cheley, S.; Bayley, H.; Gouaux, J. E. *Science* **1996**, *274*, 1859.
(520) Gouaux, E.; Hobaugh, M.; Song, L. *Protein Sci.* **1997**, *6*, 2631.
(521) Valeva, A.; Palmer, M.; Bhakdi, S. *Biochemistry* **1997**, *36*, 13298.
(522) Nguyen, V. T.; Higuchi, H.; Kamio, Y. *Mol. Microbiol.* **2002**, *45*, 1485.
(523) Kawate, T.; Gouaux, E. *Protein Sci.* **2003**, *12*, 997.
(524) Galdiero, S.; Gouaux, E. *Protein Sci.* **2004**, *13*, 1503.
(525) Donovan, J. J.; Simon, M. I.; Draper, R. K.; Montal, M. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 172.
(526) Hu, V. W.; Holmes, R. K. *J. Biol. Chem.* **1984**, *259*, 12226.
(527) Zalman, L. S.; Wisnieski, B. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3341.
(528) Montecucco, C.; Schiavo, G.; Tomasi, M. *Biochem. J.* **1985**, *231*, 123.
(529) Choe, S.; Bennett, M. J.; Fujii, G.; Curmi, P. M.; Kantardjieff, K. A.; Collier, R. J.; Eisenberg, D. *Nature* **1992**, *357*, 216.
(530) Bennett, M. J.; Eisenberg, D. *Protein Sci.* **1994**, *3*, 1464.
(531) Bennett, M. J.; Choe, S.; Eisenberg, D. *Protein Sci.* **1994**, *3*, 1444.
(532) Moskaug, J. O.; Stenmark, H.; Olsnes, S. *J. Biol. Chem.* **1991**, *266*, 2652.
(533) Zhan, H.; Oh, K. J.; Shin, Y. K.; Hubbell, W. L.; Collier, R. J. *Biochemistry* **1995**, *34*, 4856.
(534) Zhan, H.; Choe, S.; Huynh, P. D.; Finkelstein, A.; Eisenberg, D.; Collier, R. J. *Biochemistry* **1994**, *33*, 11254.
(535) Kachel, K.; Ren, J.; Collier, R. J.; London, E. *J. Biol. Chem.* **1998**, *273*, 22950.
(536) O'Keefe, D. O.; Cabiaux, V.; Choe, S.; Eisenberg, D.; Collier, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6202.
(537) Silverman, J. A.; Mindell, J. A.; Finkelstein, A.; Shen, W. H.; Collier, R. J. *J. Biol. Chem.* **1994**, *269*, 22524.
(538) Kaul, P.; Silverman, J.; Shen, W. H.; Blanke, S. R.; Huynh, P. D.; Finkelstein, A.; Collier, R. J. *Protein Sci.* **1996**, *5*, 687.
(539) Ren, J.; Sharpe, J. C.; Collier, R. J.; London, E. *Biochemistry* **1999**, *38*, 976.
(540) Zhao, G.; London, E. *Biochemistry* **2005**, *44*, 4488.
(541) Wang, Y.; Malenbaum, S. E.; Kachel, K.; Zhan, H.; Collier, R. J.; London, E. *J. Biol. Chem.* **1997**, *272*, 25091.
(542) Rosconi, M. P.; London, E. *J. Biol. Chem.* **2002**, *277*, 16517.
(543) Rosconi, M. P.; Zhao, G.; London, E. *Biochemistry* **2004**, *43*, 9127.
(544) Ladokhin, A. S.; Legmann, R.; Collier, R. J.; White, S. H. *Biochemistry* **2004**, *43*, 7451.
(545) Gerke, V.; Creutz, C. E.; Moss, S. E. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 449.
(546) Langen, R.; Isas, J. M.; Luecke, H.; Haigler, H. T.; Hubbell, W. L. *J. Biol. Chem.* **1998**, *273*, 22453.
(547) Patel, D. R.; Isas, J. M.; Ladokhin, A. S.; Jao, C. C.; Kim, Y. E.; Kirsch, T.; Langen, R.; Haigler, H. T. *Biochemistry* **2005**, *44*, 2833.
(548) Huber, R.; Schneider, M.; Mayr, I.; Romisch, J.; Paques, E. P. *FEBS Lett.* **1990**, *275*, 15.
(549) Huber, R.; Romisch, J.; Paques, E. P. *EMBO J.* **1990**, *9*, 3867.
(550) Bewley, M. C.; Boustead, C. M.; Walker, J. H.; Waller, D. A.; Huber, R. *Biochemistry* **1993**, *32*, 3923.
(551) Sopkova, J.; Renouard, M.; Lewit-Bentley, A. *J. Mol. Biol.* **1993**, *234*, 816.
(552) Langen, R.; Isas, J. M.; Hubbell, W. L.; Haigler, H. T. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 14060.
(553) Luecke, H.; Chang, B. T.; Mailliard, W. S.; Schlaepfer, D. D.; Haigler, H. T. *Nature* **1995**, *378*, 512.
(554) Isas, J. M.; Langen, R.; Haigler, H. T.; Hubbell, W. L. *Biochemistry* **2002**, *41*, 1464.
(555) Isas, J. M.; Patel, D. R.; Jao, C.; Jayasinghe, S.; Cartailler, J. P.; Haigler, H. T.; Langen, R. *J. Biol. Chem.* **2003**, *278*, 30227.
(556) Isas, J. M.; Langen, R.; Hubbell, W. L.; Haigler, H. T. *J. Biol. Chem.* **2004**, *279*, 32492.
(557) Kim, Y. E.; Isas, J. M.; Haigler, H. T.; Langen, R. *J. Biol. Chem.* **2005**, *280*, 32398.
(558) Ladokhin, A. S.; Isas, J. M.; Haigler, H. T.; White, S. H. *Biochemistry* **2002**, *41*, 13617.
(559) Ladokhin, A. S.; Haigler, H. T. *Biochemistry* **2005**, *44*, 3402.
(560) Suzuki, M.; Youle, R. J.; Tjandra, N. *Cell* **2000**, *103*, 645.
(561) Schinzel, A.; Kaufmann, T.; Borner, C. *Biochim. Biophys. Acta* **2004**, *1644*, 95.
(562) Borgese, N.; Colombo, S.; Pedrazzini, E. *J. Cell Biol.* **2003**, *161*, 1013.
(563) Wattenberg, B.; Lithgow, T. *Traffic* **2001**, *2*, 66.
(564) Brambillasca, S.; Yabal, M.; Soffientini, P.; Stefanovic, S.; Makarow, M.; Hegde, R. S.; Borgese, N. *EMBO J.* **2005**, *24*, 2533.
(565) Dempsey, C. E. *Biochim. Biophys. Acta* **1990**, *1031*, 143.
(566) Bechinger, B. *J. Membr. Biol.* **1997**, *156*, 197.
(567) Hristova, K.; Wimley, W. C.; Mishra, V. K.; Anantharamiah, G. M.; Segrest, J. P.; White, S. H. *J. Mol. Biol.* **1999**, *290*, 99.
(568) Hristova, K.; Dempsey, C. E.; White, S. H. *Biophys. J.* **2001**, *80*, 801.
(569) Ladokhin, A. S.; White, S. H. *Biochim. Biophys. Acta* **2001**, *1514*, 253.
(570) Wimley, W. C.; White, S. H. *Biochemistry* **2000**, *39*, 161.
(571) Ohki, S.; Marcus, E.; Sukumaran, D. K.; Arnold, K. *Biochim. Biophys. Acta* **1994**, *1194*, 223.
(572) Benachir, T.; Lafleur, M. *Biochim. Biophys. Acta* **1995**, *1235*, 452.
(573) Monette, M.; Lafleur, M. *Biophys. J.* **1995**, *68*, 187.

(574) Sekharam, K. M.; Bradrick, T. D.; Georghiou, S. *Biochim. Biophys. Acta* **1991**, *1063*, 171.
(575) Wimley, W. C.; White, S. H. *Biochemistry* **2000**, *39*, 4432.
(576) Ladokhin, A. S.; White, S. H. *Biochemistry* **2004**, *43*, 5782.
(577) Liu, L. P.; Deber, C. M. *Biopolymers* **1998**, *47*, 41.
(578) Liu, L. P.; Deber, C. M. *Biochemistry* **1997**, *36*, 5476.
(579) Liu, L. P.; Li, S. C.; Goto, N. K.; Deber, C. M. *Biopolymers* **1996**, *39*, 465.
(580) Booth, P. J. *Folding Des.* **1997**, *2*, R85.
(581) Booth, P. J. *Biochim. Biophys. Acta* **2000**, *1460*, 4.
(582) Booth, P. J.; Farooq, A.; Flitsch, S. L. *Biochemistry* **1996**, *35*, 5902.
(583) Lu, H.; Booth, P. J. *J. Mol. Biol.* **2000**, *299*, 233.
(584) Booth, P. J.; Riley, M. L.; Flitsch, S. L.; Templer, R. H.; Farooq, A.; Curran, A. R.; Chadborn, N.; Wright, P. *Biochemistry* **1997**, *36*, 197.
(585) Booth, P. J.; Curran, A. R.; Templer, R. H.; Lu, H.; Meijberg, W. *Biochem. Soc. Symp.* **2001**, 27.
(586) Catucci, L.; Lattanzio, V. M.; Lobasso, S.; Agostiano, A.; Corcelli, A. *Bioelectrochemistry* **2004**, *63*, 111.
(587) Curran, A. R.; Templer, R. H.; Booth, P. J. *Biochemistry* **1999**, *38*, 9328.
(588) Allen, S. J.; Curran, A. R.; Templer, R. H.; Meijberg, W.; Booth, P. J. *J. Mol. Biol.* **2004**, *342*, 1279.
(589) Nannepaga, S. J.; Gawalapu, R.; Velasquez, D.; Renthal, R. *Biochemistry* **2004**, *43*, 550.
(590) Gorzelle, B. M.; Nagy, J. K.; Oxenoid, K.; Lonzer, W. L.; Cafiso, D. S.; Sanders, C. R. *Biochemistry* **1999**, *38*, 16373.
(591) Zhou, Y.; Lau, F. W.; Nauli, S.; Yang, D.; Bowie, J. U. *Protein Sci.* **2001**, *10*, 378.
(592) Oxenoid, K.; Sonnichsen, F. D.; Sanders, C. R. *Biochemistry* **2001**, *40*, 5111.
(593) Hwang, P. M.; Bishop, R. E.; Kay, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9618.
(594) Hwang, P. M.; Choy, W. Y.; Lo, E. I.; Chen, L.; Forman-Kay, J. D.; Raetz, C. R.; Prive, G. G.; Bishop, R. E.; Kay, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13560.
(595) Sanders, C. R.; Nagy, J. K. *Curr. Opin. Struct. Biol.* **2000**, *10*, 438.
(596) Trombetta, E. S.; Parodi, A. J. *Annu. Rev. Cell Dev. Biol.* **2003**, *19*, 649.
(597) Sanders, C. R.; Myers, J. K. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 25.
(598) Nagy, J. K.; Lonzer, W. L.; Sanders, C. R. *Biochemistry* **2001**, *40*, 8971.
(599) Lorch, M.; Booth, P. J. *J. Mol. Biol.* **2004**, *344*, 1109.
(600) Nagy, J. K.; Sanders, C. R. *Biochemistry* **2002**, *41*, 9021.
(601) Nagy, J. K.; Sanders, C. R. *Biochemistry* **2004**, *43*, 19.
(602) Kadokura, H.; Katzen, F.; Beckwith, J. *Annu. Rev. Biochem.* **2003**, *72*, 111.
(603) Otzen, D. E. *J. Mol. Biol.* **2003**, *330*, 641.
(604) Plumley, F. G.; Schmidt, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 146.
(605) Yang, C.; Horn, R.; Paulsen, H. *Biochemistry* **2003**, *42*, 4527.
(606) Heinemann, B.; Paulsen, H. *Biochemistry* **1999**, *38*, 14088.
(607) Mick, V.; Eggert, K.; Heinemann, B.; Geister, S.; Paulsen, H. *Biochemistry* **2004**, *43*, 5467.
(608) Mick, V.; Geister, S.; Paulsen, H. *Biochemistry* **2004**, *43*, 14704.
(609) Kuhlbrandt, W.; Wang, D. N.; Fujiyoshi, Y. *Nature* **1994**, *367*, 614.
(610) Kuhlbrandt, W.; Wang, D. N. *Nature* **1991**, *350*, 130.
(611) von Heijne, G. *J. Membr. Biol.* **1990**, *115*, 195.
(612) Foster, W.; Helm, A.; Turnbull, I.; Gulati, H.; Yang, B.; Verkman, A. S.; Skach, W. R. *J. Biol. Chem.* **2000**, *275*, 34157.
(613) Sakaguchi, M.; Tomiyoshi, R.; Kuroiwa, T.; Mihara, K.; Omura, T. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 16.
(614) Wahlberg, J. M.; Spiess, M. *J. Cell Biol.* **1997**, *137*, 555.
(615) Denzer, A. J.; Nabholz, C. E.; Spiess, M. *EMBO J.* **1995**, *14*, 6311.
(616) Ota, K.; Sakaguchi, M.; von Heijne, G.; Hamasaki, N.; Mihara, K. *Mol. Cell* **1998**, *2*, 495.
(617) Goder, V.; Bieri, C.; Spiess, M. *J. Cell Biol.* **1999**, *147*, 257.
(618) Goder, V.; Spiess, M. *EMBO J.* **2003**, *22*, 3645.
(619) Higy, M.; Gander, S.; Spiess, M. *Biochemistry* **2005**, *44*, 2039.
(620) Monne, M.; Hessa, T.; Thissen, L.; von Heijne, G. *FEBS J.* **2005**, *272*, 28.
(621) Sakaguchi, M. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 1.
(622) Ota, K.; Sakaguchi, M.; Hamasaki, N.; Mihara, K. *J. Biol. Chem.* **2000**, *275*, 29743.
(623) Sato, Y.; Sakaguchi, M.; Goshima, S.; Nakamura, T.; Uozumi, N. *J. Biol. Chem.* **2003**, *278*, 13227.
(624) Riordan, J. R. *Annu. Rev. Physiol.* **2005**, *67*, 701.
(625) Kartner, N.; Augustinas, O.; Jensen, T. J.; Naismith, A. L.; Riordan, J. R. *Nat. Genet.* **1992**, *1*, 321.
(626) Denning, G. M.; Anderson, M. P.; Amara, J. F.; Marshall, J.; Smith, A. E.; Welsh, M. J. *Nature* **1992**, *358*, 761.
(627) Cheng, S. H.; Gregory, R. J.; Marshall, J.; Paul, S.; Souza, D. W.; White, G. A.; O'Riordan, C. R.; Smith, A. E. *Cell* **1990**, *63*, 827.
(628) Tector, M.; Hartl, F. U. *EMBO J.* **1999**, *18*, 6290.
(629) Carveth, K.; Buck, T.; Anthony, V.; Skach, W. R. *J. Biol. Chem.* **2002**, *277*, 39507.
(630) Thomas, P. J.; Shenbagamurthi, P.; Sondek, J.; Hullihen, J. M.; Pedersen, P. L. *J. Biol. Chem.* **1992**, *267*, 5727.
(631) Thibodeau, P. H.; Brautigam, C. A.; Machius, M.; Thomas, P. J. *Nat. Struct. Mol. Biol.* **2005**, *12*, 10.
(632) Lewis, H. A.; Zhao, X.; Wang, C.; Sauder, J. M.; Rooney, I.; Noland, B. W.; Lorimer, D.; Kearins, M. C.; Conners, K.; Condon, B.; Maloney, P. C.; Guggino, W. B.; Hunt, J. F.; Emtage, S. *J. Biol. Chem.* **2005**, *280*, 1346.
(633) Du, K.; Sharma, M.; Lukacs, G. L. *Nat. Struct. Mol. Biol.* **2005**, *12*, 17.
(634) Ward, C. L.; Omura, S.; Kopito, R. R. *Cell* **1995**, *83*, 121.
(635) Ward, C. L.; Kopito, R. R. *J. Biol. Chem.* **1994**, *269*, 25710.
(636) Farinha, C. M.; Amaral, M. D. *Mol. Cell. Biol.* **2005**, *25*, 5242.
(637) Hermansson, M.; von Heijne, G. *J. Mol. Biol.* **2003**, *334*, 803.
(638) Mothes, W.; Heinrich, S. U.; Graf, R.; Nilsson, I.; von Heijne, G.; Brunner, J.; Rapoport, T. A. *Cell* **1997**, *89*, 523.
(639) Bernier, V.; Lagace, M.; Bichet, D. G.; Bouvier, M. *Trends Endocrinol. Metab.* **2004**, *15*, 222.
(640) Bolen, D. W.; Baskakov, I. V. *J. Mol. Biol.* **2001**, *310*, 955.
(641) Brown, C. R.; Hong-Brown, L. Q.; Biwersi, J.; Verkman, A. S.; Welch, W. J. *Cell Stress Chaperones* **1996**, *1*, 117.
(642) Sato, S.; Ward, C. L.; Krouse, M. E.; Wine, J. J.; Kopito, R. R. *J. Biol. Chem.* **1996**, *271*, 635.
(643) Zhang, X. M.; Wang, X. T.; Yue, H.; Leung, S. W.; Thibodeau, P. H.; Thomas, P. J.; Guggino, S. E. *J. Biol. Chem.* **2003**, *278*, 51232.
(644) Tamarappoo, B. K.; Verkman, A. S. *J. Clin. Invest.* **1998**, *101*, 2257.
(645) Figler, R. A.; Omote, H.; Nakamoto, R. K.; Al-Shawi, M. K. *Arch. Biochem. Biophys.* **2000**, *376*, 34.
(646) Loo, T. W.; Clarke, D. M. *J. Biol. Chem.* **1997**, *272*, 709.
(647) Zhou, Z.; Gong, Q.; January, C. T. *J. Biol. Chem.* **1999**, *274*, 31123.
(648) Ficker, E.; Obejero-Paz, C. A.; Zhao, S.; Brown, A. M. *J. Biol. Chem.* **2002**, *277*, 4989.
(649) Morello, J. P.; Salahpour, A.; Laperriere, A.; Bernier, V.; Arthus, M. F.; Lonergan, M.; Petaja-Repo, U.; Angers, S.; Morin, D.; Bichet, D. G.; Bouvier, M. *J. Clin. Invest.* **2000**, *105*, 887.
(650) Wuller, S.; Wiesner, B.; Loffler, A.; Furkert, J.; Krause, G.; Hermosilla, R.; Schaefer, M.; Schulein, R.; Rosenthal, W.; Oksche, A. *J. Biol. Chem.* **2004**, *279*, 47254.
(651) Petaja-Repo, U. E.; Hogue, M.; Bhalla, S.; Laperriere, A.; Morello, J. P.; Bouvier, M. *EMBO J.* **2002**, *21*, 1628.
(652) Partridge, C. J.; Beech, D. J.; Sivaprasadarao, A. *J. Biol. Chem.* **2001**, *276*, 35947.
(653) Egan, M. E.; Pearson, M.; Weiner, S. A.; Rajendran, V.; Rubin, D.; Glockner-Pagel, J.; Canny, S.; Du, K.; Lukacs, G. L.; Caplan, M. J. *Science* **2004**, *304*, 600.
(654) Loo, T. W.; Bartlett, M. C.; Clarke, D. M. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 580.
(655) Song, Y.; Sonawane, N. D.; Salinas, D.; Qian, L.; Pedemonte, N.; Galietta, L. J.; Verkman, A. S. *J. Biol. Chem.* **2004**, *279*, 40629.
(656) Dragomir, A.; Bjorstad, J.; Hjelte, L.; Roomans, G. M. *Biochem. Biophys. Res. Commun.* **2004**, *322*, 447.
(657) Berger, A. L.; Randak, C. O.; Ostedgaard, L. S.; Karp, P. H.; Vermeer, D. W.; Welsh, M. J. *J. Biol. Chem.* **2005**, *280*, 5221.
(658) Wang, W.; Li, G.; Clancy, J. P.; Kirk, K. L. *J. Biol. Chem.* **2005**, *280*, 23622.
(659) Ma, T.; Vetrivel, L.; Yang, H.; Pedemonte, N.; Zegarra-Moran, O.; Galietta, L. J.; Verkman, A. S. *J. Biol. Chem.* **2002**, *277*, 37235.
(660) Pedemonte, N.; Sonawane, N. D.; Taddei, A.; Hu, J.; Zegarra-Moran, O.; Suen, Y. F.; Robins, L. I.; Dicus, C. W.; Willenbring, D.; Nantz, M. H.; Kurth, M. J.; Galietta, L. J.; Verkman, A. S. *Mol. Pharmacol.* **2005**, *67*, 1797.
(661) Pedemonte, N.; Lukacs, G. L.; Du, K.; Caci, E.; Zegarra-Moran, O.; Galietta, L. J.; Verkman, A. S. *J. Clin. Invest.* **2005**, *115*, 2564.

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