Current Topics

Peptide Models of Membrane Protein Folding[†]

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ABSTRACT: Given the central roles of membrane proteins in cellular processes ranging from nutrient uptake to cell−cell communication, as well as the importance of these proteins as drug targets, efforts to understand and control their structures are vital in human health and disease. The rational design of membrane proteins with modified properties is thus a highly desirable goal in molecular medicine and biotechnology. However, experimental data showing how individual transmembrane (TM) residues and/or segments direct the packing and folding of membrane proteins into biologically functional entities remain sparse. To address these questions in a systematic manner, helix−helix interactions between two (or more) TM segments must be identified and analyzed. Here we present an overview of the utilization of peptides as models of the TM segments of α-helical membrane proteins in uncovering the amino acid sequence motifs and interactions that build these molecules. TM peptide design and production strategies are discussed, and specific examples of the application of TM peptides to the study of membrane proteins are presented. We demonstrate that TM peptides can be routinely produced in sufficient quantities for biophysical analysis, are amenable to a variety of experimental techniques, and can effectively replicate the native helix−helix contacts and key aspects of the natural biological structures of membrane proteins.

Proteins embedded in membranes regulate the molecular traffic and information flow across biological membranes and, as such, represent attractive biomolecules for the study and/or modulation of processes in living cells. Membrane proteins of the helical type, found in eukaryotic cell membranes and in the inner membranes of bacteria and mitochondria, represent the majority of this protein group and are estimated to comprise 20−30% of open reading frames in known genomes (*1*). As well, it is estimated that approximately 70% of all current pharmaceutical targets are membrane proteins (*2*), yet despite advances in their isolation and structural characterization, membrane proteins currently represent 1−2% of the >56000 structures deposited in the Protein Data Bank (also see ref *3*), in part because of the technical challenges associated with the overexpression, purification, and structural characterization of these highly hydrophobic molecules. Disease-causing mutations that promote non-native folding are common in α-helical membrane-spanning domains, where disruption or failure of assembly constraints not only may lead to a loss of protein function but also could promote uncontrolled and potentially pathogenic polypeptide self-assembly. It is therefore crucial to elucidate the fundamental principles of how helical membrane proteins assemble into their functional biological forms in membranes.

Fortunately, gaining insight into membrane protein folding has not relied exclusively on the availability of high-resolution structures. Consideration of the individual transmembrane (TM)[1] α-helices as independently folded units has facilitated a "divide and conquer" approach to the study of membrane protein assembly, where interactions between peptides that recapitulate the membrane-embedded portions of helical membrane proteins can be studied in vitro and in vivo. Here we discuss the rationale underlying the usage of peptides encompassing single-TM segments and those with TM segment−loop−TM segment or "hairpin" sequences as models of membrane-spanning segments in proteins, present the methods used to design and produce these peptides for biophysical characterization, and then highlight the application of these peptide models to investigations of membrane protein folding and assembly.

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[1] Abbreviations: TM, transmembrane; GpA, glycophorin A; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism spectroscopy; FRET, Förster resonance energy transfer; MCP, Ff bacteriophage M13 major coat protein; CAT, chloramphenicol acetyltransferase; DAGK, diacylglycerol kinase; SMR, small multi-drug resistance protein; SPFO, sodium perfluorooctanoate.

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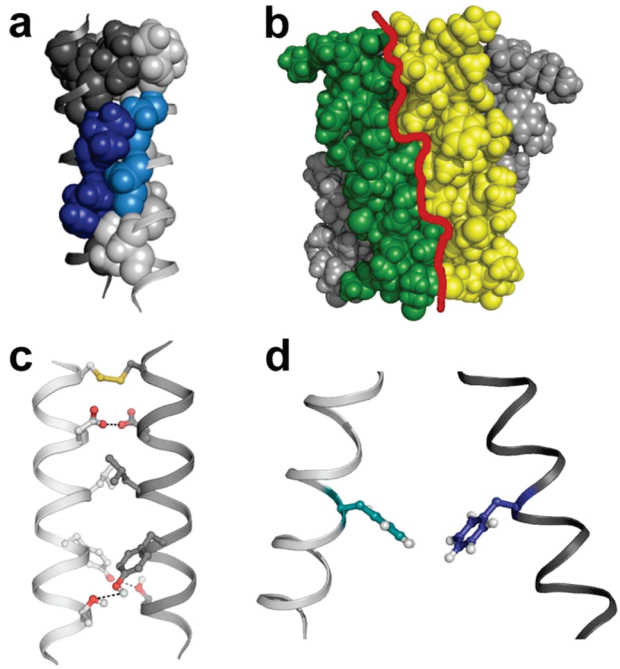

FIGURE 1: Interactions stabilizing helix−helix contacts in membranes. (a) Large Val residue "knobs" (dark blue) are in van der Waals contact with a concave helix surface, as illustrated by the Gly residue "holes" (light blue) of a GG_4 motif at the GpA dimer interface (*30*). (b) Apolar amino acid residues of the phospholamban heptad repeat are in van der Waals contact (red line) across the helix−helix interface (*102*). (c) Interhelical hydrogen bond networks between Asp-Asp and Thr-Tyr residue pairs stabilize the interface of the T-cell receptor ζ-chain dimer (*21*). (d) Edge−face cation−π interaction between Phe residues in the ErbB2 dimer (*28*). This figure was produced with PyMol (DeLano Scientific, Palo Alto, CA).

SINGLE-SPANNING TM SEGMENTS AND HAIRPINS AS BASIC FOLDING MODELS

The utility of peptides as a tool for the investigation of membrane protein folding originates from a series of early folding experiments performed on the α-helical membrane protein bacteriorhodopsin (*4*−*6*). These works showed that the native protein fold could be regenerated from various bacteriorhodopsin fragments, an indication that TM helix−helix interactions have sufficient specificity to generate tertiary structure. Subsequent studies of loop-severed or loop-deleted fragments confirmed the assembly of rhodopsin family members via helix−helix contacts (*7, 8*) and extended these findings to other TM proteins (*9*−*12*). Popot and Engelman incorporated such observations into their two-stage model of helical membrane protein folding (*13*), where the assembly process is divided into two energetically distinct categories: (i) helical secondary structure formation concomitant with insertion of hydrophobic sequences into the bilayer and (ii) tertiary and/or quaternary structure formation via lateral interactions between helices within the membrane. The folding problem then becomes a question of why two largely hydrophobic helical segments embedded in the apolar interior of the bilayer become associated when the surrounding membrane lipids could stabilize each individually. As illustrated in Figure 1, several types of interactions operate to promote dimers and higher-order oligomers of single-TM helices. For example, van der Waals forces stabilize close-packed or "knobs-into-holes" type helix−helix associations (Figure 1a,b). In addition to functional implications, polar residues in TM segments, which constitute ca. 20% of native residues in TM segments, mediate helix−helix assembly through side chain interactions; in some cases, a single "strongly polar" residue (Asp, Glu, Asn, or Gln) is sufficient for TM segment self-association (*14*−*21*), while other sequences are stabilized by interhelical hydrogen bond networks (Figure 1c) (*21*−*23*). Cation−π and/or π−π interactions between aromatic and/or basic residues also participate in TM−TM self-association in the bacterial cell membrane (*24*−*26*) and are observed in TM domain dimers (Figure 1d) (*27, 28*).

TM helix−helix interactions can be roughly grouped on the basis of sequence patterning and geometry (Table 1). Right-handed packing of helix pairs, for example, is most often characterized by an i, $i + 4$ separation of "small" residues (Gly, Ala, and Ser) along the TM sequence, alternately termed the small−xxx−small, XX_4 (where X is G, A, or S), or GAS_{Right} motif (*29*). As exemplified by the two Gly residues in the well-characterized glycophorin A (GpA) dimer, this sequence periodicity places the two small residues on the same surface of the helix, where they create a shallow groove that complements the surface of a second helix (Figure 1a). The association is stabilized by van der Waals interactions resulting from intercalation of the Gly residue holes and large residue (e.g., Leu, Ile, and Val) knobs (*30*) and possibly also by weak hydrogen bonding between $C_\alpha H$ groups on one helix and the carbonyls of the neighboring helix (*31, 32*). Left-handed helix pairings can also be stabilized by van der Waals contacts across heptad repeats of small (*33*) or large residues such as Leu and Ile, with the latter exemplified by the "Leu zipper" assembly of phospholamban (Figure 1b) (*34, 35*).

TM PEPTIDE DESIGN AND PRODUCTION STRATEGIES

The high hydrophobic residue content and ∼20-residue length of membrane-embedded helices have been utilized to identify TM segments in primary sequences via hydropathy analysis (*1, 36*−*39*) and/or pattern recognition and matching methods (*40*−*43*). Once an appropriate sequence has been selected or designed, strategies as described below have been developed to produce the often highly hydrophobic TM mimic in sufficient quantity and suitable form for biophysical studies.

Polar Residue Tagging. Placement of charged and/or polar residues at the N- and/or C-terminal ends of hydrophobic sequences, termed "polar residue tagging", allows for facile synthesis, purification, and characterization of TM-type peptides that might otherwise be intractable. Peptides that incorporate multiple Lys residues at their N- and C-termini (termed "Lys-tagged") can be synthesized in milligram amounts, and the inclusion of the positively charged residues at peptide termini improves the aqueous solubility of peptides and minimizes aggregation (*44*). Terminal Lys residues have been shown to be noninterfering with many TM core sequences and typically leave intact the native self-assembly properties of a variety of TM segments both on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and in a variety of micelles (*45*−*49*). Their inclusion in some instances, however, may unmask inherent conformational instability and/or plasticity in helix−helix interactions (*50*) and may affect self-assembly in weakly interacting segments (*51, 52*).

Table 1: Experimentally Determined Single-TM Peptide Self-Association Motifs

motif/protein[a]	TM domain sequence[b]	oligomeric state[c]	ref
	Right-Handed (GG_4/GAS_{Right})		
GpA	^{72}EITLIIFGVMAGVIGTILLISYGIRRL^{98}[d,e]	dimer	*30*, *53*, *85*
MCP	^{25}AWAMVVIVGATIGIKLFKKF^{45}[d,e]	dimer	*86*, *87*
MPZ	^{125}YGVVLGAVIGGVLGVVLLLLLLFYVV^{150}[d,e]	dimer	*88*
ErbB-2	^{651}LTSIVSAVVGILLVVVLGVVFGILI^{675}[d,e]	dimer	*28*, *89*, *90*
BNIP3	^{164}VFLPSLLLSHLLAIGLGIYIGR^{185}[d,e]	dimer	*91*−*93*
VacA	^{2}FFTTVIIPAIVGGIATGTAVGTVSGLLGWGL^{32}[e]	≥dimer	*94*
NRP1	^{857}ILITIIAMSALGVLLGAVCGVVLY^{880}[d,e]	dimer	*95*
Syn TM2	^{145}VLAAVIAGGVIGFLFAIFLILLLVY^{169}[e]	dimer	*96*
Syn TM3	^{385}VLVAVIVGGVVGALFAAFLVTLLIY^{409}[e]		
Syn TM4	^{146}VLAALIVGGIVGILFAVFLILLLMY^{170}[e]		
EphA1	^{548}IVAVIFGLLLGAALLLGILVF^{568}[d,e]	dimer	*97*, *98*
VSV-G	^{453}FFIIGLIIGLFLV^{465}[e]	≥dimer	*99*
LS49 (de novo)	LTLLTHLSCLLGGLLTG[e]	≥dimer	*100*
RPTP DEP1	^{976}VICGAVFGCIFGALVIVTVGGFIFW^{1000}[e]	dimer	*101*
	Left-Handed (heptad/GAS_{Left})		
phospholamban	^{31}LFINFCLILICLLLICIIVMLL^{52}[e]	pentamer	*34*, *35*, *102*
M2	^{22}SSDPLVVAASIIGILHLILWILDRL^{46}[d,e]	tetramer	*27*, *45*, *103*, *104*
MS1-Gly (de novo)	QTLILGLLGLALGLIGLIVIARTLYLVG[d]	dimer	*33*
ANTXR1	^{318}ILAIALLILFLLLALALLWW^{337}[d,e]	≥dimer	*105*
Hsmr TM4	^{85}VAGVVGLALIVAGVVVLNVAS^{105}[d,e]	dimer, tetramer	*46*, *106*
SerZip (de novo)	QLFAASLLLFAVSLILFIASARLRYLAG[d]	dimer	*63*
	Polar[f]		
single polar (de novo)	QLLIAVLLLIAVN/D/Q/ELILLIAVARLYLVG[d] LLLLLLLN/D/Q/E/HLLLLLLLLLLLLLLL[d,e] LLLLLLLN/LLLLN/LLLN/LLLLLL[e]	dimer, trimer	*14*−*18*
FUT3	^{17}LAALLFQLLVAVCFFSYL^{34}[e]	≥dimer	*19*
TNF5	^{26}YLLTVFLITQMIGSLFAVYL^{46}[e]	≥dimer	*19*
TCR ζ-chain	^{28}DSKLCYLLDGILFIYGVILTALFLRVKFSR^{57}[e]	dimer	*21*
Tar TM1	^{7}VVTLLVMVLGVFALLQLISGSLFF^{30}[d,e]	dimer	*22*, *45*
Ser/Thr (de novo)	SxxSSxxT, SxxxSSxxT[e]	≥dimer	*23*
AI_{toxcat} (de novo)	AIAIAIIAZAXAIIAIAIAI, where Z = W, Y, or F and X = H, R, or K[e]	≥dimer	*24*
Ii	^{30}GALYTGFSILVTLLLAGQATTAYFLY^{55}[d,e]	trimer	*107*
RET	^{635}RTVIAAAVLFSFIVSVLLSAFCI^{657}[e]	≥dimer	*108*
EpsM	^{26}MGALTVLAIAYWGIWQ^{41}[e]	dimer	*25*
	Motif Undetermined		
DAGK TM2	^{31}VDAITRVLLISSVMLVMIVEILNSAI^{50}[d]	dimer	*79*
FXYD2	^{25}TVRKGGLIFAGLAFVVGLLILLSKRF^{50}[d]	≥dimer	*109*
SARS E	^{11}TGTLIVNSVLLFLAFVVFLLVTLAIL^{34}[d]	pentamer	*49*
E-cadherin	^{562}GILALLILILLLLLFL^{577}[e]	≥dimer	*110*
A_{2A}R TM5	^{174}MNYMVYFNFFACVLVPLLLMLGVYLR^{199}[d]	dimer	*111*
PsbF	^{18}RWLAVHTLAVPSVFFVGAIAAM^{39}[e]	dimer	*112*
AZ2-Wg (de novo)	WLAALLAWLAALLAWL[e]	≥dimer	*113*
GP Ibβ	^{149}GALAAQLALLGLGLLHALLLVLLL^{172}[e]	dimer	*56*
E5	^{8}LFLGLVAAMQLLLLLFLLLFFLVYWDH^{34}[d,e]	dimer	*47*
AI_{pep} (de novo)	FAIAIAIIAWAIAIIAIAIAI[d,e]	dimer	*66*
APP	^{700}GAIIGLMVGGVVIATVIVITLVML^{723}[d]	dimer	*114*
CPT-1A TM2	^{103}NIVSGVLFGTGLWVAVIMTM^{122}[d,e]	trimer, hexamer	*115*
FGFR3	^{376}VYAGILSYGVGFFLFILVVAAVTLC^{396}[d]	dimer	*52*

[a] GpA, human erythrocyte protein glycophorin A; MCP, bacteriophage M13 major coat protein; MPZ, myelin protein zero; ErbB-2, epidermal growth factor receptor tyrosine kinase 2; BNIP3, Bcl-2/19 kDa interacting protein 3; VacA, *Helicobacter pylori* vacuolating toxin; NRP1, neuropilin-1; Syn, syndecan; EphA1, erythropoietin-producing hepatocellular receptor A1; VSV-G, vesicular stomatitis virus G protein; RPTP, human receptor-like protein tyrosine phosphatase; M2, influenza A virus M2 proton channel; ANTXR1, anthrax toxin receptor 1; Hsmr, *Halobacterium salinarum* small multidrug resistance protein; FUT3, fucosyltransferase 3; TNF5, tumor necrosis factor 5/CD40 ligand; TCR, T-cell surface glycoprotein CD3; Tar, *Escherichia coli* aspartate chemoreceptor protein; Ii, class II MHC receptor Ii protein; RET, RET receptor tyrosine kinase; EpsM, cholera toxin secretion protein EpsM; DAGK, *E. coli* diacylglycerol kinase; FYXD2, Na^+/K^+ ATPase γ-subunit; SARS E, SARS coronavirus protein E; A_{2A}R, adenosine A_{2A} receptor; PsbF, *Synechocystis* cytochrome b_{559} subunit β; GP Ibβ, platelet glycoprotein Ib β chain; E5, bovine papillomavirus E5 protein; APP, Alzheimer precursor protein; CPT-1A, carnitine palmitoyltransferase 1A; FGFR3, fibroblast growth factor receptor 3. [b] The numbering indicates start and end sites of natural TM domains; de novo-designed TMs are not numbered. Residues known to stabilize self-assembly are underlined. [c] Ranges are given where exact stoichiometry is undetermined. [d] Peptide chemically synthesized by solid phase peptide synthesis. [e] Peptide produced via fusion/recombinant expression. [f] Polar motifs can mediate right- or left-handed packing.

Expression of TM Sequences as Fusion Proteins. An alternative production approach attaches the TM peptide sequence(s) of interest to a water-soluble protein to enhance aqueous solubility and facilitate purification from bacterial expression systems. For example, fusion of individual TM segments to the C-terminus of *Staphylococcal* nuclease, a monomeric water-soluble protein (*53*), allows the resulting chimera to be readily expressed and purified from *E. coli* and used to assess the stability and stoichiometry of TM domain contacts in vitro (*53*). Our group and others have also used fusions to the water-soluble protein thioredoxin to overexpress in *E. coli* and purify TM hairpins in milligram amounts (reviewed in ref *54*), where the thioredoxin moiety is cleaved from the TM protein before characterization. A variety of other fusion protein systems for recombinant expression of TM peptides have also been developed (see refs *21*, *55*, and *56* for examples).

Choice of Membrane Mimetic. For in vitro study, TM peptides must be solubilized and maintained in a membrane-mimetic medium. Various compounds (organic solvents,

detergent/surfactant or lipid micelles, and single-layer or multilamellar bilayers) are commonly used to mimic the native membrane environment of the TM sequence of interest. The ideal membrane mimetic should recapitulate the folding behavior native to the TM peptide in vivo. Choice of solubilizing agent in some cases depends on the biophysical or biochemical technique being applied (e.g., a requirement for micellar media or UV transparency); however, as TM peptide behavior may to be influenced by the choice of membrane mimetic medium (see ref *57* for a description), its selection is an important consideration in evaluating TM peptide behavior.

BIOPHYSICAL INVESTIGATION OF HELIX−HELIX INTERACTIONS

The ability to produce model TM segments and sequence variants in milligram amounts allows for the detailed biophysical characterization of their assembly by a variety of techniques. Five classical methods are commonly employed for structural characterization.

(i) Circular Dichroism Spectroscopy. The two-stage model predicts that, as an individual domain, each TM segment should retain its helical structure when excised from its native context as a TM peptide. This prediction is classically assessed via circular dichroism (CD) spectroscopy in the membrane mimetic used for study; helical structures display characteristic CD spectra in the far-UV region, with minima in mean residue ellipticity [θ] at 208 and 222 nm (vide infra).

(ii) Tryptophan Fluorescence. The fluorescence emission spectra of Trp residues can be used to indicate if the TM peptide is properly localized in the membrane mimetic phase, as this side chain typically exhibits a blue-shifted emission maximum and increased intensity upon partitioning of the peptide from an aqueous to apolar environment (ca. 350 nm to 330 nm).

(iii) SDS−PAGE of Transmembrane Peptides. This separation technique is one of the most commonly used methods in the evaluation of TM peptide−peptide interactions. Migration of TM peptides at apparent molecular weights (MWs) greater than ~2-fold their formula MWs on SDS−PAGE is often considered to be evidence of high-affinity self-association, and SDS−PAGE can provide an accurate estimate of the oligomeric state(s) of TM peptides in some cases. However, the migration of membrane interactive peptides and proteins on SDS−PAGE depends strongly on the level of bound detergent, such that TM peptides with large SDS aggregate stoichiometry may appear to run as dimers when in reality they may be monomeric (*58, 116*).

(iv) Sedimentation Equilibrium Analytical Ultracentrifugation. This technique can be used to directly measure the mass of TM peptides solubilized in micellar membrane mimetic media and thereby determine the stoichiometry and energetics of self-association (see refs *59* and *60* for comprehensive reviews). Ultracentrifugation can be applied in isolation but is sometimes utilized as a means of separating monomeric and oligomeric TM peptides in conjunction with thiol−disulfide interchange (TDI). The premise for TDI is that oligomeric TM peptides should self-cross-link (most commonly via native or engineered Cys residues) more readily than their monomeric forms, and this technique can be applied in micellar and bilayer systems (*61*−*63*).

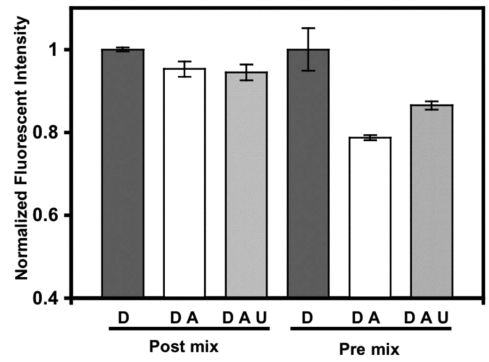

FIGURE 2: Peptide/detergent mixing order affects FRET of a de novo designed single-TM Ala/Ile-rich peptide (AI_{pep}). The AI_{pep} sequence is given in Table 1. The total fluorescence intensity of 1 μM dansyl-labeled peptide (donor) is shown in the presence of 1 μM unlabeled peptide (D), with 1 μM dabsyl-labeled peptide (acceptor, DA) or with 1 μM acceptor and 4 μM unlabeled peptide (DAU). Peptides were mixed together after each was individually solubilized in SDS (Post mix) or mixed in aqueous buffer prior to SDS addition (Pre mix). Fluorescence emission spectra were recorded from 450 to 600 nm, with excitation at 341 nm. Background fluorescence was subtracted. The fluorescence intensity was integrated between 450 and 600 nm and normalized to the fluorescence of the donor-only sample. The decrease in the fluorescence emission intensity of the donor upon addition of the acceptor indicates FRET between the two (DA). This decrease is greater with premix conditions then with postmix conditions ($p > 0.001$). With postmix conditions, there is no change in FRET between donor and acceptor fractions upon competition with unlabeled peptide (DAU; $p = 0.624$), indicating that there is no specific association between donor- and acceptor-labeled peptides. With premix conditions, there is a decrease in the level of FRET upon competition with unlabeled peptide ($p = 0.018$), indicating a specific association.

(v) Förster Resonance Energy Transfer (FRET). Here, fluorescence emission of a donor-labeled peptide is quenched and/or transferred to the acceptor molecule when the two are localized within a given radius (a property of the individual FRET pair and termed the Förster radius). This technique can be applied in membrane mimetic systems as well as intact bilayers (see refs *64* and *65*). However, FRET results must be interpreted carefully, as shown in Figure 2. We have observed that a de novo-designed TM peptide (AI_{pep}; see Table 1 for the sequence) known to oligomerize on SDS−PAGE and in bacterial membranes (*66*) reports differentially as a monomer or an oligomer via FRET in SDS micelles depending upon the mixing order of peptide and detergent. If donor-labeled and acceptor-labeled peptides are mixed from solutions that already contain SDS, specific energy transfer between the two populations is not observed, while peptide samples that are mixed prior to the addition of SDS display specific FRET that can be competed away by unlabeled peptide. These results imply that dissociation of the individual peptide−SDS complexes and/or dissociation and reassociation of TM−TM complexes in detergent have not reached equilibrium in the postmix case even after >16 h.

"MEMBRANE PROTEIN FOLDING PROBLEM": DEFINING HELIX−HELIX INTERACTION FACES

In the course of their function, membrane proteins must variously self-assemble into a monomer unit, interact with other proteins within the membrane, and/or build higher-order oligomers. How are such tertiary (folding of the

monomer) and quaternary (oligomerization of subunits) protein structures assembled and controlled in membranes? Recognition of the appropriate assembly sites for this process is complex for a TM dimer; the complexity involved in folding a seven-TM protein such as a G-protein-coupled receptor increases exponentially. Here we present several examples of how these phenomena may be investigated through the use of TM peptides.

Role of Flanking Residues in GG_4 Motif Assembly. The variety of proteins that utilize the GG_4 motif for self-assembly (Table 1) understandably exhibit a range of helix−helix interaction strengths needed for appropriate biological activity. To investigate what the sequence determinants of GG_4 motif affinity modulation could be, we studied two TM sequences that exhibit GG_4-based assembly, GpA and the Ff bacteriophage M13 major coat protein (MCP). Each of these TM segments engages their two Gly residues in right-handed interhelical contacts with crossing angles of ~40° (*67*). GpA versus MCP interaction strengths were initially assessed in the bacterial cytoplasmic membrane using the TOXCAT assay (*68*). This assay fuses the TM domain of interest at its N-terminus to the DNA binding domain of ToxR (a dimerization-dependent transcription factor) and at its C-terminus to maltose binding protein (a monomeric periplasmic anchor). Upon expression of the chimera in *E. coli*, self-association of the TM segment of interest results in the ToxR-driven activation of a reporter gene encoding chloramphenicol acetyltransferase (CAT), with the readout level of CAT expression indicating the strength of helix−helix interactions. As shown in Figure 3, GpA-wt and MCP-wt report different interaction affinities in the TOXCAT system. A hybrid sequence of the two (MCP-GpA) was additionally observed to approach, but not reach, the interaction strength of GpA in the TOXCAT assay (Figure 3b) (*19*), indicating the nuanced fine-tuning of association conferred by the helix interaction face as a whole, and highlighting that secondary factors affecting the TOXCAT signal such as expression level, linker length, and linker orientation can also influence apparent interaction strengths (*69*).

Helix−Helix Contacts in de Novo-Designed Hairpin Sequences. While single-TM peptide and chimeric fusion techniques use multimer formation between individual peptide sequences as a model for the helix−helix contacts, TM segments in polytopic (multispanning) membrane proteins are covalently connected by intervening loops that have been shown in studies of bacteriorhodopsin to modulate TM domain interactions (*70*−*74*). With a view toward addressing the influence of loops on helix−helix contacts, our laboratory has utilized helix−loop−helix (hairpin) constructs as the minimal "tertiary contact" model of two covalently linked helices (*75*). The dependence of helix self-assembly on neighboring TM sequences became evident in studies of a de novo-designed hairpin sequence composed of two identical model TM segments (sequence ^{1}KKKKKKK-^{8}FAIAIAI-IAWAXAIIAIAIAI^{28}-KSPGSK-IAIAIAIIAZAWAIIAIAIAF-KKKKKKK^{62}, where X is I or N and Z is I, N, or D), an Ala/Ile-rich design intended to promote van der Waals knobs-into-holes contacts (*66*). The single-TM helix building block of this hairpin [a Lys-tagged single-TM with residues 7−27 (X is I)] forms a strong dimer in isolation, but when the two single TMs are joined by the intervening KSPGSK polar loop, the TM-mimic sequences form intrahairpin van der Waals contacts and no intermolecular association is observed. This intramolecular association of helices was of such stability that inclusion of noninterfacial strongly polar residues (Asn, Asp, and Gln) did not "pull apart" the van der Waals interface of the hairpin but rather caused the assembly of a four-helix bundle composed of a dimer of hairpins (*66*).

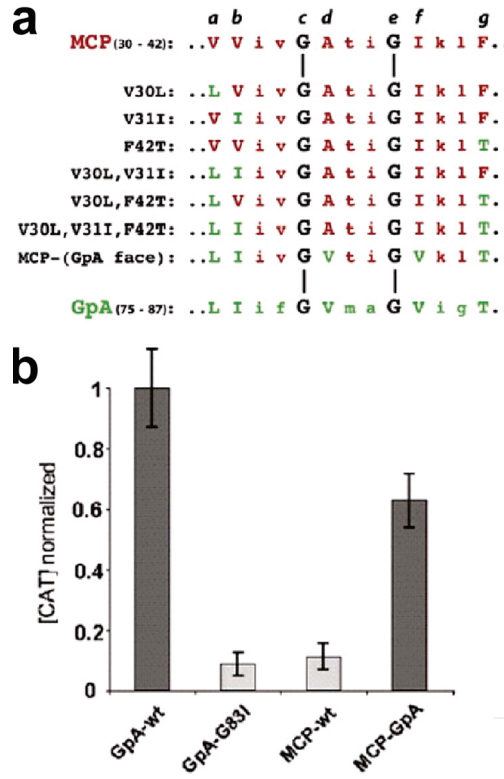

FIGURE 3: Residues that flank the GG_4 motif modulate dimer affinity in MCP vs GpA. (a) Alignment of MCP (red) and GpA (green) sequences studied in the TOXCAT assay. Residues at the dimer interface are indicated in uppercase. Note that the MCP-GpA sequence has each native interfacial residue of MCP substituted with the corresponding GpA residue. (b) Dimerization of GpA vs MCP sequences. All CAT expression levels are normalized to that of wild-type GpA. GpA-G83I is a monomeric mutant used as a negative control for dimerization. The interaction strength of the MCP sequence is significantly increased when all of its interfacial residues are changed to those of GpA (MCP-GpA), but this mutant does not recapitulate the interaction strength of wild-type GpA. Adapted from ref *67*.

Activity Modulation by TM Peptides. Even in cases where the precise residues mediating peptide self-association are not known (see Table 1 for examples), TM-mimic peptides can be used to modulate protein−protein interactions within the membrane. Signaling molecules [e.g., G-protein-coupled receptors (*76, 77*)] and the self-assembly/function of other proteins [e.g., integrin αIIbβ3 (*78*)] are susceptible to inhibition by externally added natural and/or de novo-designed TM peptide analogues. Specific inhibition of enzymatic activity of the homotrimeric *E. coli* diacylglycerol kinase (DAGK) by a TM segment analogue has also been demonstrated (*79*). A Lys-tagged peptide corresponding to the DAGK TM2 sequence (Table 1) was able to self-assemble and form heterodimers with full-length DAGK on PAGE and also inhibited the protein's activity in a dose-dependent manner, whereas peptides derived from TM1 and TM3 were benign toward DAGK assembly/function. Although poor aqueous solubility and in vivo degradation

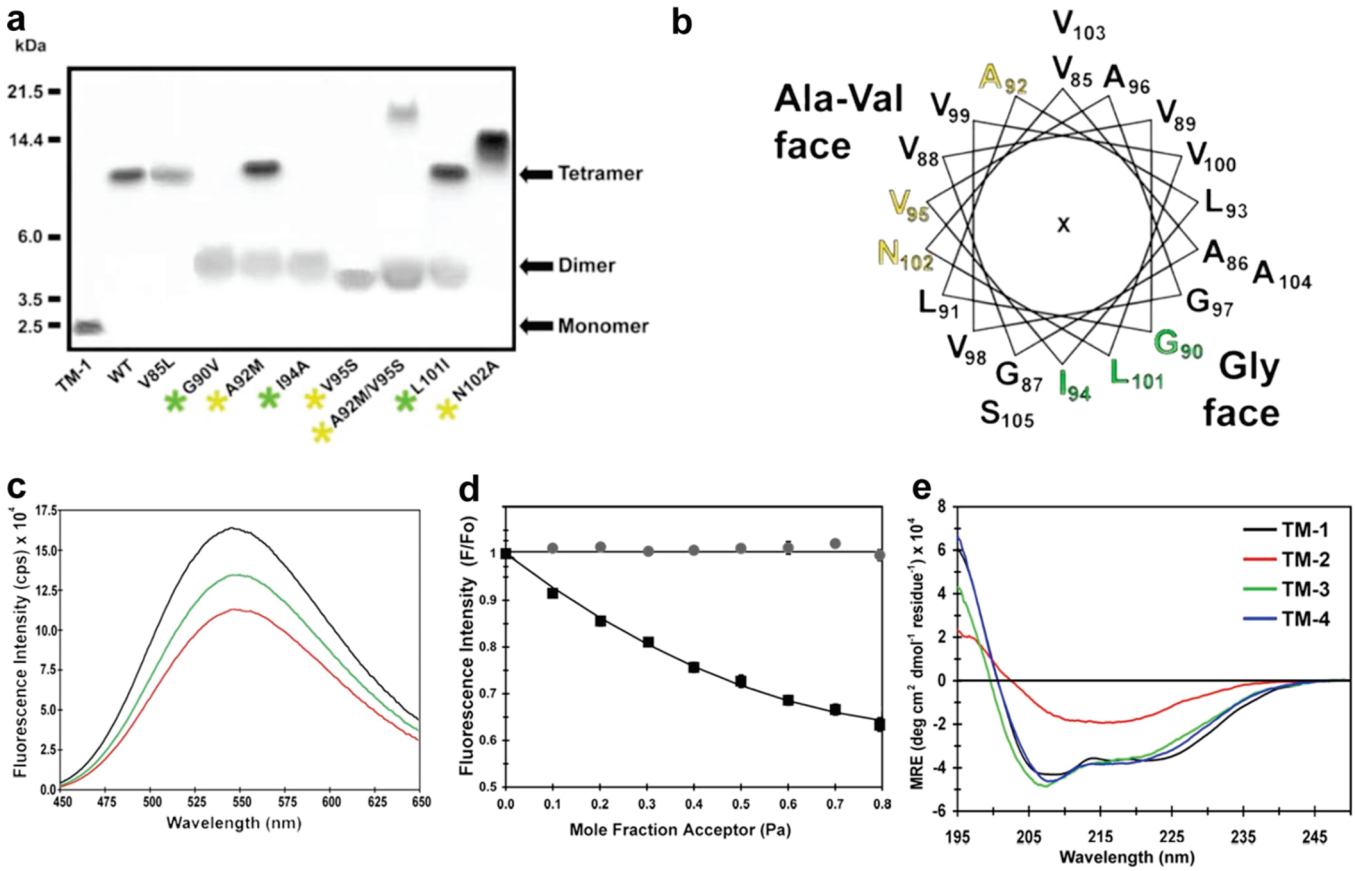

FIGURE 4: Self-assembly of Hsmr single-TM peptide TM4. See Table 1 for peptide sequences. (a) Electrophoretic separation of wild-type and mutant Hsmr TM4 peptides in SPFO demonstrates varying oligomeric states of the peptide library. Native TM4 (indicated as WT) migrates as a tetramer. (b) Residue replacements made on the Ala-Val and Gly faces of TM4, depicted in a helical wheel projection, are colored yellow and green, respectively. (c) FRET spectra of TM4 peptides in SDS micelles. Fluorescently tagged TM4 exhibits unquenched fluorescence when mixed in a 1:1 ratio with unlabeled peptide (black line); fluorescence reduction in a 1:1 mixture of fluorescently tagged TM4 peptide with a quencher-tagged TM4 peptide (red line) indicates that FRET has occurred and the two peptides self-associate. Upon addition of the unlabeled peptide, FRET is reduced in magnitude (green line), indicating that self-association is not an aggregative process. (d) FRET of TM peptides in SPFO micelles. Emission from a fluorescently tagged TM4 peptide is reduced as the mole fraction of quencher-tagged TM4 peptide increases (black squares); fits to this curve indicate a TM4 self-association stoichiometry of 3.2 ± 0.4. A corresponding TM1 donor/acceptor FRET pair exhibits no quenching (gray circles), indicating that the TM1 peptide is monomeric. (e) Hsmr TM peptides (except TM2) display helical circular dichroism (CD) spectra in SPFO. Adapted from ref *46*.

remain challenges in the therapeutic application of TM peptides, peptide-based drug molecules, which that may be synthesized using D-enantiomers to reduce degradation, have shown promise in animal models (*80, 81*).

Folding of Small Multidrug Resistance Proteins. The demonstrated utility of TM sequences to mimic native helix−helix interactions led us to examine the basis of assembly of small multidrug resistance proteins (SMRs), four-TM segment prokaryotic drug/proton antiporters that confer clinically significant resistance via their ability to efflux toxicants (reviewed in ref *82*). As relatively small proteins (ca. 110 residues), SMR monomers must self-assemble with at least dimeric stoichiometry to confer drug resistance. To discover the loci of assembly, four TM peptides corresponding to the individual TM segments of SMR family member Hsmr were synthesized and examined for their interactivity in micelles (*46*). Among these single-TM peptides, Hsmr TM4 (Table 1) formed discrete dimers in SDS and assembled into tetramers in the more permissive detergent sodium perfluorooctanoate (SPFO) (Figure 4a). This phenomenon is geometrically explicable only if two mutationally defined binding faces of the single-TM peptide participate in its self-assembly (Figure 4b). Association of such a "two-faced" TM4 peptide into a largely tetrameric form in SPFO (while TM1 remains monomeric) was supported by FRET experiments (Figure 4c,d), while CD spectra confirmed TM4 helicity in SPFO micelles (Figure 4e). This work suggests that disruption of TM4 interactions might provide the most readily accessible target for peptides that could inhibit SMR-based drug resistance in bacteria.

The geometric constraints in peptide assembly displayed by the TM4 peptides of Hsmr, particularly the notion of the utilization of two faces of a single-TM helix to build oligomeric structures, raised the possibility that certain geometric and sequence cues for self-regulated TM peptide assembly should be discerned in the extant membrane protein structural database (*83, 84*). By considering each TM segment as a rigid body (*83*), we found that (i) three classes of assembly describe all available membrane protein structures (Figure 5, top) and (ii) the proteins that sort into each class are distinguished by stoichiometry and sequence (see Figure 5, bottom, for examples). For example, type III structures have >2-fold higher stoichiometry than type II proteins, whereas type II structures have ~4-fold more TM segments per monomer unit. This research further showed that two of these types segregate

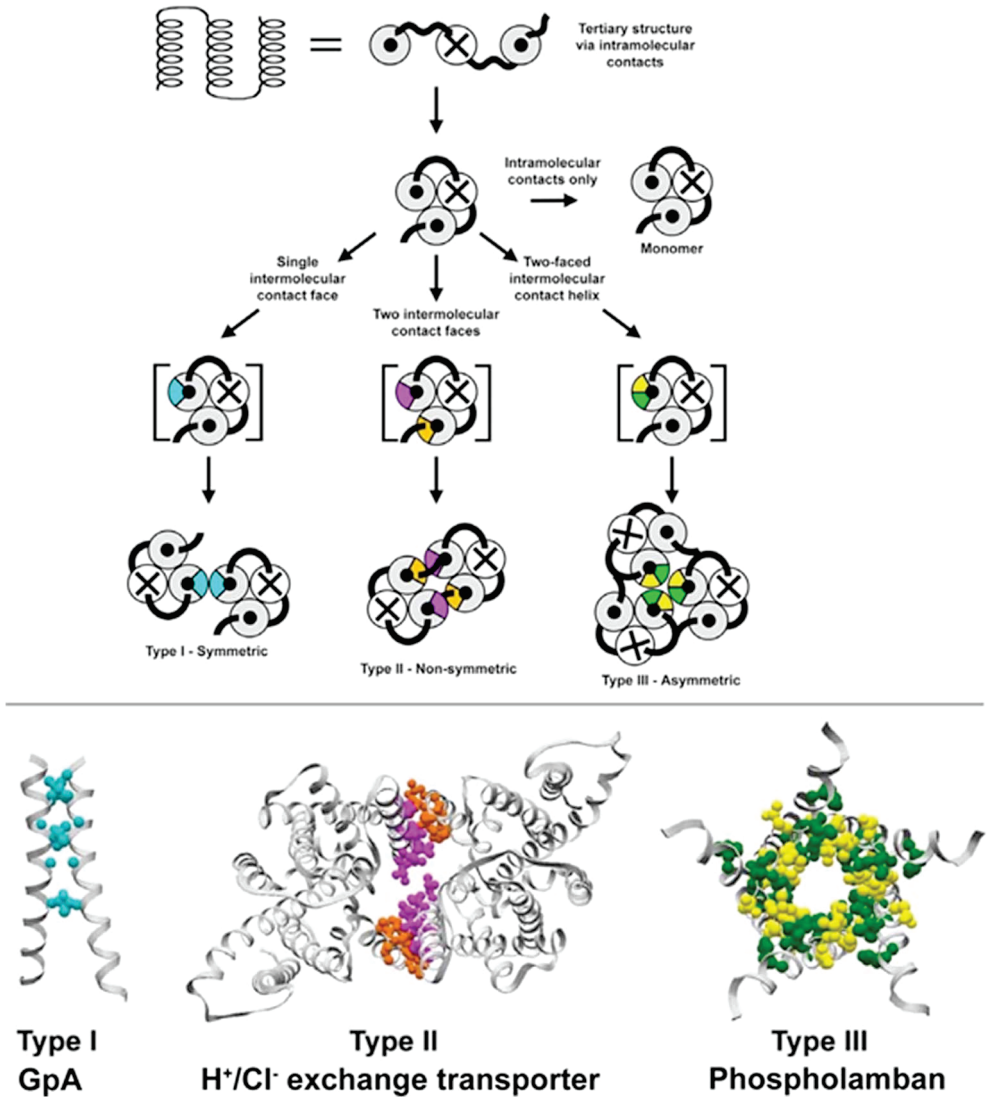

FIGURE 5: Three categories of helix−helix contacts describe membrane protein dimers and oligomers. In the top panel, assembly is diagrammed with a model three-helix bundle monomer, where circles represent end-on views of single-TM helices, connecting loops are shown as solid lines, dots (•) indicate a helix orientation with the C-terminus pointing out of the page, and crosses (×) indicate N- to C-terminal helix orientation with the N-terminus pointing toward the reader. Gray shading indicates helices with their C-termini toward the reader. Helix faces bridging chains are rendered in various colors. In type I (symmetric complexes), a single helix face (blue) contacts its counterpart on an equivalent/homologous helix. Type II structures (nonsymmetric complexes) assemble via contacts between faces (orange and purple) of two or more nonequivalent/nonidentical helices. In type III (asymmetric complexes), two distinct interactive surfaces (green and gold) found on the same equivalent helix make "two-faced" contacts such that each retains an interaction-competent face when matched with its counterpart in another monomer. In the bottom panel are examples of proteins assembled according to classification types I−III. Residues that mediate intermonomer contacts of each protein are color-coded to correspond to their type classification. Note that type III (asymmetric) corresponds to the "two-faced" assembly mode suggested to underlie Hsmr self-association. Adapted from ref *83*.

with protein function: Type II produces energy-dependent transporters, while type III builds channels for passive diffusion.

CONCLUSION

Production of individual TM sequences as peptides has greatly facilitated the study of the sequence determinants and interactions that stabilize membrane protein assembly. The advantages of these peptide models of membrane protein folding lie in their ability to replicate native helix−helix contacts, in their ability to be produced in sufficient quantities for biophysical analysis, and in their amenability to structural studies by a variety of experimental techniques. As shown here, TM peptides not only provide specific tools for probing the roles of protein TM domains but also constitute an attractive approach for the design of therapeutic membrane protein modulators. It should be noted, however, that the self-association behavior of a single-TM peptide excised from a polytopic membrane protein may not always reproduce its behavior in its native protein context. Despite this caveat, peptide models may still be successfully applied in experimental methodologies not readily accessible to full-length polytopic membrane proteins. In concert with the judicious design of peptide sequence and choice of appropriate membrane-mimetic media, the helix−helix interactions observed in these peptides may be expected to closely approximate the TM domain interactions in their native structures.

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