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Review

Helix formation and stability in membranes ☆

Matthew J. McKay[1], Fahmida Afrose[1], Roger E. Koeppe II, Denise V. Greathouse*

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, USA

ARTICLE INFO ABSTRACT

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In this article we review current understanding of basic principles for the folding of membrane proteins, focusing on the more abundant alpha-helical class. Membrane proteins, vital to many biological functions and implicated in numerous diseases, fold into their active conformations in the complex environment of the cell bilayer membrane. While many membrane proteins rely on the translocon and chaperone proteins to fold correctly, others can achieve their functional form in the absence of any translation apparatus or other aides. Nevertheless, the spontaneous folding process is not well understood at the molecular level. Recent findings suggest that helix fraying and loop formation may be important for overall structure, dynamics and regulation of function. Several types of membrane helices with ionizable amino acids change their topology with pH. Additionally we note that some peptides, including many that are rich in arginine, and a particular analogue of gramicidin, are able passively to translocate across cell membranes. The findings indicate that a final protein structure in a lipid-bilayer membrane is sequence-based, with lipids contributing to stability and regulation. While much progress has been made toward understanding the folding process for alpha-helical membrane proteins, it remains a work in progress. This article is part of a Special Issue entitled: Emergence of Complex Behavior in Biomembranes edited by Marjorie Longo.

1. Overview

Integral membrane proteins fold into their active conformations in a complex milieu dictated by the lipids of a bilayer cell membrane. Membrane proteins, estimated to make up 30% of the proteins encoded by the human genome, are vital to numerous biological functions, including signal transduction and the transport of ions and other molecules across cellular and organelle membranes. Membrane proteins are also implicated in many human diseases, and therefore are targets for over 50% of marketed drugs [1]. Although many membrane proteins rely on the translocon and chaperone proteins to fold correctly, others can be folded into a functional form in the absence of any translation apparatus or other aides [2]. Nevertheless, the spontaneous folding process is not well understood at the molecular level.

2. General features of alpha-helical membrane protein folding

A lipid membrane environment imposes a strict set of rules that must be followed by molecules residing within it. Proteins found in this environment must not only adhere to the requirements of the membrane, but also retain the ability to perform elaborate transport and signaling functions. The structures of membrane proteins therefore need to adapt accordingly to accommodate these demands. Generally, membrane proteins can be categorized into two classes: α-helix bundles and β-barrels. This review will focus on the more abundant α-helix bundle class of membrane proteins [3,4]. As the membrane milieu presents challenges for obtaining the structures of membrane proteins, structure prediction may provide a promising option [5,6]. To this end, establishing and understanding basic principles for folding functionally active proteins within the complex membrane environment can lead to more reliable methods for structure prediction.

The properties of lipid bilayer membranes have been studied extensively. Bilayers are composed of closely packed amphiphilic phospholipids and may include, in addition to the membrane proteins, other hydrophobic components such as cholesterol, sphingolipids, and cardiolipin. The polar head groups protect the acyl chains of phospholipids from water, while the hydrophobic interactions of the acyl chains stabilize the overall bilayer structure. The thickness of a bilayer may vary

Abbreviations: GWALP23, acetyl-GGALWLALALALALALALWLAGA-amide; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; pHLIP, pH driven low insertion peptides; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-snglycero-3-phospho-L-serine

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* Corresponding author.
E-mail address: dgreatho@uark.edu (D.V. Greathouse).
[1] These authors contributed equally.

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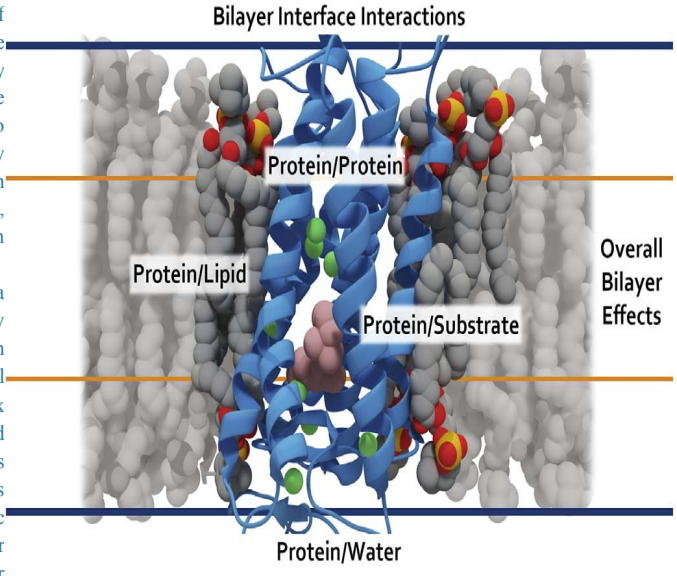
but is usually in the range of 25–35 Å [7]. The length of the core hydrophobic region of a transmembrane protein must match the thickness of the lipid membrane to avoid the unfavorable exposure of hydrophobic side chains to water. To minimize the free energy in the case of hydrophobic mismatch, the membrane may deform to accommodate hydrophobic protein segments or the protein itself may adjust its folding or orientation to match the bilayer thickness [8,9]. The polarity of the lipid head groups also varies. Many polar head groups require hydration, thus limiting the amount of available free water in the lipid head group region. The amphipathic membrane interface, therefore, encompasses a region capable of diverse interactions for stabilizing specific protein conformations.



The bilayer landscape changes from nonpolar to polar and then to bulk water over a very short distance, therefore the energetics of protein folding are likely to vary accordingly. Membrane proteins are equilibrium structures whose interactions with lipids can be described using experimentally available pathways [10]. Particularly useful is a four-step model for partitioning, folding, insertion and association of an alpha-helix into a lipid bilayer [10]. An unfolded polypeptide chain cannot easily exist in a lipid membrane due to the unfavorability of partitioning the backbone peptide amide bonds into the hydrophobic environment. The initial formation of the α-helical structure is largely controlled by this constraint and the favorable free energy of the hydrophobic effect [11,12]. The extent of the hydrophobic effect relies on dehydration of the nonpolar surface upon membrane entry. A nonpolar molecule partitioning within the bilayer interface is exposed to both polar and nonpolar regions and thus is never completely dehydrated. This effect reduces by half the free energy advantage offered by the hydrophobic effect at a lipid bilayer interface compared to that of, e.g., water-saturated octanol [11]. The energy cost of partitioning an unfolded peptide bond into the interface can be offset if hydrogen bonds form to stabilize a helical secondary structure [13]. The free energy advantage offered by partitioning a hydrogen bonded peptide backbone into the interface is estimated to be −0.4 kcal mol^{-1} per residue [14] and is the driving force for helix formation either before or concomitant with insertion into the bilayer interface. Hydrogen bond formation is consequently a primary alpha-helical stabilizing interaction. More deeply within the membrane, the lipid hydrocarbon core contains few water molecules, such that even within the context of backbone hydrogen bonding, the cost of dehydrating a peptide bond as it is partitioned into the hydrocarbon core of a lipid bilayer is unfavorable [15]. To overcome the cost of partitioning the backbone, transmembrane protein domains must contain primarily hydrophobic amino-acid side chains with favorable free energies of transfer. The folding problem associated with biological membrane proteins is even more complicated because the interactions of multiple membrane spanning α-helices, influenced by their amino acid sequences, must also be taken into account.

Transmembrane segments often can be identified from their sequences as long stretches of hydrophobic amino acids. These regions tend to form α-helices that may oligomerize into “bundles” linked together by flexible loop regions, which protrude from the membrane. The average length of transmembrane helices is 17.3 residues or 26 Å, just long enough to span the average bilayer's hydrophobic core region [16,17]. Once inserted into the membrane, numerous stabilizing physicochemical interactions come into play, as illustrated in broad terms in Fig. 1 [11,18]. Typical amino acids found in α-helical transmembrane domains include those with hydrophobic side chains, such as Leu, Ile, Met, Val and Phe, which are able to interact favorably with the lipid acyl chains in the core of the bilayer [19]. Alanine, while less hydrophobic, often is present in transmembrane sequences due to its high propensity for helix formation [20]. Another important feature of membrane proteins is the preferred association of aromatic amino acids at the interface [21,22]. Such interactions have been confirmed with transmembrane peptide models [11,23,24]. The presence of proline or glycine residues in transmembrane α-helices may lead to coiled or

Fig.1.Summaryofinteractionsthatstabilizefoldedmembraneproteinsinlipidbilayers.(Bluelinesrepresentinterfaceboundariesandorangelinesrepresenttheboundariesofthehydrophobiclipidcore.)Theproteinstructuredepictedisthatofrhodopsin(bluepolypeptide)at2.6Åresolution(PDBcode1L9H)withboundretinal(pink).Lipidhydrocarbonsaredepictedingrayandwatermoleculesareshownasgreenspheres.Source:Figureinspiredby[11].

distorted regions within helix [18,25,26]. Glycine (G) and other small residues such as alanine or serine can also be involved in helix-helix interactions. For example, the [GXXXG] motif creates a grooved region for the association of helices containing the same structural motif [27,28]. Polar amino acids, while less common, are often highly conserved when present in transmembrane domains. Residues with long side-chains such as arginine and lysine are capable of “snorkeling” in order to interact at the interface with the polar lipid headgroups and water [29,30]. This effect, together with bilayer deformation [31,32], is responsible for keeping the arginine-rich voltage-sensing domains of channel proteins in a transmembrane conformation [33,34]. Mutations that introduce polar residues into transmembrane domains often come with severe consequences, such as the oncogenic rNeu Val ➔ Glu mutation [35], highlighting how subtle changes in the amino acid sequence of a transmembrane helix can alter a protein's function and its interaction with the membrane.

The co-translational folding process for α-helical proteins typically involves membrane insertion via a translocon as the polypeptide elongates from the N to C terminus. Use of the translocon complex avoids the unfavorable thermodynamic consequences of exposing particular nonpolar domains to the aqueous environment [36]. The initial sequence of events results in the insertion and organization of α-helices across a lipid membrane and is the first stage in a “two-stage model” derived from Popot and Engelman's work with bacteriorhodopsin [37]. The protein's orientation can usually be determined by the “positiveinside” rule [38]. The second stage is then the formation of the three-dimensional protein structure, based on interactions among the already inserted transmembrane helices. A modified model adds a third step in which oligomerization and post-translational modifications may occur, or parts of the emerging polypeptide may preferentially associate with the membrane interface instead of the translocon. The latter would drive the partitioning of transmembrane helices into the bilayer by taking advantage of the free energy transfer between the aqueous and lipid environments [11,39]. Recent folding simulations of completely unfolded membrane proteins agree with both models [1]. Experimentally, the three-step model draws support from evidence showing the free energies of insertion are similar for both translocon-assisted and spontaneous transmembrane insertion [40]. Furthermore, recent studies have established that a lipid membrane by itself, in cell-free systems that are absent translocon components, is sufficient to allow the co-translational folding of several α-helical membrane proteins [41,42]. These findings demonstrate that membrane protein folding and insertion can be thermodynamically driven based on the amino acid sequence alone. Popot and Engelman [2] further elaborate on the

argument that while some transmembrane properties offer stability and regulation, even these may not be required for a membrane protein to achieve its basic tertiary structure. This concept is supported by findings [43] that membrane proteins are capable of folding and oligomerizing in synthetic polymers called "amphipols" which share few chemical and physical similarities with lipids. Consequently, while the final folded protein structure is sequence-based, the likely role for lipids is to offer stability and regulation.

3. Fraying of model transmembrane helices

Membrane proteins are among the most fascinating biomolecules; however, characterizing their structure in the complex heterogeneous environment of biological membranes presents numerous challenges. Many vital cell functions depend on properties of lipid bilayer membranes, such as their composition, fluidity and hydrophobic thickness. A wide range of interactions at the lipid/water interface, including hydrophobic, electrostatic, dipolar and hydrogen bonding interactions, influence and regulate the local conformations of membrane proteins. Changes in these interactions may cause membrane-embedded α-helices to alter their tilt, to turn, to shift position, or to unravel. In recent years, designed synthetic model peptides have established principles for examining transmembrane peptide and protein behavior [20]. The accumulated results tend to align with the folding behavior of natural proteins, leading to the extensive use of these systems for understanding the effect of amino acid identity, solvation and secondary interactions in the folding and unfolding process [44,45]. The model systems also allow specific changes in peptide sequence in addition to modifications to the lipid environment, widening the scope of protein-lipid interaction analysis.

Among the model systems, the WALP-like peptides [20] have proven to be useful for elucidating fundamental principles. In particular, the second-generation peptide GWALP23, formyl-GGALW $(LA)_6$LWLAGA-amide, [46,47] has proven to be a useful host framework for characterizing protein-lipid interactions. Notably, GWALP23 exhibits a well-defined tilted transmembrane orientation [48]. The magnitude of the GWALP23 helix tilt scales with the bilayer thickness,
[47] and the helix undergoes only modest dynamic averaging about a principal transmembrane orientation [49,50]. Interestingly, the presence of more than two interfacial Trp or Tyr residues tends to increase dramatically the extent of the motional averaging [50–52].

Comparisons among similar transmembrane helices with interfacial Trp, Tyr or Phe residues [51–53] have raised questions about factors other than interfacial aromatic residues that might help to define and stabilize particular orientations for neutral transmembrane helices lacking charged side chains. Experiments with deuterium labels on Ala residues near the ends of transmembrane helices have revealed that the fraying of helix terminals is commonly observed and may provide additional stabilization for particular helix orientations [54]. Indeed, when peptide bonds engage in H-bonding, the free energy of partitioning unsatisfied peptide backbone amide bonds to the membrane/ water interface is energetically favorable [14,55], and thus could be more important than interfacial tryptophan and tyrosine aromatic rings for defining helix orientation and dynamics. Particular combinations of side chains in turn could govern the extent of helix unwinding.

Alanines 3 and 21 of GWALP23, when deuterated, are particularly sensitive probes for helix fraying [54]. Located 18 residues apart, Ala3 and Ala21 will be separated by exactly five helical turns in a "perfect" α-helix [56]. Their geometric positions would be on top of one another and hence, regardless of the peptide tilt, their side chains would yield the same angle with respect to an external magnetic field and the quadrupolar splittings $|\Delta\nu_q|$ of their methyl group would be identical. Different magnitudes of the alanine 3 and 21 CD_3 quadrupolar splittings would therefore indicate the fraying or unwinding of one or both peptide ends beginning from that alanine or from some former residue [57]. Indeed, when this hypothesis is tested with the single-Trp peptides

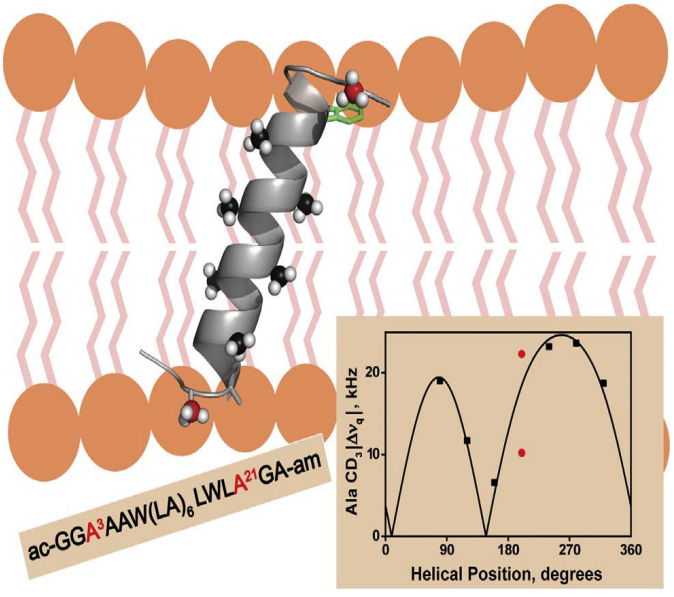


Fig. 2. Quadrupolar wave plot and model representation of helix unwinding of $A^{4,5}$GWALP23 in a lipid bilayer, including the sequence of the peptide. Labeled core (black) and terminal alanines (3 and 21; red) are shown as spheres in a model alpha helix and as squares or circles in a quadrupolar wave plot. Source: Redrawn from Chembiochem 2016,17,462–465.

$F^{4,5}$- and $A^{4,5}$GWALP23, the ^{2}H signals from the labeled Ala3 and Ala21 side chains show very different quadrupolar splitting values, which in turn markedly deviate from the quadrupolar wave plot for the core $F^{4,5}$ helix, implying the helix unwinding of both ends of -and $A^{4,5}$GWALP23 [54]. A model representation of $A^{4,5}$GWALP23 with frayed ends as well as the deviation of the alanine 3 and 21 quadrupolar splittings from the core helix quadrupolar wave plot is shown in Fig. 2. GWALP23 itself furthermore shows partial unwinding of two ends in DMPC and DOPC membranes [47,54]. Indeed, the helices are rarely ever intact from end to end, as the terminal fraying is becoming a generally observed property for isolated transmembrane helices [57].

Introduction of a single glutamic acid residue into the core of GWALP23 confers some interesting local variations in the helix integrity [57]. For example, the respective peptide terminals, A3 and A21, of GWALP23-E14 respond differently with pH variation. The ^{2}H quadrupolar splitting magnitude $|\Delta\nu_q|$ for the A3 side chain remains unchanged with pH, while that for the A21 side chain changes significantly. Additionally, a pH sensitivity is noted for the ^{2}H signal from A17, which is one helical turn above E14 [57]. These observations indicate major changes in the orientation and extent of unwinding of the C-terminal of GWALP23-E14 at high pH, but little influence on the N-terminal. As A17 is located on the adjacent helical turn of residue E14, its response to pH could be due to its proximity to E14.

When a Glu residue is present at position 16, some of the properties of GWALP23-E16 are similar to those of GWALP23-E14, although some differences are noted. In the case of E14, while residue A17 remains unwound from the helix core regardless of pH, E16 keeps that residue as part of the core helix up to pH 12. Above that pH, using ether lipids that are not subject to hydrolysis, A17 as well as A15 becomes unwound, likely reflecting the titration to ionize E16 [57]. So, the resulting shortened peptide with a long-unwound C-terminal portion (all the way to residue 15) embraces an orientation with decreased tilt when the aqueous pH surrounding the DLPC bilayers is above 12.

Residue 12 is effectively within a tryptophan indole "cage," on the same helix face as residues 5 and 19 (see below). Unlike GWALP23-E14 and -E16, that permit access of the Glu residue to the interfacial layer at high pH, GWALP23-E12 shows no inclination to change with pH. Rather, GWALP23-E12 prefers the same single transmembrane helical orientation, with modest fraying of residues 3 and 21, throughout the pH range [57].

A question remains as to what fundamental characteristics are responsible for unwinding that make the helix termini and even sometimes a portion of helix backbone vulnerable by exposing them to the

membrane-water interface? A possible explanation could be hydrogen bonding ability. Partial unwinding of a transmembrane helix would allow the peptide backbone to act as a stalk by providing an uncoiled segment with a large contact area to form hydrogen bonds with water molecules and lipid head groups at the membrane-water interface. A secure hydrogen bonding network among the backbone groups of unwound helix and polar lipid head groups would be energetically favorable [55]. This type of hydrogen bond, involving the unwound peptide terminal segment with three or more residues, can help to explain the high stability and low dynamics of a neutral helix such as $A^{4,5}$GWALP23 and related peptides. These peptides do not have any aromatic groups or candidate side chains, other than the indole ring of Trp 19, that can direct the core helix toward a particular tilt or azimuthal rotation, yet several such helices still maintain a well-defined single transmembrane orientation that is specific for each peptide se

$A^{4,5}$

quence and each lipid membrane [54]. As neither -nor $F^{4,5}$GWALP23 has any side chains with hydrogen bonding ability near the N-terminal, it is possible that hydrogen bonding between peptide backbone and lipid head groups or water is vital for defining the preferred tilted transmembrane orientation and limiting the global dynamics. The more complicated helix transitions observed with Glu residues at positions 14 or 16 in GWALP23 are possibly related to the ionization state of the Glu side chain.

GWALP23 family peptides are not the only class of peptide that exhibit helix end unwinding, suggesting that helix end fraying may be a more general feature of transmembrane helices. For example, the designed amphipathic antimicrobial peptide LAH4 also shows evidence of helix unwinding with different solvent systems and pH. Although the results vary with different conditions, the N and C-termini of LAH4 remain undetectable by 2D NMR spectroscopy in both DPC micellar solution and membrane-mimetic TFE-PBS solvent mixtures [58], which could be indicative of helix end fraying similar to GWALP23. In addition to the ends, there is evidence for an unstructured region in the center of LAH4, separating two helical domains, that converts to a continuous helix in a pH dependent-fashion.

Helix discontinuities, connecting loops and other inter-helical segments are suggested as important features that multi-span proteins such as G protein-coupled receptors may use to adjust to a surrounding membrane of differing hydrophobic thickness [59,60]. Significant unwinding in a cytoplasmic segment of opsin TM5, for example, moves Tyr 223, in the sequence motif of Px_3Ix_3Y, from an exposed position on the surface of rhodopsin into the interior of opsin. Three conserved residues of the opsin Px_3Ix_3Y motif, Pro215, Ile219 and Tyr223, are more aligned in the helical wheel after the unwinding of TM5, allowing for a new interaction between Arg135 and Tyr223 [61]. The unwinding of TM5 also modifies the positions of Gly224 and Gln225, forming a more regular α-helix in opsin compared with rhodopsin, and thus may play a crucial role in the characteristic elongation of opsin upon photoconversion [61]. Additionally, as part of a voltage response, a change in helicity is observed to be coupled to sodium channel gating. Namely, an unwinding of about seven residues from an approximately 25–30 residue long C-terminal domain helix appears to link with channel opening, with provision for an ion exit pathway [62,63]. These and other findings suggest that helix fraying, helix-coil transitions and loop formation, at the ends or middle of a helix and possibly H-bonded at the interface, are not passive but rather active and critical stabilizing factors that help each multi-span transmembrane protein adopt a defined resting geometry that is poised to respond to specific signals, as appropriate for particular biological functions.

4. Peptides exhibiting pH-dependent membrane topology

Polar and ionizable residues, when present in the α-helical core of membrane proteins, are often highly conserved and critical to function [64]. For instance, titratable histidines in transmembrane helices can exhibit pH-dependent behavior, thereby acting as pH “sensors” for the protein [65]. Indeed, a single conserved histidine in the influenza A virus M2 channel is responsible for channel activation [66] and proton-selective conductance [67]. In the T domain of diphtheria toxin A, the protonation of one histidine triggers a conformational change, leading to its translocation across the endosomal membrane, and a strategically located histidine in the aquaporin channel has been identified as a pH-modulated gate [68].

The insertion and folding of a protein into a membrane environment is a complex process where many details remain poorly understood at the molecular level (see above Section 2, General Features of Alpha-Helical Membrane Protein Folding). Regardless of the mechanism of insertion, the folding and stability of membrane proteins are governed by the formation of secondary structures on the surface or inside the bilayer that depend on a balance of hydrophobic, polar and electrostatic interactions [69]and hydrogen bonds [54,70]. When amino acids with ionizable side chains are present in the membrane domains of proteins, their pKa values often differ from the canonical values reported in aqueous solution [53,65,71–73]. Membrane-activepeptidescan serveasuseful modelsfor characterizing the effects of ionizable residues on the folding and insertion of proteins into membranes. Peptides with combinations of hydrophobic and ionizable residues may be unstructured in water, but upon interaction with membranes they often fold into α-helical conformations on the membrane surface [10,74]. Under specific conditions, such as a change in peptide concentration [74], a change in pH [65], or the addition of cholesterol [75], a helix may transition between a membrane surface/interface location and a transmembrane topology [76,77]. Here we review the properties of several model peptide-lipid systems that undergo changes in membrane topology with pH.

4.1. Histidine-rich LAH4 peptides

LAH4 peptides are a family of synthetic antimicrobial peptides, designed based on naturally occurring frog peptides such as PGLa and magainin, that adopt reversible pH-dependent surface and transmembrane alignments [69][78]. The LAH4 peptides also possess cell-penetrating properties, with an ability to transport cargo [79] (see below, Section 5, Crossing the Bilayer). The 26 amino acids of LAH4 ($KKALLALAL^{9}_{HH}LAHLALHLALALK^{24}_{KA}$) include four histidines strategically positioned among Leu and Ala residues to allow the peptide to fold into an amphipathic α-helix. Lysine residues were included at the N-and C-termini to ensure good water solubility and facilitate membrane anchoring [80–82]. In aqueous solution the LAH4 peptides are approximately 24% α-helix with ~22% β-structure. Upon binding to neutral POPC membranes, the helix content increases to 52%, and then to 88% in the presence of 25% anionic POPS. At high peptide concentration, above a peptide:lipid ratio of about 1:150, however, the conformational change to α-helix can be suppressed by peptide crowding [81].

NMR chemical shift changes of the imidazole protons measured in dodecylphosphocholine micelles have revealed average pKa values of 5.4–6.0 for the His side chains [69]. At low pH, LAH4 aligns on the bilayer surface with the positively charged His side chains located on one side of an amphipathic helix [58,78,81]. At intermediate pH (~6), a flexible hinge involving the H10–A13 sequence is thought to mediate membrane insertion [58,78]. Similar wedge-like bent structures have been reported for the highly lytic bee venom melittin [83] and the antimicrobial magainin peptides [84], and have been modeled as a voltage-gating mechanism of alamethicin [85]. Finally, at pH 7.8, when the His side chains have lost their charge, LAH4 adopts an α-helical transmembrane alignment encompassing residues L4–L21, with disordered N-and C-terminal residues 1–3 and 22–26 [58,69,78] (similar to the helix fraying noted above in Section 3).

4.2. pH driven low insertion (pHLIP) peptides

The36-residuepH-lowinsertion peptide(pHLIP) (GGEQNPIYWARY-ADWLFTTPLLLLDLALLVDADEGT), derived from bacteriorhodopsin helix

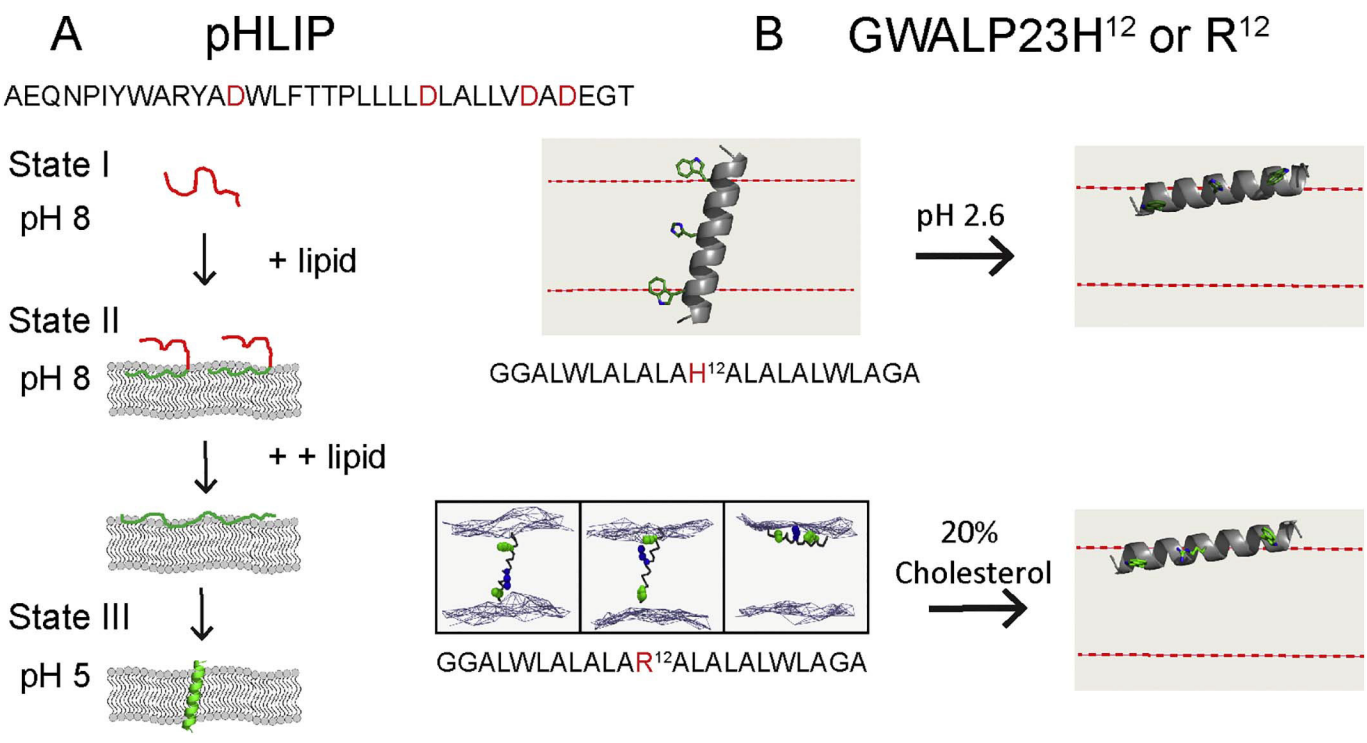


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Fig. 3. A) Schematic representation of pHLIP peptides at pH 8 in aqueous solution (top), and in the presence of a lipid membrane (bottom 3 panels). Above pH 8 in aqueous solution pHLIP is unstructured. With addition of a lipid membrane at pH 8 and at high peptide concentration (high peptide:lipid ratio), pHLIP partially adsorbs to the membrane interface. Upon addition of more lipid (lower peptide:lipid ratio) pHLIP fully adsorbs to the membrane interface in an extended conformation. Additional stages, for example a wedge-shaped α-helix in the interfacial head group region, may be involved between pH 7.0 and 6.4 (see text). Finally, as the pH is lowered to 5 and the Asp and Glu residues become protonated, pHLIP inserts into the bilayer as a transmembrane α-helix. B) Top Panel, Illustrations revealing changes in membrane topology of GWALP23-H12 in response to a change in pH in DOPC. Above pH 2.6 the uncharged peptide exists as a tilted transmembrane helix. Below 2.6, the His becomes protonated, causing the peptide to exit the bilayer and assume a surface bound orientation. B) Bottom Panel, GWALP23-R12^{+} exists in DOPC in 3 major states, two transmembrane with the charged R12^{+} side chain snorkeling toward either the C or N terminal ends, and a surface bound state. The addition of 10–20% cholesterol causes GWALP23-R12^{+} to exit the membrane to assume a single surface bound orientation like that of charged GWALP23-H12^{+}.

C, senses subtle changes in pH upon protonation of Asp and Glu residues [86,87]. In contrast to many typical amphipathic peptides, the binding of pHLIP to the membrane itself does not promote folding. Instead pHLIP forms a stable, unstructured conformation at a bilayer surface. Subsequent protonation of charged Asp residues increases the hydrophobicity, inducing a coil ➔ helix transition and peptide insertion into the membrane. The formation of an interfacial helical intermediate may reduce the free-energy penalty associated with the partitioning of the peptide backbone into the low dielectric environment of the bilayer [88]. pHLIP peptides furthermore may carry cargo for important biomedical applications [86,87,89] (see below, Section 5, Crossing the Bilayer).

At neutral pH, pHLIP is largely unstructured; nevertheless, upon sequential protonation of Asp31, Asp33, Asp25, and Asp14 (pKa values of 6.5, 6.3, 6.1, and 5.8) the equilibrium shifts toward a membrane-spanning α-helical conformation (Fig. 3A) [88,90][91][87]. At least three states have been defined for the folding and insertion of pHLIP into the membrane. State I, at low concentration (< 7 μM) in aqueous solution above pH 7.4, is an unstructured monomer [70]. State II is an unstructured monomer, adsorbed to the membrane surface through N-terminal interactions [92]. State III is a monomeric α-helix that partitions into the bilayer when the Asp and Glu residues become protonated below pH 6.0 [70,86,93]. Solid-state NMR measurements, of ^{13}C-labeled pHLIP between pH 6.4–7.0, reveal additionally a wedge-shaped α-helix embedded interfacially in the membrane head group region [87]. A kink at T19-P20 may cause dynamic perturbation of the outer leaflet of the membrane, thereby facilitating membrane disruption and/ or insertion, in a similar manner to the LAH4 peptides. Subsequent protonation resulting in neutralization of the remaining Asp residues 25 and 14 causes pHLIP to sink deeper into the membrane at pH 5.3, eventually parting the lipids of the inner leaflet to achieve full insertion of the membrane-spanning helix [87].

Time-resolved stopped-flow fluorescence and circular dichroism experiments [94] indicate that helix formation from the unstructured surface bound peptide (State II) begins within 1 s after a drop in pH from 8.0 to 4.0. This is followed 1500 times more slowly over the next 100 s by membrane insertion and helix maturation. The slow insertion process is due to the time required for the polar C-terminus to cross both leaflets of the membrane and for the lipids to reorganize around the transmembrane helix. As has been noted above for the LAH4 pep-tides, peptide crowding can lead to a "parking problem," inhibiting membrane adsorption and helix formation [86]. Notably, the distinctly different unfolding/exit helix-coil transition is about 400 times faster than the folding/insertion pathway [88].

4.3. Cationic GWALP23 peptides

Designed to traverse membranes as α-helices, GWALP23 peptides are second-generation "WALP" family peptides [20] characterized by having only two interfacial Trp residues, an alternating $(Leu\text{-}Ala)_n$ core, and blocked termini (acetyl-GGALW^{5}LALALALALALW^{19}LAGAamide) (see above Section 3, Fraying of Model Transmembrane Helices). In lipid bilayers, the core GWALP23 helix maintains one preferred and well-defined tilted transmembrane orientation with low dynamic averaging [48,49,95]. The helix tilt and dynamics are sensitive to changes in membrane environment, rendering the system an excellent framework for investigating the influence of guest residues on the properties of a well-characterized membrane-spanning helix. To this end, GWALP peptides have been used to compare the ionization behavior of arginine [32,75], lysine [53], and histidine [65] side chains in lipid-bilayer membranes. Substitution of Leu 12 or Leu 14 in GWALP23 with Arg, His or Lys produces a remarkable diversity of properties for the transmembrane helix. Notably residue 14 is on the opposite helix

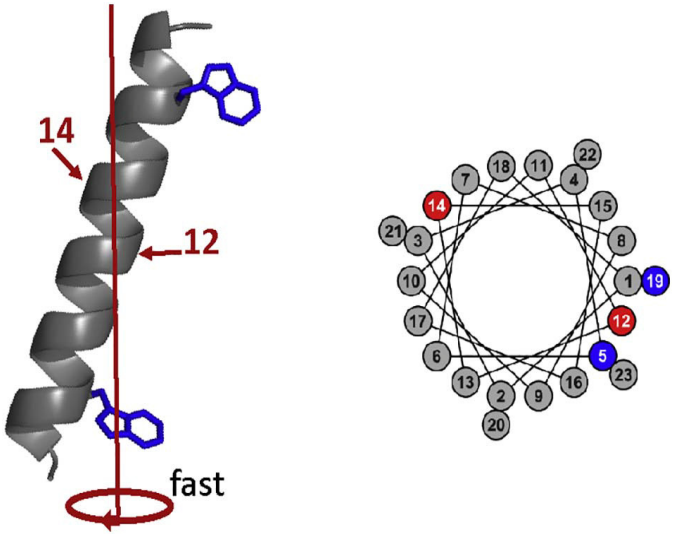

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Fig.4.PymolandhelicalwheelrepresentationsoftheGWALP23α-helix,showingthe"cage"formedbyTrp5and Trp19.Leu12resideswithinthecagewhereasLeu14doesnot.

face from tryptophans 5 and 19 (Fig. 4), whereas residue 12 is essentially "sandwiched" between the two tryptophan indole rings, seven residues away from each of them (Fig. 4). When Leu14 is replaced with Lys, Arg or His, although the transmembrane helix tilt (τ) and azimuthal rotation (ρ) may change in response to changes in pH, the conformation always remains transmembrane with single-state behavior. For example, when Leu14 is replaced with Arg14 in GWALP23R14, the transmembrane helix responds by increasing its tilt ~10° and by rotating ~75°, with the guanidinium group remaining charged from pH 2–13 [75]. Coarse-grained simulations furthermore suggest that the bilayer thins and the peptide helix about 3 Å is displaced to "lift" the charged guanidinium toward the bilayer surface [32]. By contrast, GWALP23-R12, with the arginine located between the Trp indole rings, exhibits multiple states in slow exchange on the NMR time scale. Coarse-grained molecular dynamics simulations also reveal three distinct α-helical orientations for GWALP23-R12, in agreement with experiment. While two of these orientations are transmembrane, the third population is a surface bound helix [32]. If the tryptophans are moved outward and to different radial positions, to open the Trp "cage" [96], then the GW^{3,21}ALP23-R12 helix does not seek the membrane surface. By contrast (Fig. 3B, Bottom panel), the addition of 10–20% cholesterol shifts the equilibrium so that ~85% of the GWALP23-R12 helices populate the surface-bound state [75]. The presence of 10–20% cholesterol, nevertheless, has minimal impact on the orientation of the GWALP-R14.

Lysine, in contrast to arginine, at position 12 or 14 of GWALP23, is observed to titrate within the DOPC membrane [53]. GWALP23-K14 responds to pH by changing its tilt and rotation. Three distinct orientations are observed, depending on whether the position-14 side chain is nonpolar (L14), neutral polar (K14^{0}), or charged (K14^{+}). In the case of GWALP-K12, with the lysine located between the two Trp indole rings, the peptide helix once again exhibits multiple states at low pH, similar to GWALP23-R12. When the pH is raised above 8.2, the K12 side chain titrates to release a proton and the helix adjusts by shifting to a single major tilted orientation that indeed is identical to that of GWALP23-L12. Therefore, at position 12 of GWALP 23, within the Trp indole "cage", it makes no difference whether a non-polar leucine or a neutral but polar lysine residue is present. The experimental pK_a values for the lysines in GWALP23-K14 and -K12 in DOPC bilayers, derived from solid-state ^{2}H NMR observables, are both below 7, approximately four units less than the value aqueous solution [53]. By contrast, the Arg pK_a has not been observed to shift in lipid bilayers, although a shift of four units "would" be observable if it occurred [65,75].

Similar results are observed when L14 or L12 in GWALP23 is substituted with histidine in DOPC bilayers. Notably, when the H12 side

chain is charged, below pH 2.6, the helix moves primarily (85%) to a surface orientation (Fig. 3B Top Panel), identical to that of GWALP23R12 in the presence of 10% cholesterol [65,75]. The His pK_a furthermore is observed to be sequence and bilayer position-dependent, with the magnitude of the change being about 2–3 pH units, namely somewhat less than the change observed for Lys when comparing the aqueous and bilayer environments [65]. The titration behavior of the His imidazole side chain, therefore, depends on its location within the helix and the membrane.

In summary, when a buried non-polar amino acid is replaced with a polar or charged residue in a transmembrane α-helix, the helix may respond, or the side chain may respond. The helix may adjust by changing its tilt, rotation or distribution of orientations, or by exiting the bilayer. The pK_a values for His and Lys side chains will be position-dependent and up to four pH units lower than in aqueous solution, although a titration of an arginine side chain has not been observed in a lipid bilayer. Instead, if located near the center of a transmembrane helix, the Arg guanidinium group will seek hydration by snorkeling as well as global helix translocation and rotation, or will exit the bilayer but it will not titrate to give up its proton. The noted interactions are important because amino acids with cationic side chains play central roles in many membrane proteins, including Arg residues in the sensor domains of voltage-activated channels [97–101] and His residues in cytochrome c oxidase [72,73], the influenza A M2 channel [66,102–104], and the transmembrane domain of the photosynthetic reaction center [105]. It is hoped that better understanding of the fundamental biophysics of lipid-protein interactions will facilitate better understanding of biological function.

5. Crossing the bilayer

While a lipid-bilayer membrane is often a barrier for proteins and peptides, occasionally some particular peptides are able to cross spontaneously, without facilitation. We discuss two examples of passive peptide transfer across a lipid bilayer.

5.1. Gramicidin

The peptide gramicidin A from Bacillus brevis [106], with sequence formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp9-D-Leu-L-Trp11-D-Leu-L-Trp13-D-Leu-L-Trp15-ethanolamine, forms trans-membrane channels that are selective for monovalent cations. The channels are dimers, consisting of two gramicidin (gA) subunits that assemble from within opposing leaflets of a lipid bilayer [107]and are held together by hydrogen bonding. The subunits are anchored by the four Trp residues due to the affinity of the Trp indole ring for the membrane/water interface (Fig. 5B). The interfacial affinity prevents individual subunits from crossing the membrane and requires that gA subunits be introduced to both sides of a lipid bilayer in order to facilitate channel formation [21].

Remarkably, when Trp residues 13 and 15 have been replaced with Phe, the subunits of the new gramicidin, designated [Phe13,15]gA, are able to cross lipid membranes with relative ease [108]. Indeed, [Phe13,15]gA forms membrane-spanning dimeric channels when added to only one side of diphytanoylphosphatidylcholine membranes [108]. How is this possible? What causes the significant change in molecular properties? Considerations of gramicidin conformational plasticity can address these questions. It turns out that gramicidin can adopt double-stranded as well as single-stranded β-helical conformations, as explained below. Apparently, then, a subtle interplay between different helical conformations governs not only channel formation but also membrane crossing.

Besides the single-stranded conformation of the subunits in the dimeric gramicidin channel (Fig. 5B and C), gramicidin A can fold into a variety of double-stranded conformations [109], particularly when dissolved in organic solvents [110,111]. One of these double-stranded

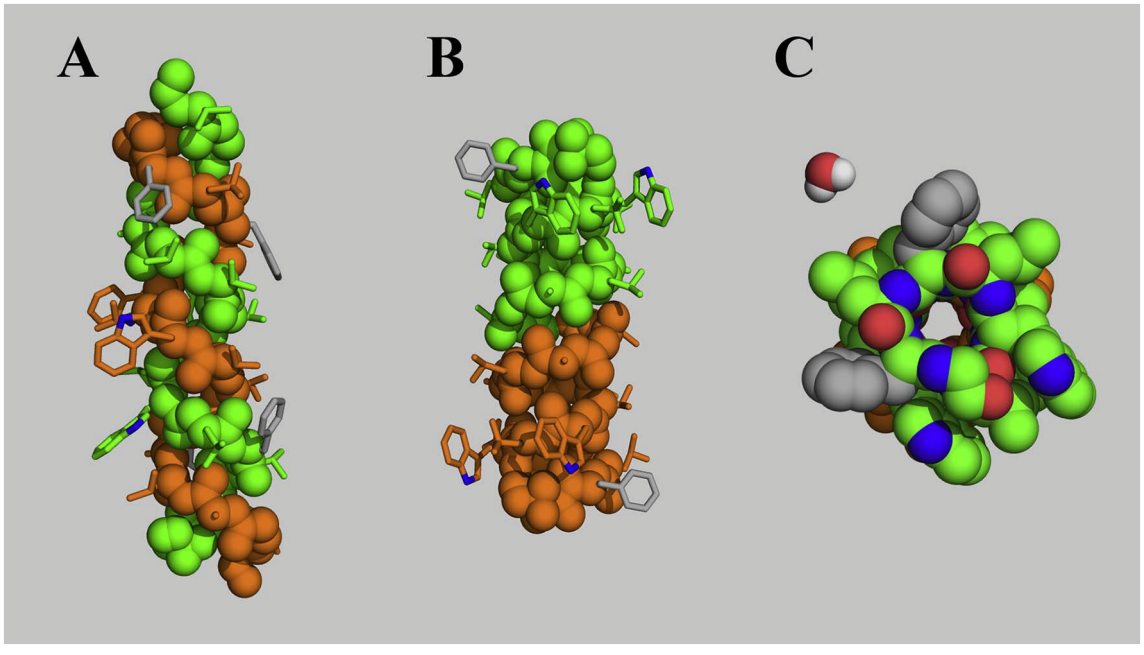

Fig. 5. Models for membrane crossing and channel conformations of [F[13,15]]gA. Subunit one is orange and subunit two is green. Hydrogen atoms are not shown. The side chains of F13 and F15 are gray, while the indole nitrogens of W9 and W11 are blue. To show the helical patterns, backbone atoms in panels A and B are depicted with arbitrarily small radii of 1.3 Å.
A. A double-stranded conformer is proposed to cross the bilayer. The model is based on structure 1ALZ from the Protein Data Bank [110], with arbitrary orientations for the substituted Phe side chains.
B. The channel conformation shown here was built from first principles [122] and is consistent with NMR data [123,124] and MD simulations [125,126], with arbitrary orientations for the substituted Phe side chains.
C. An end view of the channel conformation, rotated 90° from panel B, using full van der Waals radii, to illustrate the pore diameter with respect to the size of a water molecule. In this view, oxygens are red, nitrogens blue, and the phenyl rings remain gray. The indole rings of W9 and W11 "sandwich" the side chain of D-Leu-10 (lower right), consistent with NMR data [127].

conformations is depicted in Fig. 5A. In a lipid membrane environment, nevertheless, gA refolds into the single-stranded channel conformation [112,113].

The folding and conformational preferences for gramicidin subunits furthermore vary with the number and placement of Trp and Phe residues among positions 9, 11, 13 and 15 in the sequence [108,114]. Notably the [Phe13,15]gA population with tryptophans 9 and 11 is ~75% single-stranded, whereas the population of the [Phe9,11]gA isomer with tryptophans 13 and 15 is ~75% double-stranded [108]in vesicles of DMPC. The reasonable interpretation [108] is that a minor population (25%) of double-stranded [Phe13,15]gA (Fig. 5A) crosses bilayer membranes and then releases individual single-stranded subunits as the preferred (75%) conformers for forming channels as depicted in Fig. 5 B and C. We envision the dynamic sequential processes as membrane crossing by a double-stranded conformer (Fig. 5A), followed by subunit release and assembly of a channel from single-stranded subunits (Fig. 5B, C). Conversely, the subunits of [Phe9,11]gA do NOT form channels when added to only one side of a diphytanoyl-phosphatidylcholine bilayer [108]. Apparently the bias (~75%) in favor of double-stranded dimers is large enough to preclude channel formation under such conditions with the [Phe9,11]gA subunits (as the respective monomer population would be dilute under the conditions for channel detection).

Given the subtle differences in the relative positions of the interfacial Phe and Trp residues, the drastically different outcomes for the sequence isomer [Phe13,15]gA and [Phe9,11]gA subunits with respect to the coupled activities of membrane crossing and channel formation are striking.

5.2. Passive cell-penetrating peptides

A wide variety of peptides, many of which are arginine-rich peptides [115,116], are able to enter the interior of living cells in cell culture by means of either active or passive membrane translocation mechanisms [117]. Some of these peptides are able to translocate passively at low concentration, without endocytosis or significant membrane disruption. Notable examples are peptides such as PLIYLRLLRGQF [118] that spontaneously cross vesicle bilayers or eukaryotic cell membranes at concentrations below 2 mM and peptide:lipid ratios < 1:1000, without permeabilizing the membranes to other substances. The passive and essentially "silent" translocation occurs apparently without peptide clustering or significant membrane disruption [119]. The internal sequence within PLIYLRLLRGQF contains a hydrophobe(ϕ)-arginine motif, ϕRϕϕR, for example LRLLR, that also is evident and repeated in many voltage sensor helices [120]. Indeed, the ϕRϕϕR motif is likely to be responsible for the passive translocation. Nevertheless, unlike the case of [Phe13,15]gA (above), specific proposals for changes in the peptide folding or topology that may drive the peptide transfer are lacking at this time. Additional mechanistic understanding is needed.

Many other categories of arginine-rich "cell penetrating" peptides can enter cells by a variety of other active, endocytotic or membrane disruptive mechanisms [115–117] that vary from case to case and will not be discussed here. Indeed, the rather sparse examples of peptides that spontaneously and passively cross lipid bilayer membranes at low concentration – peptides such as PLIYLRLLRGQF and [Phe13,15]gA – are remarkable.

6. Perspective

The assembly of membrane proteins is vital for numerous biological functions. Key steps involve the insertion of protein domains into lipid membranes, the formation of hydrogen-bonded alpha and beta secondary structures, and the association of transmembrane segments into the final folded proteins. Although an understanding of the entire assembly process is improving, it yet remains a work in progress. The interfacial and juxta-membrane loops that connect adjacent or

consecutive transmembrane motifs should not be ignored, as they may be important for overall structure, dynamics and regulation of function. Changes in the integrity and extent of helices sometimes link to a membrane crossing or the onset or termination of a specific biological function.

Looking forward, there exist notable opportunities for addressing computational predictions, biological function and tissue targeting. Cases where helix-coil transitions might be crucial for biological function, such as the gating of sodium channels [63], will be further characterized. The promising characteristic of pHLIP to target metastases as well as primary tumor tissue, due to the inherent acidity of cancer cells, can be increasingly developed to advance medical treatment [121]. Helix unwinding or helix-coil transitions present challenges for molecular dynamics simulations, as the time scales are difficult for all-atom simulations while, on the other hand, coarse-grain methods often impose a secondary structure and may not examine alternate backbone conformations. Advancements in methods to crystallize membrane proteins in native-like environments, combined with molecular simulations using experimentally derived structures, nevertheless, will allow the prediction of molecular functional properties within increasingly complex and native-like lipid-protein systems [6].

Conflict of interest

The authors declare no conflict of interest.

Transparency document

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