The role of tryptophan side chains in membrane protein anchoring and hydrophobic mismatch

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Tryptophan (Trp) is abundant in membrane proteins, preferentially residing near the lipid–water interface where it is thought to play a significant anchoring role. Using a total of 3 μs of molecular dynamics simulations for a library of hydrophobic WALP-like peptides, a long poly-Leu α-helix, and the methyl-indole analog, we explore the thermodynamics of the Trp movement in membranes that governs the stability and orientation of transmembrane protein segments. We examine the dominant hydrogen-bonding interactions between the Trp and lipid carbonyl and phosphate moieties, cation–π interactions to lipid choline moieties, and elucidate the contributions to the thermodynamics that serve to localize the Trp, by ~4 kcal/mol, near the membrane glycerol backbone region. We show a striking similarity between the free energy to move an isolated Trp side chain to that found from a wide range of WALP peptides, suggesting that the location of this side chain is nearly independent of the host transmembrane segment. Our calculations provide quantitative measures that explain Trp’s role as a modulator of responses to hydrophobic mismatch, providing a deeper understanding of how lipid composition may control a range of membrane active peptides and proteins.

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1. Introduction

Membrane proteins are vital for a wide spectrum of cellular functions [1–3], making them highly significant from a pharmacological perspective [3,4]. Membrane protein stability and function can be highly sensitive to membrane composition [5–7], owing to the interplay between the hydrophobic transmembrane (TM) protein segments and the host lipid membrane, as well as specific interactions between lipid moieties and protein residues that may anchor the protein in the membrane. In the accompanying study [8], we have explored the possible responses and energetics associated with hydrophobic mismatch [9,10]. In this study we explore the roles of tryptophan (Trp), commonly found to interact with the membrane interface, in stabilizing membrane proteins and contributing to hydrophobic mismatch responses.

Our goal is to provide fundamental understanding of how the interactions between the membrane and protein can control activity, using model protein segments that may reveal common mechanisms. Due to the unusual abundance of Trp in membrane proteins [11,12], existing inside the membrane near the membrane–water interface [13–15], it is widely accepted that these amino acids play an important role as membrane anchors [13,16–19]. This non-random preference [20,21] is evident, for example, in photosynthetic reaction centers [12], porins [22,23], ion channels [24] (e.g. the prototypical gramicidin A, gA [25,26]), helix-bundle proteins such as cytochrome c oxidase [27] and a range of other membrane proteins [20]. Trp has been found to play important roles in protein folding [28,29] and in the structures and activities of different peptides and proteins. e.g. gA [30–32], the HepG2 glucose transporter [33], ACh receptors [34,35], the outer membrane protein OmpA [36], and the human anti-HIV antibody 4E10 [37].

For membrane proteins, an important factor governing protein stability and function is the hydrophobic mismatch that arises from a difference in the hydrophobic length of the TM segment and the thickness of the surrounding lipid bilayer [9,17,38,39]. Hydrophobic mismatch can lead to structural perturbations [8,17] such as helix tilting [40] and membrane thickness changes [41–43]. These perturbations have associated energetic costs that control stability and regulate conformational changes in the TM domains of proteins. In the accompanying study, the Trp residues have been observed to modulate the responses to hydrophobic mismatch [8], in a series of Trp-Ala-Leu peptides (based on the common model WALP peptides) [44] with a varying number of Trp side chains, as well as the gA peptide, in dipalmitoylphosphatidylcholine (DPPC) lipid bilayers. In that study, peptides were observed to increase their tilt (and, to a lesser extent, stretch and bend) when positively mismatched to the bilayer, with a corresponding small increase in the local membrane thickness, while negatively matched peptides led to a significant thinning of the
lipid bilayer. Responses were seen to be sensitive to the number and placement of the Trp side chains on the peptide, especially for the tilt of positively mismatched peptides, arising from asymmetry in the placement of the Trp side chains around the helical axis and the multiple Trp–lipid interactions. The influence of flanking side chains on mismatch response has also been observed experimentally by Killian and co-workers [16]. They observed that the distance between two flanking Trp residues can sometimes affect the mismatch response more than the hydrophobic length of the peptide [16,45] and that even those Trps that are furthermost from the interfacial region can control mismatch response [45]. These computational and experimental evidences support the case that the Trp side chains play a significant role in modulating helix tilt and membrane deformations.

Experimental measures of the partitioning of amino acid side chains from the water to the bilayer interface [46], suggests that Trp is the most interfacially associated amino acid side chain (by ~2 kcal/mol), supported by NMR studies that observe Trp side chains residing within the glycerol backbone region [14,47]. The propensity of Trp to localize around the bilayer interface explains its role as an interfacial anchor [8,15,16], and has been attributed to the size and shape of the indole moiety [14,48–50], hydrogen (H)-bonding between the indole donor and lipid carbonyl acceptor [13,30,47,51–53], the interaction between the indole dipole moment and the bilayer electric field [30,48] and the aromaticity of the indole ring that gives rise to cation–π interactions [34,47,51,54,55]. The interfacial preference of Trp has been suggested previously based on molecular dynamics (MD) simulations of Trp analog molecules [48,50,56]. Though acknowledging sampling limitations, Grossfield and Woolf [56] found a 10 kcal/mol minimum in free energy at ~10 Å from the bilayer center for indole. Unbiased histograms of indole in a lipid bilayer have suggested binding by 4.3 kcal/mol at ~15 Å from the bilayer center [48], while more recent Umbrella Sampling [57] simulations have suggested a 5.3 kcal/mol minimum at 12 Å [50]. Although considerable variation exists between these studies, it is clear that the indole moiety is interfacially active. In this present study, we carry out free energy calculations covering a range of different Trp side chain analogs and TM segment models: a free analog (3-methylindole); Trp attached to the middle of a long translationally-invariant α-helix that spans the bilayer; and as anchoring side chains within a series of TM WALP-like peptides, similar to those used experimentally to study protein–lipid interactions [48,50,56,58,59]. In total we report observations from ~3 μs of simulation, with a comparison of different models allowing us to understand the anchoring property of the Trp side chains and our observations of modulated hydrophobic mismatch response, with widespread implications for membrane protein structural and functional studies.

2. Computational methods

2.1. Molecular dynamics models

MD simulations were used to calculate the free energy profiles for moving a Trp side chain across a DPPC bilayer using 3 different series of model systems, illustrated in Fig. 1: 1) a series of 20 different WALPs [44] (Trp Ala Leu peptides) or WALP-like peptides (collectively referred to as WALPs in this paper) [8]; 2) a long (93-residue) poly-leucine α-helix with one central Trp at residue 47 (TRP-HELIX), similar to that used previously to explore the thermodynamics of charged amino acid movement in lipid membranes [58]; and 3) the free Trp analog 3-methylindole (3-MIND). Each system is composed of the peptide or peptide analog, and a fully hydrated DPPC bilayer. The free energy profile for the series of WALP peptides was calculated from 1 μs of unbiased histograms of the z-position of the center of mass of the Trp side chain relative to the center of mass (COM) of the lipid bilayer, while those for the TRP-HELIX and 3-MIND systems were calculated...
from biased distributions using Umbrella Sampling [57], as explained below.

The CHARMM program [60] was used for this study employing the PARAM27 force field [61–63] including CMAP dihedral cross-term corrections for proteins [64]. The waters were modeled using TIP3P parameters [65] and all bonds to hydrogen atoms were maintained with the SHAKE algorithm [66]. The cutoff for constructing the non-bond pair list was 16 Å and the real-space cutoff for Lennard-Jones was 10 Å. The particle-mesh Ewald (PME) method was used to calculate electrostatics [67]. Simulations were performed under constant temperature (300 K) and normal pressure (1.0 atm) conditions using Nosé–Hoover methods [68,69] and the Langevin piston [70], respectively. The temperature was chosen to ensure lipids were in the fluid phase.

2.2. Structural and interaction analyses

Membrane thickness perturbations were calculated as the difference between the average bilayer thickness in the inner shell (defined to include the number of lipids within the first minimum of the radial distribution function for lipid carbonyl oxygen atoms around peptide COM) and the average bilayer thickness in the outer shells of lipids [8], referred to as membrane deflection.

Trp solvation by oxygen atoms of water, lipid phosphate and carbonyl groups was calculated around the indole N–H hydrogen, including the number of oxygen atoms within 2.8 Å of the indole H atom based on a radial distribution analysis. For counting the number of solvating choline groups, a radius of 7 Å was used [51] for the nitrogen around the COM of the indole group. The number of H-bonds formed between the indole N–H hydrogen and the oxygens of water, phosphate and carbonyl moieties was determined using a radial cutoff of 3.4 Å and an angle cutoff of 60°. For cation–π interactions, a radial cutoff of 7 Å around the indole COM to the choline nitrogen was used together with an angle cutoff of 60°, as in Ref. [50], with the angle defined between the indole COM–choline nitrogen vector and the normal to the indole group.

To avoid any truncation of interactions between the Trp side chain and the components of the lipid bilayer, all interaction energies were computed based on the energy difference between the complex (e.g. indole + carbonyl) and the interacting fragments (e.g. indole and carbonyl) using the same non-bonded method as used in MD simulations (i.e. with PME electrostatics). The carbonyl selection was the glycerol ester moiety, while for the phosphate, the dimethylene-phosphate moiety was used.

2.3. Free energy calculations

The potential of mean force (PMF), or free energy profile, \( W(z) \), representing the reversible work to move the Trp side chain along the coordinate, \( z \), has been computed for each system. For the PMF of moving a Trp side chain analog (3-MIND) across the membrane, the chosen order parameter was the position of the molecular COM along the \( z \) axis parallel to the membrane normal, relative to the COM of the lipid bilayer. For the WALP systems, the same definition was applied. In the case of the TRP-HELIX model, the coordinate chosen was the COM of the entire helix, relative to the membrane, and this approximately corresponds to the position of the \( \beta \)-carbon of the Trp side chain.

For the TRP-HELIX and 3-MIND systems, Umbrella Sampling [57] was employed to ensure thorough sampling along a range of \( z \) completely spanning the bilayer, reaching bulk reference positions where the indole ring did not interact with lipids, as detailed above. Distributions of \( z \) were unbiased using the Weighted Histogram Analysis Method (WHAM) [73]. For both the TRP-HELIX and 3-MIND systems, a biasing harmonic potential of 2.5 kcal/mol/Å\(^2\) was used to hold the COM of the helix or analog at the desired depth.

In the case of the WALP peptides, the Trp side chain PMF as a function of its COM \( z \)-position (relative to the bilayer), was computed from a total of 1 ps of unbiased trajectories from 20 independent WALP simulations. Using the average unbiased histograms, \( \rho(z) \), from all the WALP simulations, the PMF was calculated via, \( W(z) = -k_B T \ln \rho(z) + C \), where \( k_B \) is Boltzmann’s constant, \( T \) is the temperature, and \( C \) is a constant. Due to the vast trajectories available for these models, biasing was not required to sample the reaction coordinate. The influence of the host peptides on the Trp distribution in the bilayer is expected, and is the motivation for this analysis. However, the Trp positions near the center of the bilayer, or far outside the interface in solution, correspond to rare events on highly mismatched peptides, as reflected by increased error bars in those regions.
The relationship between the PMF and the mean force allows for the decomposition of the reversible work into contributions from different interactions (\(\alpha\)),

\[
W_\alpha(z) = - \int_z^{z_0} d\zeta F_\alpha(\zeta) \tag{1}
\]

where \(F_\alpha(\zeta)\) is the instantaneous force on the helix or Trp COM due to the component, \(\alpha\). Such a decomposition of reversible work was carried out for 3-MIND and the WALP systems by an analysis of the mean forces as a function of position.

3. Results and discussion

3.1. Membrane deformations and hydrophobic mismatch

In the accompanying paper [8], we have described the various hydrophobic mismatch responses that occur when a TM segment interacts with the surrounding bilayer, and the role the number and position of the Trp side chains play in that response. Fig. 2a, based on that study, shows the average tilt of the different WALP-like peptides and how it is affected by the peptide hydrophobic length and number of the Trp residues. The general trend is that tilting will increase as the mismatch increases. Increasing the number of Trps does not significantly affect the tilt of short peptides, but for longer peptides (hydrophobic lengths 25.5–31.5 Å), Trp clearly exerts its influence.

3.1.1. WALPs

Fig. 2b shows the average membrane deflection (local thickening/thinning) of the bilayer caused by the WALPs, with large negative deflections occurring for short peptides, and smaller positive deflections for long peptides, reduced due to helix tilting [8]. Varying the number of Trps has a most profound effect on bilayer perturbation for the negatively mismatched peptides. In this regime, the \((W_3)_2\) and \((W_4)_2\) peptides (i.e. flanked by 3 or 4 Trps at each end) appear to have a different influence compared to the \((W_1)_2\) and \((W_2)_2\) peptides (flanked by 1 or 2 Trps at each end). This can be attributed to the fact that \((W_3)_2\) and \((W_4)_2\) peptides exhibit a more symmetric arrangement of the Trp side chains around the helical axis, while for \((W_1)_2\) and \((W_2)_2\), the Trps at each end exist on different faces of the helical surface. Furthermore, the peptides with more flanking Trp residues also result in less membrane deflection due to the spreading of the Trps over a greater length of the peptide [8]. Clearly the Trps play an important modulating role in mismatch perturbations.

3.1.2. Single side chains (TRP-HELIX and 3-MIND)

Because there is an interplay between the effects of hydrophobic mismatch and the anchoring effects of Trp, we now examine the 3-MIND and TRP-HELIX models to isolate the effects of the Trp side chains themselves on the membrane. Compared to the WALPs, the 3-MIND and TRP-HELIX systems show reduced membrane deflections (only of the order of 1 Å) as the Trp side chain enters and moves across the bilayer (see Fig. 3a and b). We can make some observations that can help to explain this reduced membrane perturbation.

When the single side chain crosses into the membrane core it eventually loses contact with the glycerol backbone and lipid head groups and leads to a loss of bilayer perturbation. Even in the case of the short WALP peptides, the membrane remains perturbed around the highly mismatched peptide and the indole stays coordinated by interfacial lipid moieties. We also note a second difference between these models, which is that the WALPs deform two leaflets of the bilayer simultaneously, leading to possibly double the...
membrane deflection. However, it is clear that the overall perturbative power of a WALP (even a singly-Trp flanked WALP), with its hydrophobic mismatch, is several times greater than that of a single Trp side chain. We remark that, in the case of the TRP-HELIX model, the long helix spans membrane and water and leads to some meniscus formation that may influence the inward motion of the lipid head groups. Whereas this effect was less evident in previous studies of a highly perturbative Arg side chain [58,74], it may cause artifacts for studies of Trp–interface interactions, compared to our 3-MIND analog model, although the membrane perturbations remain comparable.

### 3.2. Trp position and orientation on TM helices

The comparison of an isolated Trp and a short WALP peptide with a single flanking Trp is already a strong evidence that membrane perturbations are not determined solely by the Trp anchors. The fact that the lipid deflections around the WALP peptides are primarily governed by mismatch to the hydrophobic segment, with the number of Trp side chains only modulating the response, further demonstrates this fact. However, this may also be associated with the distribution of Trps around the helix and interfacial spreading. Another difference between the TRP-HELIX and WALP simulations, is the effect of the host peptide in determining the preferred orientation of the indole, that could influence the pulling strength of the side chain on the membrane interface.

#### 3.2.1. WALPs

Fig. 4 shows the rotameric states visited by the Trp side chain during the course of simulation of the WALP systems, sampling all allowed rotamers (e.g. see energy maps in Fig. S1a and c for a Trp located near the C- and N-terminals, respectively) during the microsecond combined trajectory. Rotamers 1, 2, 5 and 6 correspond to orientations where the plane of the indole ring is approximately parallel to the membrane normal with the N–H bond for 1 and 6 pointing towards the interface, while 3 and 4 are orientations where the plane of the aromatic ring is somewhat perpendicular to the membrane normal.

#### 3.2.2. TRP-HELIX

Fig. 5 shows the dihedral angle distribution of the Trp side chain for the TRP-HELIX system in four general regions: in bulk water (±30–35 Å), the phosphate/choline region (±18–23 Å), the carbonyl region (±13–17 Å) and the bilayer center (0±3 Å), based approximately on the distributions of bilayer components for a DPPC lipid bilayer [75]. It can be seen that, while sampling is fairly thorough, rotamers 1 and 2 are not visited by the Trp side chain of the TRP-HELIX system and it visits only the rotamers shown in Fig. S1b for a Trp in the middle of a helix. The absence of two side chain rotamers is likely due to steric blocking of these rotamers for a Trp in the middle of the helix.
To provide a more direct comparison of the TRP-HELIX and WALP observations, Fig. S2 shows the time evolution of rotameric states visited by the Trps of the \((W1)_2\) series of the WALP-like peptides only (i.e. those peptides with only one flanking residue; Trp 2 and Trp \((n-1)\), where \(n\) is the number of residues). The simulations for the WALP peptides were started with the Trp 2 in rotamer 1 and Trp \((n-1)\) in rotamer 3 (which would be outwardly directed at the N terminal in the bottom leaflet). Except for Trp 2 of \((LA)_8\)L and \((LA)_{10}\)L, the Trp side chains visited similar rotameric states to that observed for the Trp in the TRP-HELIX model. With \((LA)_8\)L and \((LA)_{10}\)L, being longer, the closer proximity of the Trp side chains to the interface likely made it more favorable for them to retain their original orientations.

The Trp orientation relative to the membrane z-axis for the TRP-HELIX model is shown in Fig. 6a. The orientation is described by the cosine of the angle that the indole dipole (defined from the indole nitrogen in the five-membered ring to C-5 in the six-membered ring \([30]\)) makes with the membrane normal. In the bulk water region (± 30–35 Å), the indole ring has an average cost of approximately 0, indicating a fairly random distribution of orientations (as opposed to being orthogonal to the membrane normal), because there is little pull from the interface in this region. As the indole ring enters the interface (headgroup region, ±19–23 Å; and glycerol backbone region, ±13–17 Å) the Trp side chain becomes oriented (with an angle approaching ~40° from the membrane normal in each leaflet). This preferential orientation of the indole ring is consistent with experimental evidence that it would tend to be directed parallel to the membrane normal when in the interfacial region \([47]\). Within ~5 Å from the bilayer center, as interactions with the head group components weaken, the indole dipole starts to approach a more random distribution once again. We note from the error bars in Fig. 6a, that the indole fluctuates extensively, regardless of its position, despite the pull by the interface that affects its average orientation.

\[\cos(\theta) = \frac{\text{dipole component parallel to normal}}{\text{dipole magnitude}}\]

Fig. 6. a) Indole ring orientation for the TRP-HELIX system characterized by the angle that the indole dipole makes with the membrane normal. Error bars represent the standard deviation. Snapshots of the interaction of the indole ring with different lipid head group components: b) H-bonding with carbonyl oxygen, c) H-bonding with phosphate oxygen and d) cation–π interaction. The carbon atoms are in gray, nitrogen atoms are in blue, hydrogens in white, oxygens in red and phosphorus in orange.

3.3. The interactions of the flanking Trp side chains of the WALP peptides with lipids

Lipids may interact with Trp in several ways, as illustrated in Fig. 6b, c and d. This includes H-bonding between the indole N–H hydrogen and the carbonyl (Fig. 6b) and phosphate oxygens (Fig. 6c), and the cation–π interaction of the aromatic ring with the choline group of the PC lipid (Fig. 6d, although cation–π to a PC lipid is expected to be weak \([51]\)), as well as the interactions with the lipid hydrocarbon tails (not shown). In addition, it is anticipated that there will be a hydrophobic driving force created by water pushing the bulky indole group into the membrane core (i.e. due to the greater cost of cavity formation in water than in hydrocarbon). We begin here by reporting the interactions encountered by the Trp side chains in the WALP systems, before analyzing the isolated Trp models.

Fig. 7 (left) shows the coordination (a), H-bonding or cation–π interaction (b) and energy of interaction (c) of the Trp side chains of the WALP systems with the bilayers. At the outer reaches of the interface, Trp interacts with both interfacial water and the lipid choline moiety. The choline groups are the lipid moieties that reside, on average, furthermost from the bilayer center \([75]\) and may interact with the indole via charge–dipole interaction, as well as through a weak cation–π interaction \([51,76]\), though which may play a greater role in phosphatidylethanolamine bilayers \([51]\). The Trp is in contact with ~1.5 choline moieties on average in this outer region of the bilayer (Fig. 7a, green curve), and experiences up to ~7 kcal/mol attractive interactions with that group (Fig. 7c, green curve). Of these choline groups, almost all can be characterized to be in a cation–π interaction with the indole ring. As the side chain enters deeper into the membrane interface, the attractive interaction with choline becomes repulsive, owing to the direction of the indole dipole when inside the membrane (Fig. 6a).

When the Trp side chain resides deeper into the interface, it encounters the oxygen atoms of the phosphate, with which H-bonding can occur. We observe in Fig. 7a (magenta curve) that ~0.2 phosphate oxygen atoms coordinate the indole N–H on average and, in Fig. 7b we observe that essentially all of these oxygen atoms were H-bonding. Surprisingly, the coordination by phosphates persists deep into the membrane for the WALP peptides, with up to ~10 kcal/mol interactions inside the core (Fig. 7c). We will demonstrate in the following section that this is due to the membrane deformations associated with the negatively mismatched WALP peptides.

For the Trp residing deeper inside the membrane, interactions with carbonyl groups begin to occur, with up to ~0.2 carbonyl oxygens coordinating the indole N–H (Fig. 7a, blue curve), which are almost exclusively H-bonding (Fig. 7b, blue curve). The extent of H-bonding to the indole by the glycerol backbone carbonyls is
comparable to the phosphate H-bonding, suggesting potentially significant contributions from both lipid moieties in directing the Trp side chain to the interface, whereas previous studies have reported H-bonding only to the lipid carbonyls [51, 76]. In fact, Fig. 7c (magenta and blue curves) shows that the strength of the interaction with the phosphates is greater than that with the lipid carbonyls (by a factor of two; $-10$ vs. $-5$ kcal/mol). However, such an analysis maybe misleading because it neglects the choline contribution to the indole–zwitterion interaction which would be almost canceling. From the sum of the phosphate and choline interactions (black dashed line) it can be observed that only in the outer regions of the bilayer do both phosphate and choline provide attractive interactions and this suggests that the head groups may play a role in attracting Trp to the interface from the bulk water, whereas lipid carbonyls may play a greater role in stabilizing Trp within the membrane (see also free energy calculations below).

The Trp side chain remains well hydrated when in the outer regions of the membrane interface, with the N–H hydrogen surrounded by ~1 water molecule on average (Fig. 7a, red curve) forming up to 0.9 H-bonds (Fig. 7b, red curve), compared to 1.0 in the bulk water.

Inside the interface, water competes with phosphates, (and with lipid carbonyls deeper into the interface) for H-bonding, explaining a drop to around 0.5 H-bonds to water there; though the extent of H-bonding to water is greater than that to lipid glycerol and phosphate moieties as a result of the comparative ease of reorienting water molecules. The interaction energy between the indole and the water gradually weakens from around $-12$ kcal/mol to zero at the bilayer center (Fig. 7c, red curve). However, as noted previously [56, 76], the water interactions remain significant deep into the bilayer core, being of the order of $-5$ kcal/mol in the glycerol backbone region. In the region close to the bilayer center, the interactions with the hydrophobic lipid tails (gray curve) become predominant due to the loss of interactions with water and the head groups.

The right panel of Fig. 7 (Fig. 7d, e, f) shows the solvation number, H-bonds and interaction energies for just the (W1)$_2$ series (the singly-flanked WALPs). This comparison allows us to judge how multiple flanking side chains influence how Trp side chains anchor to the membrane. The results are broadly similar to those in Fig. 7a, b and c, with the main difference being the reduced range sampled by the Trps on these peptides, not exploring the outer reaches of the membrane.
bilayer–water interface, or deep inside the membrane core. We do observe an increase in the interaction with the phosphate groups in the absence of competition with other Trp side chains, yet all other interaction energies are consistent with the calculations for the complete set of WALP peptides. These results suggest that the Trps interact with the bilayer in a manner that appears to be fairly independent of the details of the host peptide, which we now explore further.

3.4. The role of the host helix in Trp–membrane interactions

To reveal the role of the host helix, we need to isolate the interactions of the membrane with the Trp side chain itself. We now report solvation, H-bond and interaction analyses for a single Trp side chain on a long α-helix (TRP-HELIX model shown in Fig. 1c; Fig. 8, left) and an isolated Trp side chain analog (3-MIND shown in Fig. 1b; Fig. 8, right).

The features of the curves in Fig. 8 generally reproduce those analyzed for the WALP systems, but with differences that allow us to resolve the roles played by the side chain and the presence of a helix. The comparison of a Trp on a long host helix (Fig. 8, left) serves the purpose of eliminating the contributions from helix tilting and membrane deformations owing to the hydrophobic matching of the WALP peptide. Comparison to the isolated analog (Fig. 8, right) reveals the inherent solvating and membrane perturbative effects of the side chain itself, without the restrictions that the host helix places on the side chain.

We focus on the comparison between the WALP peptides that possess a single flanking Trp side chain, (W₁)₂ (Fig. 7, right), without the complications of multiple Trp effects. We observe a range in lipid solvation of the Trp side chains within the (W₁)₂ peptides extending deeper into the bilayer, compared to the isolated Trp models (Fig. 8). For these WALP peptides, the finite TM segment deforms the membrane such that the Trp side chains always reside interfacially (with random tilting and bobbing of the peptides allowing a broad range of positions to be sampled while interacting with the interfacial lipid components). In contrast, the isolated side chain passes through the membrane interface into the membrane core and loses contact with the interface. As a result, the solvation of the Trp side chain in the TRP-HELIX or 3-MIND models, is closer to what one might anticipate based on the distribution of lipid components in an unperturbed bilayer [75], where phosphate and choline groups contribute only near the interfacial region, carbonyls in the glycerol backbone region,

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**Fig. 8.** Mean first-shell Trp solvation numbers (a and d), H-bonding of the indole N–H hydrogen (b and e) and interaction energies (c and f) of the indole with lipid carbonyls, phosphates, tails and water are shown for the TRP-HELIX (left) and 3-MIND (right) models.
and dehydration is more rapid as the Trp side chain is immersed in the hydrocarbon core. In contrast, membrane deformations around the WALPs, owing to the mismatch of peptides, lead to a penetration of interfacial components deeper into the membrane core. In fact, carbonyl and phosphate oxygen solvation on the \((W_1)_2\) peptides occurs 6–8 Å closer to the bilayer center than for the single Trp on a long helix.

A host helix may limit access to solvating species, restrict conformational freedom and, as suggested above, may have membrane-perturbative effects that influence the solvation of the side chain. We observe from Fig. 8 (TRP-HELIX, a,b and c, compared to 3-MIND, d, e and f) that the solvation, H-bonding/cation–π and interaction energies are reduced due to the presence of the host helix, and with that by the carbonyl oxygens and choline groups being particularly affected. We notice that the lipid phosphates contribute to solvation deeper inside the membrane for the 3-MIND model compared to the TRP-HELIX, presumably due to the absence of an imposed direction, which is the case for the Trp side chain by the helix. We also observe a reduction in solvation by lipid chains due to the presence of the helix (compared in Fig. S3). Yet the overall similarity in solvation and interaction analyses points to a consistent mechanism of Trp anchoring that exists regardless of the host helix.

3.5. Work done by lipid components in moving the Trp side chains within the membrane

Interaction energies do not solely govern stability as these are not free energies. We have calculated the reversible work associated with side chain movements within the bilayer, with mean force decompositions helping to better describe the roles of bilayer components in controlling side chain anchoring. We first describe the contributions for the isolated side chain and then how they are influenced by the host peptides.

3.5.1. 3-MIND

For the isolated side chain analog (Fig. 9a), in moving from the bulk water towards the interface of the bilayer, we see competing forces. Upon entering the membrane interface, water (red curve) presents a barrier of ~4 kcal/mol as a penalty for leaving the bulk water and entering a region of aligned water molecules that stabilize the lipid head groups. The increase in energy associated with Trp disrupting the favorable water and head group interactions leads to a force that expels the side chain from the interface, into the hydrocarbon region, illustrating the hydrophobic effect on this bulky side chain. Once inside the membrane core (at around 10 Å from the bilayer center), the force from water molecules vanishes, leading to a plateau in the water contribution to the free energy, representing a net drive from water to the membrane core of around \(-10\) kcal/mol.

Competing with this hydrophobic driving force are the head group interactions, a magnified view of which is shown in Fig. 9b. The phosphate (magenta curve) and choline (green curve) groups together (sum of phosphate and choline contributions not shown) serve to pull the indole ring into the bilayer interface from the bulk water, by around \(-3\) kcal/mol, countering much of the 4 kcal/mol penalty imposed by the interfacial water molecules there. Carbonyl groups (blue curve) exert little force in the interfacial region. However, when the Trp side chain moves deeper into the membrane, all interfacial lipid components become unfavorable, especially in the glycerol...
backbone region with carbonyl groups pulling the indole group back to the interface. Whereas the force from the carbonyls extends to near the bilayer center, the pull by choline and phosphate groups is shorter-ranged and disappears by z~15 and 7 Å, respectively. Overall, however, the drive to the membrane core is dominated by attractive work contributions due to water and lipid hydrocarbon tails (hydrophobic effect). The net result of all interactions (black curve in Fig. 9a), is a binding inside the interface, near ±10 Å from the bilayer center, by ~5 kcal/mol. This position of the minimum is not surprising, given the fact that the indole has a tendency to reside within the hydrocarbon core, as well as to H-bond with polar glycerol and interfacial components [48]. This depth of the minimum is similar, but stronger than the experimental partitioning of 3-methylindole between water and cyclohexane (~2.3 kcal/mol [77]), owing to attractive interfacial interactions. The breadth of the minima is surprising, as it extends across much of each leaflet. This suggests that while the Trp side chains are favored in the glycerol backbone region, they may explore much of the bilayer and thus do not provide a tight anchoring that may, for instance, be expected to hold highly polar segments in the membrane.

3.5.2. WALPs

The force decomposition for the WALP systems (Fig. 9c) is quantitatively different, owing to the maintenance of Trp-head group and Trp-glycerol carbonyl interactions for these TM helices. This decomposition was calculated from the unbiased simulations of all the WALP-like peptides, with the Trp positions extending only to ±22.5 Å. The drive of the Trp side chain from water (red curve) is again evident, but since the Trp side chains always maintained contact with the interfacial lipid components, no “entrance” barrier is observed. This partly explains the larger work contribution due to the water compared to the isolated Trp side chain model in Fig. 9a, because the reference (zero) free energy is defined at the interface for the WALP systems, not in the bulk water. Overall a similar picture of the work decomposition is seen for the WALP systems, governed by a balance of hydrophobic effect and interfacial binding, but the magnitudes appear to be greater, especially due to the lipid head groups and water. We attribute this to the fact that interfacial components remain in contact with the Trp side chains, experiencing stronger forces of interaction, while also extending deeper into the bilayer, integrating to give larger work contributions. The sum of all work contributions (black curve) follows the same shape as for the 3-MIND model, importantly with a similar binding location (despite the influence of the host peptide), driven by a balance of the hydrophobic and interfacial binding contributions. We note that a quantitative comparison of the sums of all membrane work contributions for the 3-MIND and WALP peptides in Figs. 9c/d is complicated by the fact that the WALP calculation does not include any protein contribution, which is expected to be large and opposing for the case of the symmetric TM WALP peptides. The total PMF that includes this contribution is presented below.

3.6. Comparison of free energy profiles for different models

The PMF for the movement of a Trp side chain within the membrane, when attached to a WALP peptide has been obtained from the equilibrium distribution of the Trp positions obtained from all Trp side chains within the complete set of WALP peptides. It is compared to that from simulations of a single Trp side chain on a long helix (TRP-HELIx) and an isolated Trp analog molecule (3-MIND) in Fig. 10, where the 3-MIND PMF has been set to zero in the bulk water, while the WALP PMF is matched at the minimum within the bilayer.

The PMFs are all broadly similar, with the depth of the free energy well being ~5 kcal/mol, consistent with previous models [48,50]. In the WALP and 3-MIND model systems, we consistently observe the Trp side chain binding just inside the membrane core (z~10–12 Å), driven by a balance of hydrophobic forces and H-bonding to the lipid head group and glycerol backbone moieties. We observe that the PMF for the TRP-HELIx system exhibits broader wells at the interfaces further from the center of the bilayer. We attribute this to the positive mismatch caused by the long helix, and choose to now focus instead on the comparison of the 3-MIND analog to the WALP models.

One would anticipate significant differences in the PMFs for the analog molecule and the side chain due to the presence of the host peptide. The peptide (of varying hydrophobic lengths, leading to varying helix orientations and membrane perturbations) would be expected to place a bias on the positioning of the side chain in the membrane, alter its solvation environment, and restrict its conformational freedom (influencing its interactions and also reducing the entropic costs of entering the membrane). The similarities between the WALP and 3-MIND PMFs are therefore surprising. Notwithstanding the variety of host TM helices included in the WALP calculation (with hydrophobic lengths ranging from 10.5 to 31.5 Å, and with 1–4 flanking Trp side chains), overall, the presence of the host helices does not appear to greatly influence the PMF (on the order of kT or less across the membrane). In fact, Fig. 5a shows that the PMFs obtained by separate analysis of only peptides with 1, 2, 3 and 4 flanking Trp side chains are essentially the same. Furthermore, Fig. 5b shows PMFs from a separate analysis of peptides of different hydrophobic lengths, revealing free energy wells that are again very similar, only being significantly different for the extremely negatively mismatched peptides. The overall invariance to the nature of the host helix, combined with the fact that the 3-MIND and WALP models produce such similarity in the thermodynamics of moving a Trp side chain within a membrane, leads to an important implication for the role of Trp in anchoring TM segments. It suggests that it is the Trp itself that is primarily determining its location within the membrane, explaining why the Trp side chains can play a significant modulating role in mismatch phenomena in the accompanying study [8], as also suggested experimentally [16].

3.7. Explaining the role of Trp side chains in hydrophobic mismatch responses

For the Trp side chains to influence the responses to hydrophobic mismatch (membrane thickness changes and helix tilting in particular), they must bind to interfacial components with energies comparable to or greater than the energies required for a mismatch response. From the PMFs for the tilting of TM helices, calculated in the accompanying paper [8], force constants for helix tilting away from their equilibrium

![Fig. 10. Symmetrized PMFs for the WALPs (blue curve), 3-MIND (red) and TRP-HELIx (gray) systems. Unsymmetrized PMFs were converged to within 1 kcal/mol asymmetry (e.g. that for 3-MIND is shown in Fig. 9c).](image-url)
values were found to range from $-0.005$ to $0.03$ kcal/mol/Å$^2$. When projected onto the membrane normal as an effective “mismatch stiffness” (explained in the accompanying study [8]), they corresponded to force constants in the range of $0.03$ to $-0.2$ kcal/mol/Å$^2$ for the longer peptides ([LA]$_3$ L and [LA]$_{10}$ L) which experience significant tilting that is modulated by the number and position of the Trp side chains. As comparison, force constants around the minima of the Trp PMFs in this study, obtained by fitting parabolas to the minima, yield values of $0.06$ kcal/mol/Å$^2$ ([WALPs]), $0.04$ kcal/mol/Å$^2$ (TRP-HELIX) and $0.09$ kcal/mol/Å$^2$ (3-MIND). This similarity of the Trp movement and helix tilting energetics is not so surprising because a change in the helix length parallel to the membrane normal due to tilting maybe expected to involve a vertical movement of the flanking Trp side chains, and suggests that the flanking Trps may indeed be the controlling factor in helix tilting response to mismatch (at least for positively mismatched peptides). In contrast, tilting of shorter peptides, especially those of the ([LA]$_3$ L and [LA]$_{5}$ series, is less dependent on the number of the Trp side chains, with projected force constants ranging from 2 to 14 kcal/mol/Å$^2$, being too great compared to that for the Trp anchoring to allow for a significant effect.

In the case of the membrane thickness perturbations, the force constants range from 0.2 to 0.5 kcal/mol/Å$^2$ [8], which are of the same order, but several times that of the force constants from the Trp PMFs (especially for longer peptides). This says that the Trp movement around its equilibrium position (within its broad well in the glycerol backbone region) is similar or somewhat cheaper than the cost of deflecting the lipids, indicating that membrane thickness perturbations are not likely originating from the Trp placement within the membrane (as the Trp side chains could adjust more easily than lipids), and that variations in this response due to the placement of the Trp side chains may be moderate (especially for longer peptides where tilting dominates). This is supported by our mismatch response analysis, and especially by our observation that an isolated Trp alone does not cause bilayer perturbation at the levels observed due to a mismatch of the hydrophobic segment.

4. Conclusions

The amino acid Trp is described as normally rare but enriched in membrane proteins [3]. This fact, together with its interfacial preference, to point to the important role that Trp plays in the stability and conformational changes of TM protein segments. In this study, we have examined the interactions of Trp side chains with lipid bilayers, using three different model systems. We have found that the preference for Trp side chains to reside deep in the membrane interface (in the glycerol backbone region) is fairly strong ($-4$ kcal/mol) and is driven by a balance of the hydrophobic effect and interfacial binding to polar lipid moieties. This binding is strong enough so as to be relatively unaffected by its attachment to a hydrophobic TM segment, and to provide a significant modulating effect on hydrophobic mismatch responses.

Interactions with interfacial and glycerol backbone polar moieties, in particular, H-bonding with lipid carbonyl and phosphate components, were seen to create forces that pull the Trp side chains towards the interface. H-bonding of Trp with lipids has typically been discussed in terms of the lipid carbonyls, but we have shown that phosphate oxygens play an important role in hydrogen bonding with the N-H hydrogen in the outer interface, though canceled to some extent by choline interactions. However, deeper inside the interface, near the free energy minimum, H-bonding to the lipid carbonyl oxygens dominates (and as such, is the dominant H-bonding interaction in localizing Trp in the membrane). The ability of the lipid interfacial components to interact with Trp over a broad range of depths in the membrane leads to a strong, but broad free energy minimum. While this free energy minimum may be sufficient to ensure that hydrophobic peptides reside trans-membrane and control their orientations, it is likely insufficient to anchor highly polar segments across the membrane with any precision, as suggested by recent NMR experiments that have introduced arginine into the WALP peptides [78].

This preference for Trp to reside towards the bilayer interface can play a role in controlling the stability and orientation of a TM segment. We have shown that helix tilting is the most accessible response to positive hydrophobic mismatch [8]. For these long peptides, the free energy required to tilt the TM segment is comparable to the energy required to move the Trp side chain, leading to a strong coupling between the Trp placement on the peptides and their tilting response. For shorter peptides (negative mismatch), however, the interfacial preference of Trp corresponds to a small restoring force in comparison to that for the helix tilting, leading to a negligible role in the negative mismatch regime. Membrane thickness changes (which dominate the response to a negative hydrophobic mismatch), however, are influenced by the number and placement of Trp side chains to some extent, owing to the stiffness of the membrane thickness being similar (although somewhat greater) to that for Trp movement, but is diminished in the positive mismatch regime due to extensive helix tilting.

Hydrophobic mismatch plays a major role in effecting changes to both a TM segment and the membrane structure. These studies have shown that the presence of Trp side chains serves not only to anchor the hydrophobic TM segment across the membrane, but also to modulate the structural perturbations associated with hydrophobic mismatch. This suggests a determining role of Trp–lipid interactions in the function of the membrane proteins and membrane-active peptides, as observed experimentally (e.g. [30]). Moreover, we have revealed that while the Trp side chains themselves do not dominate the membrane perturbative effects of a TM peptide, they do act to guide the equilibrium orientation of the peptide, to the extent that the equilibrium distribution of the Trp side chains is almost independent of the host protein segment. These studies have described the key interactions leading to Trp anchoring of TM protein segments, as well as the balance of forces that determine structural responses due to a hydrophobic mismatch. It is the knowledge of these fundamental rules governing the membrane protein structure that will enable improved predictions of protein function and its modulation by a membrane composition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.09.009.

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