Peptide-based studies of hydrophobicity in membrane environments

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Department of Biochemistry

University of Toronto

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Abstract

Hydrophobicity – fear of water – drives the folding of soluble proteins into their final folded state. However, within the lipid bilayer, an environment devoid of water, the concept of hydrophobicity, and its role in protein folding, becomes more obscure. Utilizing synthetic peptides of length and hydrophobic content typical of nascent transmembrane (TM) segments, in this thesis we systematically assessed how altering amino acid sequence while maintaining composition impacts peptide-membrane interactions. We used four distinct sequence patterns: scrambled sequences, with hydrophobic character dispersed along the sequence length; clustered (or block) sequences, with a continuous stretch of hydrophobic character; amphipathic sequences, with one polar face and one hydrophobic face; and lipopathic sequences, with two hydrophobic faces where one is more hydrophobic than the other. Biophysical techniques, including circular dichroism and fluorescence spectroscopy, and HPLC retention times, were employed to measure peptide properties in a range of membrane mimetics. This versatile approach enabled us to relate specific features of sequence and environment to observed peptide-membrane interactions. We found that peptides containing a hydrophobic block or face were
deemed most hydrophobic in membrane mimetics. Comparison to \textit{in vivo} protein-assisted membrane insertion revealed that the translocon machinery could lower the energetic barrier to membrane integration, allowing a hydrophobic block to ‘carry’ polar residues into the bilayer. As well, local hydrophobic and helical patterning, including interchange of leucine and isoleucine residues, were found to play central roles in mediating peptide-membrane interactions. We concluded that the lack of water in the membrane does not render TM sequences any less “hydrophobic”; rather, the accessibility of a protein’s irregular surface to lipid acyl chains becomes a measure of “lipophilicity/lipopathy”, where the overall TM sequence produces the mix of protein-membrane and protein-protein interactions that fold a membrane protein into its final biologically-functional structure.
Acknowledgements

I am exceedingly grateful to my supervisor Dr. Charlie M. Deber, who’s encouraging and warm nature has made the completion of my doctoral degree an enjoyable experience. It has been a great privilege to work under such an exemplary scientist and advocate of fundamental science, who always strongly supported and promoted my academic growth in both research and teaching.

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My deepest gratitude to my family and close friends. Particularly my parents, Leanne and Gary Stone, and my grandmother, ‘Nana’. This thesis is dedicated to them. It is through their unconditional support that I have been able to pursue my dream of becoming a scientist. I will continue to think ‘square’.

Lastly, thank you to Greg for his endless support. You have been an ongoing source of encouragement and inspiration to me in science and more.

*Ad astra per aspera*
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$\Delta G_{\text{app}}$ exp., experimentally derived free energy of membrane insertion; $\Delta G_{\text{app}}$ pred., predicted free energy of membrane insertion; 6,7-dibromoPC, 1-palmitoyl-2-(6,7-dibromo)stearoyl-$sn$-glycero-3-phosphocholine; 9,10-dibromoPC, 1-palmitoyl-2-(9,10-dibromo)stearoyl-$sn$-glycero-3-phosphocholine; 11,12-dibromoPC, 1-palmitoyl-2-(11,12-dibromo)stearoyl-$sn$-glycero-3-phosphocholine; BSA, bovine serum albumin; CD, circular dichroism; CMC, critical micelle concentration; DCE, 1,2-dichloroethane; DCM, dichloromethane; DIEA, $N,N'$-diisopropylethylamine; DMF, $N,N'$-dimethylformamide; DMPC, 1,2-dimyristoyl-$sn$-glycero-3-phosphocholine; DPC, dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-$sn$-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-$sn$-glycero-3-phosphocholine; ER, endoplasmic reticulum; FRET, Förster resonance energy transfer; Fmoc, fluorenylmethoxycarbonyl; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; LASA, lipid accessible surface area; MRE, mean residue ellipticity; PEG-PAL, (aminomethyl-3,5-dimethoxyphenoxyvaleric acid)-polyethylene glycol-polystyrene; POPC, 1-palmitoyl-2-oleoyl-$sn$-glycero-3-phosphocholine; ProtK, proteinase K; RP-HPLC, reversed-phase high performance liquid chromatography; rpm, revolutions per minute; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol, TIPS, triisopropylsilane; Tm, transition melting temperature; TM, transmembrane; Tris, tris(hydroxymethyl)aminomethane.
1 Introduction
1.1 Hydrophobicity and Protein folding

The simple concept of ‘like dissolves likes’ is one of the most fundamental features of chemistry and biology. The preferred interaction of electrochemically similar molecules with one another over those that are different enables an array of biological processes to occur, including protein folding, enzymatic reactions, and cellular compartmentalization for the creation of microenvironments within the cell. The classical definition of hydrophobicity describes a non-polar molecule or hydrophobe that ‘fears’ water. Academically, hydrophobicity is ‘a measure of relative solubility in water versus a solvent of lower polarity’ [1]. This general feature when applied to biological systems ensures that molecules or macromolecular domains of lower polarity are driven together by their insolubility in a surrounding solvent that is more polar [2]. This insolubility is the driving force responsible for the coalescence of oil in water, the cleaning action of soaps, the spontaneous formation of cellular membranes, and the favorable folding of most soluble proteins [2,3]. During cellular synthesis of soluble proteins, non-polar amino acids such as those depicted in Figure 1.1 are sequestered together into what becomes the interior of the protein, shielded from the polar solvent of the cytosol, in an entropically driven process known as the hydrophobic effect [2].

However, considering that some protein environments lack water, such as the lipid bilayer, hydrophobicity can be viewed in a more dynamic manner where the given solubility of a molecule in another solvent is heavily dependent on protein sequence context and local environment. For these reasons hydrophobicity will often be referred to as ‘effective hydrophobicity’ within this thesis, defined as ‘the hydrophobicity exhibited by an individual amino acid residue in a given sequence and solvent environment.’ [1].
1.1.1 Measures of hydrophobicity

Quantification of hydrophobicity has long been sought after to aid in the characterization of protein sequences and the prediction of protein folds and cellular localization. However, the medium dependent nature of hydrophobicity has made these measurements challenging and has led to the development of numerous quantification techniques and a considerable number of available hydrophobicity scales, a few of which are depicted below (Table 1.1). Despite the large number of scales derived from very different means, comparison of various hydrophobicity scales does show a convergence, depicting similar trends, albeit with varied exact values [4–6]. Remarkably, values derived from simple *in vitro* partitioning techniques carried out in the absence of any cellular machinery are similar to values derived from *in vivo* integration into the membrane [4,7]. While hydrophobicity remains a moving value, broadly the various hydrophobicity scales capture the nature of hydrophobicity. There are four main methods to measure/quantitate hydrophobicity as discussed and contrasted below.

![Models of three hydrophobic amino acids commonly found within transmembrane domains](image)

**Figure 1.1. Models of three hydrophobic amino acids commonly found within transmembrane domains.** Leucine, isoleucine, and valine are depicted. Despite similarities in side chain composition and volume, the three amino acids exhibit variation in their reported hydrophobicity values (see Table 1.1).
Table 1.1. Amino acid hydrophobicity values derived from various methods

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<th>LD&lt;sup&gt;a&lt;/sup&gt; Exp.</th>
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<sup>a</sup>Liu-Deber hydrophobicity scale experimentally derived from retention times and helicity in n-butanol of 20-mer peptides [8].<sup>b</sup>Wimley & White hydrophobicity scale experimentally derived from partitioning of penta-peptides between an aqueous phase and lipid bilayers [9].<sup>c</sup>Punta & Maritan hydrophobicity scale derived from the propensity for amino acids to be found within membrane spanning domains [10].<sup>d</sup>Hessa, White, and von Heijne biological hydrophobicity scale experimentally derived from translocated/inserted equilibriums of α-helical proteins passed through the mammalian translocon [11].<sup>e</sup>Moon and Fleming hydrophobicity scale experimentally derived from the reversible folding of a β-barrel, containing single amino acid mutants, into membranes [12]. The relative rankings of amino acids, with the most hydrophobic as 1 and least hydrophobic as 20, are listed in parenthesis.

1.1.1.1 Partitioning

Early methods for measuring hydrophobicity involved the partitioning of molecules between two different phases [13–17], typically water and an organic solvent. Initial experiments
were carried out on single amino acids followed by short peptides of increasing complexity and structure. The partitioning of these amino acids or short peptides between water and organic solvents such as cyclohexane, ethanol, and octanol resulted in the earliest hydrophobicity scales, some of which remain in use today [14,17]. However difficulties in finding organic solvents that closely mimic biological membranes and the slight but unpreventable mixing of some solvents and water [4,18] led to the use of lipid bilayers in later partitioning experiments [19]. The exchange of organic solvents for lipid bilayers led to the development of novel hydrophobicity scales (Table 1.1), capable of capturing features unique to peptide-membrane interactions, such as the preference of aromatic amino acids for the bilayer interface [19,20].

1.1.1.2 Chromatographic separations

Reverse phase (RP) chromatography provides a method for measuring the adsorption of peptides to a nonpolar stationary phase. Retention times derived from RP chromatography can provide detailed information on the relative hydrophobicity of any given set of compounds. Additionally, peptides traversing a RP-HPLC column typically adopt α-helical conformations, providing a measure of hydrophobicity within the context of a folded helix [8,21], unlike some of the earlier scales measuring partitioning between aqueous and organic phases [17]. The practice of RP chromatography for the determination of a compound’s hydrophobicity remains commonly in use today (Table 1.1) [22,23].
1.1.1.3 Amino acid localization within a protein

Advances in high resolution structure determination allowed for assessment of the differential localization of amino acids within a protein’s hydrophobic core vs. the aqueous exposed surface [24–27]. Considering general principles of protein folding and the hydrophobic effect, amino acids found to localize within the center of a soluble protein are deemed more hydrophobic than those located on a protein’s surface. In a similar manner, structural data of membrane proteins can be used to classify amino acids commonly found spanning the membrane as hydrophobic over those that are typically excluded from TM domains (Table 1.1) [10,28].

1.1.1.4 Protein integration into biological membranes

Development of new hydrophobicity scales over the years has contributed greatly to our understanding of what constitutes a hydrophobic amino acid, with scales often combining data from various techniques to produce more robust scales [13,15]. However, problems arise when hydrophobicity values derived from single amino acids or short unstructured peptides partitioning between water and organic phases are extrapolated and averaged to apply to a folded helical domain inserting into a native membrane. The need for a ‘biological’ hydrophobicity scale remained.

A scale measuring hydrophobicity as a function of membrane integration utilizing the cellular insertion machinery and native membranes was developed, providing the first molecular code for transmembrane recognition (Table 1.1) [11,29]. In eukaryotes, glycosylation occurs solely within the endoplasmic reticulum (ER) via oligosaccharyl transferase. Glycosylation sites
(namely Asn-X-Thr/Ser) may be engineered up/downstream of a potential TM sequence and the exposure of the glycosylation site to the ER lumen used to probe whether a protein sequence is translocated across the ER or inserted into the membrane [30]. Hessa et al., utilized a modified form of the membrane protein, leader peptidase (Lep), in which they placed a sequence of study downstream of the two native Lep TM domains, flanked by two glycosylation sites (Figure 1.2A) [11]. In this manner, differential glycosylation of the Lep construct provided information on the insertion status (hydrophobicity) of the given sequence (Figure 1.2B). This ‘biological’ hydrophobicity scale provided vast insight into how the cell selects helical TM domains through an in-depth look at the energetics of membrane insertion and amino acid positional biases along the length of a TM segment [11,29].

![Diagram](A) Inserted Singly glycosylated

![Diagram](B) Translocated Doubly glycosylated

![Image](A) ER lumen

![Image](B) Cytoplasm

**Figure 1.2. Schematic of the differential glycosylation of inserted/translocated sequences in the Lep construct.** (A) The two native TM domains of leader peptidase are depicted in black and the sequence of study in grey. Two glycosylation sites, denoted G1 and G2, flank the sequence of interest. If the sequence inserts into the membrane, only G1 is exposed to the ER lumen, becoming singly glycosylated. If the sequence is translocated across the membrane into the ER lumen, both G1 and G2 become glycosylated. (B) The difference between unglycosylated (○), singly (●), and doubly glycosylated (●●) constructs can be detected by migration rates on SDS-PAGE. Shown in the gel are examples of constructs treated in the absence of ER lumen (-control) and those that are fully translocated, inserted, and 50% translocated/50% inserted.
More recently, Moon et al., developed a hydrophobicity scale that quantifies the impact a single amino acid substitution within a common β-barrel protein has on the reversible and spontaneous insertion into the membrane (Table 1.1) [12]. Unlike the Hessa et al., scale, here partitioning is assessed in the absence of any insertion machinery and from a fully unfolded, denatured state, thus providing insertion free energy values that more closely mimic a true equilibrium state [31].

1.2 Biological membranes

Biological membranes are complex, dynamic structures composed of a large variety of lipids and proteins that together provide essential barriers between cellular compartments and the exterior of the cell. Once considered a passive participant in cellular processes, biological membranes have been shown to play active roles in modulating numerous critical functions, including the establishment of nutrient, waste, and proton gradients [32,33]. A major component of biological membranes are the amphipathic lipid molecules that spontaneously associate in water to form bilayers [34], with hydrophobic acyl chains sandwiched between polar head groups [35]. The hydrophobic core of the bilayer prevents the non-specific passage of polar molecules, thus forming an effective barrier to most solutes.

1.2.1 Lipids

In eukaryotes, the lipid bilayer is composed primarily of glycerophospholipids, consisting of a glycerol backbone, two acyl chains, and one phosphate group that may be additionally
bound to a headgroup moiety [36]. Acyl chain length, saturation, and headgroup composition may all be varied, increasing the diversity of lipid features and roles in membrane biology [37]. The extensive favorable hydrophobic forces between lipid acyl chains in the core of the bilayer are contrasted by the unfavorable repulsion between acyl chains and lipid headgroups. The large functional variety of lipids enables exquisite control over the physical properties of the bilayer, including membrane curvature, thickness, fluidity, and internal lateral pressure as outlined in Figure 1.3 [37].

Membrane curvature is established by the overall shape of the lipid, determined by the relative comparison of the size of lipid headgroups to the acyl chains (Figure 1.3A) [37,38]. Cylindrical lipids (e.g. phosphatidylcholine) with similar width headgroups and lipid acyl chains, produce a flat bilayer, while lipids with smaller headgroups have an inverted cone-like shape that induces negative membrane curvature (e.g. phosphatidylethanolamine) (Figure 1.3A) [37]. Lipids with larger headgroups have cone-like shapes that induce positive membrane curvature (e.g. phosphatidylinositol) (Figure 1.3A) [37]. The introduction of inverted cone or cone shaped lipids induces negative and positive curvature, respectively, to the bilayer which correspondingly alters the lateral pressure profiles within the bilayer (Figure 1.3A; red arrows) [38]. Altering acyl chain length and saturation both impact bilayer thickness. Longer lipid acyl chains contributing to thicker bilayers and conversely shorter lipids producing thinner membranes (Figure 1.3B). Membrane fluidity is controlled by the degree of attractive forces between lipid headgroups and acyl chains. Large amounts of van der Waals forces and low thermal motion within the bilayer result in a tightly packed, thicker membranes of lower fluidity while decreasing van der Waals forces and increased thermal motion increases the fluidity of the bilayer while decreasing the thickness (Figure 1.3C) [36].
Figure 1.3. Biophysical properties of lipid bilayers. (A) Inverted cone shaped lipids induce negative bilayer curvature and increase lateral pressure near the core of the bilayer, as indicated by the double headed red arrow. Cylindrical shaped lipids result in flat bilayers. Cone shaped lipids induce positive bilayer curvature and increase lateral pressure near lipid headgroups (double headed red arrow). (B) Shorter chain lipids produce thinner bilayers and longer chain lipids produce thicker bilayers. (C) Lipids in the gel phase are closely packed, resulting in a thicker bilayer. In the liquid-crystalline phase acyl chains are more disordered, resulting in a thinner bilayer.
1.3 Membrane Proteins

Membrane-associated proteins are unique in their structures and functions, often carrying out vital roles in communication, cell-cell identification, the transduction of signals, nutrient/waste/ion transport, and establishment of proton gradients for generation of energy. These proteins belong to one of two groups: peripheral membrane proteins or integral membrane proteins. Peripheral membrane proteins associate with membranes, residing on the membrane surface and do not span the width of the lipid bilayer. They often associate with the membrane through electrostatic interactions with lipid headgroups and/or other membrane embedded proteins or through the covalent attachment to membrane embedded lipids or the insertion of hydrophobic loops.

Integral membrane proteins, or transmembrane (TM) proteins, span the width of the lipid bilayer, making significant contacts with the membrane interface and hydrophobic core. The impenetrable barrier of the lipid bilayer ensures that cells must rely on membrane embedded proteins for the transport of polar molecules and the transduction of cellular signals from the extracellular matrix.

1.3.1 β-barrels and α-helical transmembrane proteins

Integral membrane proteins span the lipid bilayer through one of two types of structures: a closed β-barrel or α-helical TM domains (Figure 1.4). Transmembrane proteins found within the plasma membrane of eukaryotes and the inner membrane of bacteria are α-helical in nature,
while β-barrels are restricted to the outer membrane of bacteria and double membrane organelles (mitochondria in eukaryotes and chloroplasts in plants).

**Figure 1.4. Representative examples of β-barrel and α-helical membrane protein structures.** (A) The structure of outer membrane protein A (OmpA; PDB ID: 1BXW) from *E. coli* at 2.5 Å resolution [39]. The β-barrel consists of 8 β-strands hydrogen bonded together with the N-terminal strand (dark blue) bonded to the C terminal strand (red). Extended inter-strand loops are seen connecting the β-strands on the extracellular side and short compact loops on the periplasmic side. (B) The structure of bacteriorhodopsin (PDB ID: 1X0S) from *H. salinarum* at 2.5 Å resolution [40]. The protein contains 7 independent α-helices connected by short extracellular and periplasmic loops (TM1 in blue, TM 7 in dark orange). Retinal can be seen bound in the center of the helical bundle, rendered in black. Boundaries of the lipid bilayer are roughly depicted by the two yellow lines.

β-sheet-type hydrogen bonding occurs intermolecularly between the backbones of adjacent β-strands, resulting in exposure of the polar polypeptide backbone to the surrounding environment. Therefore, the only permissive form of β-strands within the lipid bilayer are self-sealed barrels in which the initial β-strand is hydrogen bonded to the terminal β-strand.
The unique secondary structure imparted by the α-helix is responsible for its ubiquitous role in spanning the lipid bilayer. Within the helical structure, the polar atoms of the polypeptide backbone are engaged in hydrogen bonding between the C=O at position i and the N-H at position i + 4 within the sequence. This network of intramolecular hydrogen bonds between backbone carbonyls and amides effectively shields the polarity of the peptide backbone while projecting the amino acid side chains towards the surrounding environment [9]. The relatively linear structure of the α-helix also allows for easy accommodation in-between the linear acyl chains of the lipid bilayer.

Transmembrane helices act as independently folded domains, increasing the structural complexity α-helical membrane proteins may adopt, some proteins containing as little as one TM spanning helical domain, others more than 24 [41]. The diverse number of helical structures available corresponds to an equally diverse set of biological functions ranging from simple lipid anchors (e.g. cadherins) to cell-cell recognition (e.g. Glycophorin A) to molecular transport (e.g. ion channels) to complex components in signaling pathways (e.g. G-protein coupled receptors) [41].

The critical roles membrane proteins play in communication across the cell has attracted the attention of pharmaceutical companies, with membrane proteins making up to ~60% of all current approved drug targets [42]. The importance of these proteins as drug targets and maintaining cellular homeostasis makes the understanding of how these proteins fold and function crucial.
1.3.2 Properties of transmembrane segments

The average α-helical TM domain contains 24 (± 5) amino acids, sufficient length to span the approximate 30 Å width of a standard hydrocarbon bilayer core [43]. However, α-helical TM domains as short as nine amino acids and as long as 43 have been reported [43]. Such TM domains that are significantly shorter or longer than the bilayer core induce hydrophobic mismatch that can be accommodated through membrane deformations (e.g. shrinkage, stretching) and/or helix tilt as described in more detail in section 1.4.4.2 [44]. Notably, organelle specific TM domains are found to have helix lengths corresponding to the observed membrane thicknesses of their respective organelles within eukaryotic cells [45].

The hydrophobic environment of the lipid bilayer has imposed a strong bias on the amino acid composition of typical TM domains. Hydrophobic amino acids such as Leu, Ala, Val, Ile, Phe, and Met make up almost 60 % of the amino acids within TM domains, with Leu and Ala being particularly prevalent at ~ 16 and 11 % respectively [43]. The remaining ~ 40 % is composed of polar and charged amino acids, the most prevalent being Gly, Ser, and Thr (8.7, 5.2, and 5.3 %, respectively) [43]. While these amino acids may appear unfavorable in a TM domain, they often play important roles in the linings of pores and channels as well as in initiating helix-helix contacts. Glycine in particular is commonly found to participate in helix-helix associations, the minimal side chain allowing close packing between two TM helices [46]. Ser and Thr have also been shown to participate in helix-helix interactions, shielding the polar nature of their side chains in the helix-helix interface [46–48]. Further, the energetic penalty for membrane burial of Ser/Thr side chains is proposed to be smaller than anticipated due to the ability of the side chain -OH to hydrogen bond with the polar backbone [34,48,49].
remaining polar and charged amino acids make up less than 5 % of amino acids found within TM domains [43].

The positioning of hydrophobic and polar/charged amino acids along the length of a TM domain follows the general physical properties of the membrane [29]. Strictly hydrophobic amino acids such as Leu, Ile, and Val tend to exhibit only a small positional bias for the center of the TM helix, placing them near the core of the lipid bilayer [29,50]. Polar, charged, and aromatic amino acids strongly favor positions close to the TM helix termini where they may have access to charged/polar lipid head groups, as well as the lipid bilayer-water interface [29,50,51].

Subtler positional biases can be observed for organelle specific TM domains as a result of varied lipid composition between organelles and even between bilayer leaflets [45]. For example, structural studies of single pass membrane proteins across the plasma membrane, endoplasmic reticulum, and the Golgi apparatus have shown variations in TM domain length and positional biases for large vs. small amino acids [45]. Such biases mirror the physical properties of the given membrane [45]. The plasma membrane – being thicker with more lipid packing in the outer leaflet relative to the inner leaflet – contains longer than average TM domains with a preference for smaller amino acid side chains in the more-well-packed portion of the bilayer. Similarly, the Golgi membrane – being thinner and less-well-packed in general – contains shorter than average TM domains, more abundant in bulkier amino acid side chains. The impacts of the physical properties of the lipid bilayer on the composition and structural design of TM domains is clear in structural studies of membrane proteins, albeit there are exceptions to the general rules outlined above.
1.3.3 Marginally hydrophobic transmembrane α-helices

A subset of TM sequences are not predicted *a priori* to reside within the lipid bilayer, often having relatively low average hydrophobicity and a high abundance of polar/charged amino acids. Such TM domains are classified as marginally hydrophobic and are typically found in multi-pass membrane proteins [52]. How these non-typical TM domains are selected to reside within the membrane and subsequently stably inserted is unclear. Hedin *et al.*, have shown for some marginally hydrophobic TM domains that the presence of neighboring polar loops or upstream/downstream native TM helices can aid in their stable membrane integration [52]. However, in some cases, the insertion of these marginally hydrophobic TM domains remains a mystery [52]. A subset of helices, found within the cores of soluble or secreted proteins, exhibit high average hydrophobicity, exceeding the threshold for spontaneous membrane insertion [8], marking them as candidates from membrane insertion, and further complicating the detection of soluble vs. membrane bound domains [51]. Such ‘hidden TM domains’, termed by our lab as “delta helices”, despite having sufficient average hydrophobicity, are unlike native TM domains in that they lack a positional bias for charged amino acids and are generally shorter in length [51]. However, subtle changes to the sequences of select delta helices readily converted them into TM domains [53]. This ease with which a soluble secreted protein may be altered to a membrane protein and vice versa implies that a fine balance exists between the secretion and insertion of protein segments on the hydrophobic edge [53].
1.4 Transmembrane protein folding

The unique environment of the lipid bilayer imparts constraints on the folding of proteins embedded within the membrane. Relative to the folding of soluble proteins within the cytosol of the cell, membrane protein folding is complicated by the hydrophobic, anisotropic, and restrictive environment of the lipid bilayer. The two-stage folding model, first proposed by Popot and Engelman in 1990, suggested a reductionist model for the folding of membrane proteins [54]. TM domains first insert into the lipid bilayer independently, as discrete folded helices that once membrane embedded, may laterally associate to form tertiary and quaternary contacts [54,55]. While the model represents an over-simplification, it captures the nature of membrane protein folding, providing insight into how proteins are inserted and fold within the confines of the lipid bilayer. Further details on the intricacies of membrane protein folding are discussed below.

1.4.1 Non-protein assisted bilayer insertion

A subset of membrane proteins and membrane interactive peptides undergo spontaneous membrane insertion without the assistance of any cellular machinery [56]. This unguided insertion into the membrane relies on the given protein segment having sufficient length and hydrophobicity to stably span the bilayer. What constitutes “sufficient hydrophobicity” is often measured by averaging hydrophobic character along the length of the helix. If the average hydrophobicity exceeds a given ‘threshold’ value for spontaneous membrane insertion, the segment will readily partition into the membrane [8]. Such hydrophobicity ‘threshold’ values have been extensively studied using in vitro biophysical and in vivo biological techniques. A
poly-Ala segment of ~20 amino acids in length is often considered the consensus for the boundary of membrane insertion [8].

1.4.2 Protein-assisted bilayer insertion

The bilayer insertion of more complex membrane proteins, including multi-spanning membrane proteins containing marginally hydrophobic TM helices, cannot rely on simple partitioning from the cytosol, but rather requires assistance to adopt the correct topology and three-dimensional fold. While bacteria contain numerous secretion systems for the transport of molecules outside of the cell, only a few proteins have been shown to be directly involved in mediating the integration of membrane spanning domains into the lipid bilayer [57,58]. Insertion of β-barrels into the outer membrane of bacteria is primarily coordinated by the β-barrel assembly machinery (BAM) complex and the translocation and assembly module (TAM) [59]. The insertion of helical proteins into the inner membrane of bacteria is mediated by two main protein insertases: YidC and SecY [57]. While these two insertases may cooperate for the insertion of helical membrane proteins [60–62], the two proteins are capable of functioning as stand-alone insertases of differing structure, mechanism of action, and preference for TM domains [58].

1.4.2.1 β-barrel assembly machinery (BAM) and translocation and assembly module (TAM)

The main component of the BAM complex, responsible for facilitating the insertase activity, is BamA, a 16 stranded β-barrel located in the bacterial outer membrane [63]. Structural
data has shown the first and terminal β-strands of the barrel to be precariously hydrogen bonded together with the C-terminal strand seen separating from the N-terminal strand and collapsing inwards in molecular dynamic simulations [63,64]. This opening in the side of the barrel domain may allow newly forming β-barrels to template onto the exposed β-strands, inserting the putative barrel into the membrane one β-hairpin at a time [64].

TAM consists of two proteins, TamA and TamB, which span the outer and inner bacterial membranes respectively [59]. TamA, much like BamA, is a β-barrel from the Omp85 superfamily, while TamB spans the inner membrane through a single helical TM domain [59]. Although not essential, TAM is proposed to aid in the translocation of autotransporters to the cell surface and has, as of yet, an undefined role in assisting the BAM complex in the insertion and folding of β-barrels into the outer membrane [59]. Structural similarities between TamA and BamA suggest a similar mechanism of action with nascent β-strands possibly templating onto the terminal strands the TamA β-barrel [65].

1.4.2.2 YidC insertase

YidC is found exclusively in the inner membrane of bacteria [57], and consists of a set of five TM helices loosely arranged on the cytoplasmic side of the inner membrane, forming a deep cytosol and lipid exposed groove [66]. The groove is lined with polar residues and contains a highly conserved, essential arginine residue [66]. TM membrane insertion is hypothesized to occur through interaction of negatively charged periplasmic loop regions with the conserved arginine in the hydrophilic groove. The membrane potential could then aid in transferring the negatively charged loop from the polar groove of YidC across the membrane to the periplasm, resulting in a TM orientation of the protein [66]. Unsurprising in light of the potential insertion
mechanism, YidC has been shown to exhibit preferences for inserting TM domains containing negative charges and higher than average polar character [67,68].

1.4.2.3 The translocon protein complex: SecYEG insertase

SecYEG (Sec61αϒβ in eukaryotes and archaea), also known as the translocon protein complex, is a heterotrimeric protein found in the inner membrane of bacteria and the plasma membrane of eukaryotes and archaea [69]. SecY/Sec61α is the main protein component of the heterotrimeric complex [70], composed of ten TM helices that form a channel to provide a conduit through the membrane for proteins destined for secretion, and a pathway into the lipid bilayer for membrane integration (Figure 1.5; grey helices) [69,71]. The TM helices are arranged in a clam-shell like manner, with one side of the channel functioning like a hinge to allow opening of the opposite side [72]. TM helices 2 and 7 (highlighted orange in Figure 1.5) form the opening or ‘lateral gate’ of the channel, that parts to allow exposure of the channel interior to the lipid bilayer [72]. A narrowed region within the channel, denoted as the constriction ring, is composed of closely interacting hydrophobic amino acids (six Ile in E.coli; V82, I86, I181, T185, M294, and M450 in yeast) that along with a flexible cytoplasmic plug domain help maintain the integrity of the lipid bilayer when the channel is in a resting state (Figure 1.5; constriction ring in yellow space filling spheres, plug domain in green) [69,73].

SecE (Sec61ϒ), containing minimally a single TM domain but more often three separate membrane spanning regions, is located at the back side or ‘hinge’ of the SecY channel where it functions in lateral gate opening (Figure 1.5; black helices) [58]. SecE (Sec61ϒ) is an essential protein in vivo, with deletion leading to instability and rapid degradation of SecY (Sec61α) [58].
The final protein component of the heterotrimeric complex is distinct between bacteria and eukaryotes, consisting of SecG and Sec61β, respectively (Figure 1.5; blue helix). SecG, composed of two TM domains, has been shown to be non-essential and is predicted to play a role in ATPase driven protein translocation [58].

Figure 1.5. Crystal structure of SecYEG from *Methanococcus jannaschii*. TM helices of the SecY subunit are rendered in grey and helices from the SecE subunit in black and SecG in dark blue. TM helices 2 and 7 of the lateral gate are highlighted in orange, the plug domain in green, and the amino acid side chains that make up the central constriction ring in yellow, space filling spheres. A side view looking into the lateral gate (closed position) is shown alongside a top-down and bottom-up view to emphasize positions of the constriction ring and plug domain respectively.

1.4.3 Translocon-mediated membrane insertion

Protein sequences transiting through the aqueous channel of the translocon may be exposed to the interior of the lipid bilayer through the opening of the lateral gate between TM helices 2 and 7 (Figure 1.5; highlighted orange) [69,72,74,75]. Structural evidence has emerged to indicate that opening of the translocon lateral gate is dependent on the sequence of the translocating polypeptide [76], *viz.*, sequences identified as membrane-targeted induce the lateral
gate to open, exposing the passing segment simultaneously to the aqueous translocon channel and to the hydrophobic core of the bilayer [77]. Ribosome and SecA ATPase docking, during co-translational and post-translational insertion, respectively, as well as signal sequence translocation, have also been reported to open the lateral gate, priming the translocon for the insertion of membrane domains [78,79].

Passing TM segments may sample both the aqueous environment of the channel and the hydrophobic interior of the lipid bilayer. If the sequence is sufficiently hydrophobic it may then laterally diffuse out of the translocon channel, partitioning into the lipid bilayer, becoming membrane-embedded [71]. While the translocon has been proven to be an aqueous water filled channel [80], molecular dynamic simulations have shown that the environment within the channel does not resemble that of bulk water, with water molecules moving more slowly and exhibiting more order [81].

The energetics of translocon assisted membrane insertion is closely mirrored in simple partitioning assays between water and lipid bilayers [4,7]; however, noteworthy differences exist. Interestingly, partitioning measurements of peptides between water and lipid bilayer interfaces, rather than the lipid bilayer core itself, most closely match experimentally derived insertion free energy values [4,77]. Such observations have led to the novel idea that TM helices do not truly transverse the translocon channel but rather ‘slide’ along the lateral gate helices, partitioning into the bilayer interface before becoming transmembrane [77]. The translocon alone or in conjunction with neighboring assistant membrane proteins known to associate with the translocon channel (e.g. TRanslocating chain–Assisted Membrane protein, TRAM [71]; TRanslocon–Associated Protein, TRAP [82]; YidC [83]) may lower the energy barrier to
membrane integration, providing an environment intermediate between the translocon channel and the lipid bilayer core [4,71,84].

The role – active or passive – of the translocon channel itself during membrane integration remains debated. TM domains have been shown to contact specific points within the translocon channel in a sequence-specific manner [85] and remain bound to the translocon for extended periods of time [86,87]. Sequences with lower overall hydrophobicity (i.e. marginally hydrophobic TM domains) have been shown to be sensitive to translation/translocation rates [88]. Slower translation rates and/or the presence of longer flanking soluble domains, extends the time translocating segments interact with the translocon channel, increasing the likelihood for membrane integration of marginally hydrophobic TM domains [88]. Additionally, alterations to the translocon protein have been shown to affect the hydrophobicity threshold for membrane insertion [89–92]. Specifically, polar/charged mutations to the lateral gate [93] or the native six hydrophobic amino acids in the constriction ring [92] have been shown to lower the hydrophobicity threshold for TM membrane integration, enabling insertion of marginally hydrophobic domains that would otherwise be translocated. Further, the constriction ring was found to impart a functional asymmetry on membrane integration [94]. Sequences with bulky hydrophobic amino acids clustered together and positioned close to the constriction ring experience a raised energy barrier to insertion vs. sequences where bulky hydrophobic residues are positioned away from the constriction ring [94].

1.4.4 Transmembrane protein folding within the bilayer

The folding of membrane proteins within the lipid bilayer is complicated due to the
diverse environments these proteins encounter. Most membrane proteins make contact to some degree with the translocon, an aqueous water containing channel, prior to entering the apolar lipid bilayer core, an environment devoid of water [77]. While the hydrophobic effect is therefore operative to some extent within the channel, the absence of water within the bilayer core ensures the hydrophobic effect is no longer a major force in the folding of a membrane protein; rather, side chain-side chain helix/helix packing, and side chain-lipid interactions must play these roles [1].

1.4.4.1 Helix-helix interactions

The adoption of correct protein tertiary and quaternary folds within the membrane environment relies on the stable formation of the correct helix-helix associations. These types of interactions are mainly mediated through van der Waals forces and hydrophobic interactions involving the close packing of two or more helices together [95]. The GG4 or small-small helix-helix association motif is the most commonly occurring in TM domains [95,96], as exemplified by the well described GpA dimer (TM sequence: \textsuperscript{76}IIFGVMAGVIGT\textsuperscript{87} [motif underlined]) [97]. The positioning of two small residues at positions i and i+4 (i.e. G-XXX-G) places them on the same face of the helix, producing a concave surface into which bulky hydrophobic residues such as Ile or Val may fill in a ‘knobs-into-hole’ type packing [96]. Similar in nature is the “heptad repeat” packing motif that uses two small residues placed seven amino acids apart (i.e. G-XXXXXX-G) as found in the fourth TM domain of the small multidrug resistance (SMR) family \textit{(Halobacterium salinarum}, Hsmr TM4 sequence: \textsuperscript{85}VAGVVLAL\textsubscript{105}VAGVVVLNVAS [motif underlined]) [98]. Additionally, polar amino acids have been shown to drive helix-helix association through the favorable burial of the polar atoms in the helix-helix interface via
formation of inter-helical hydrogen bonds [47,99–102], cation-pi, and pi-pi interactions [103–105].

1.4.4.2 Helix-lipid interactions

Helix-lipid interactions play equally important roles in defining the final folded structure and function of membrane proteins [106]. The composition of the lipid bilayer can be essential to membrane protein structure, activity, and stability [107]. Some proteins fail to adopt the correct tertiary fold or oligomeric states when placed in membranes of the incorrect lipid composition [108,109]. In some cases, specific lipids can act as chaperones for folding or bound ligands for function [110]. A notable example includes the lactose permease, LacY, whose topology is highly sensitive to the content of phosphatidylethanolamine in the bilayer [111].

Both helix length and amino acid composition impact how TM helices interact with lipid bilayers [44]. Unfavorable protein-lipid interactions can manifest as ‘hydrophobic mismatch’ between TM domains and the bilayer, which can be resolved through changes in bilayer thickness, helix tilt, and/or orientation of side chains as outlined in Figure 1.6 [44]. Specifically, changes to bilayer width to accommodate shorter or longer than average TM domains may be accomplished through alterations to lipid packing, with decreased ordering of lipid tails resulting in thinning of the membrane core and increased ordering of lipid tails in bilayer thickening (Figure 1.6B, D respectively). Segments as short as ten leucine residues have been seen to undergo bilayer insertion, but accompanied by some deformations to the membrane [112,113]. Polar and charged side chains in shorter helices may be accommodated through side chain ‘snorkeling’ to allow polar contacts with the lipid-water interface [112] (Figure 1.6C). The degree of hydrophobic mismatch between TM domains and the bilayer can also strongly
influence TM helix orientation within the bilayer [114,115]. Longer TM domains may become tilted relative to the bilayer normal to ensure hydrophobic domains remain properly buried, despite disruptions to lipid-lipid packing [44] (Figure 1.6E). Such structural rearrangements of the bilayer come at an energetic cost that is balanced by the favorable burial of the hydrophobic residues within the TM domain.

Figure 1.6. Lipid bilayer and TM adaptations to minimize hydrophobic mismatch. (A) TM domains of average length span the membrane with minimal disruption to the bilayer. Shorter TM domains may be accommodated through membrane thinning (B) or through re-orientation of polar amino acid side chains towards the bilayer interface. (C) Longer TM domains may be accommodated through thickening of the membrane (D) or through tilting of the helix (E) [44].
Despite efforts to minimize hydrophobic mismatch, the lipid bilayer is not always capable of fully solvating TM domains. The idea of membrane proteins as ‘impurities’ within the bilayer, or lipids as a poor solvent, explains the driving force behind membrane protein folding [1]. Protein surfaces incompatible with lipid (or ‘lipophobic’) are driven to associate in favorable helix-helix interactions [116,117]. Subtle alterations to the surface of a TM domain has the capability of impacting helix-helix associations by either favoring new interactions with lipid and thus decreasing helix-helix association or vice versa [118,119]. For example, vast changes in dimer strength of GpA occur upon slight changes to the lipid accessible surface area of the two helices [118,119].

1.5 Peptides as models for membrane protein folding

The α-helix as the minimal folding unit of helical membrane proteins enables the use of TM peptides in a ‘divide and conquer’ approach to study membrane protein folding [120,121]. Peptides as simplified models for the study of membrane proteins are amenable to a variety of biophysical techniques that would otherwise be difficult carried out on a full-length membrane protein [44,120]. In vitro chemical synthesis allows for the easy incorporation of non-natural components such as peptoids, β-amino acids, amino acids with altered side chains, fluorophores, etc. [122]. Further, peptides have been shown to recapitulate native protein oligomeric states [123] and structures [124] in many membrane mimetic systems.
1.6 Membrane mimetics

Working with native membranes is often challenging and/or unfeasible in a laboratory setting, due to their complex composition and heterogeneity, and so various mimetics that capture the physical properties of native membranes are used in their place. Membrane mimetics range in complexity and composition from simple organic solvents (e.g. trifluoroethanol, octanol) to complex mixtures of lipids (e.g. mixed composition liposomes). The membrane mimetic of choice is dependent on the technique being used and the protein being studied and is therefore often empirically chosen.

1.6.1 Organic solvents

The most primitive of membrane mimetics would be the use of small organic molecules such as trifluoroethanol (TFE), methanol, and octanol (Figure 1.7A). Such hydrophobic organic solvents can induce adoption of an α-helical fold, but being a homogenous isotropic mixture, may fail to capture the subtle nuances of native membranes.
Figure 1.7. Solvation of a TM helix in membrane mimetics of varying complexity. (A) Organic solvents such as TFE are capable of solvating simplistic TM peptide models and capturing secondary structure characteristics. (B) Detergent micelles provide a hydrophobic interior that can coat a TM domain while still allowing easy access to the micelle surface. (C) Lipid bilayers provide a more organized, uniform, and tightly packed environment than organic solvent and detergent micelles.

Organic solvents can also act as excellent solvents for small, simple TM domains making them valuable for assessments of effective hydrophobicity and propensity for secondary structure. However, their unfolding action on soluble domains and ability to completely ‘dissolve’ or solvate a membrane domain often makes them unfeasible for further structural and functional characterization. Nevertheless, over 10% of reported membrane protein structures solved using solution state NMR were done using organic solvents (Protein Data Bank: [125]).
1.6.2 Detergent micelles

One of the most commonly used membrane mimetics are detergents [126]. These molecules generally contain a polar head group and a single hydrophobic tail, making them amphipathic in nature and conical in shape (Figure 1.7B) [126]. In aqueous solutions, above a critical concentration (critical micelle concentration, CMC), detergent molecules will spontaneously assemble into a roughly spherical micelle structure with hydrophobic tails buried in the center and polar head groups exposed to the aqueous environment (Figure 1.7B) [34]. Broadly, the hydrophobic core of the micelle mimics the hydrophobic core of the lipid bilayer, albeit with a more isotropic environment. The micelle is a malleable structure capable of maximizing favorable contacts between detergent and protein hydrophobic domains while permitting contacts between polar protein regions and the micelle surface or aqueous environment (Figure 1.7B; Figure 1.8) [127]. This permissive ‘escape to water’ behavior enables a TM domain to sample the hydrophobic micelle interior and polar surface in a manner representative of the equilibrium event experienced during membrane insertion in vivo (Figure 1.8) [1]. Further, such protein contacts with the micelle surface and exterior aqueous environment has been shown to accurately recapitulate the structures [128] and hydrophobic burial of TM domains found lining aqueous pores and channels [124].
Figure 1.8. Schematic diagram of the range of helix-detergent contacts available in micellar systems. Depicted above are examples of helix-micelle interactions, including scenarios in which the helix is fully/partially solvated and lying atop the micelle surface. The permissive nature of detergent micelles enables a large range in types of interactions including contact with water [1].

While primarily used in the extraction of membrane proteins from native membranes during purification, detergents have the added benefit of being highly soluble in aqueous solutions, making them amenable for membrane protein characterization [126]. An exhaustive number of detergents are available, varying in head group composition, size, charge, and hydrophobic tail length, exhibiting equally variable solubilizing behavior [126]. Detergents are typically classified based on their ‘harshness’ which is defined by how well the detergent can solubilize a TM domain while maintaining native structures and helix-helix contacts [126]. Often specific detergents are well suited for the purification and characterization of specific membrane proteins or biophysical techniques making the choice of the ideal detergent a matter of ongoing experimentation.

The ease of use, high solubility of protein-detergent complexes, and their relatively small size make detergents well suited for many biophysical characterizations including SDS-PAGE,
circular dichroism spectroscopy, fluorescence spectroscopy, oligomeric state determination, and even high-resolution structure determination. Many detergents have proven capable of maintaining native tertiary and/or quaternary structure [124,128,129], with the majority of current membrane protein structures utilizing detergent micelles (Protein Data Bank: [125]).

However, detergents are not without drawbacks. Not all membrane proteins are stable and capable of maintaining proper tertiary and/or quaternary structure in detergent micelles [126,128]. Structural features unique to lipid bilayers are sometimes required for correct folding and function of membrane proteins, particularly large multi-spanning proteins. Further, use of detergents on membrane proteins with large soluble domains can pose problems through unwanted unfolding of these susceptible domains [126]. The more isotropic nature of micelles relative to bilayers may not fully capture the hydrophobicity gradient observed across native bilayers, nor can micelles truly mimic the complexities of lipid packing and corresponding lateral pressure profiles. In these ways, detergent micelles are overly permissive, lacking some of the restrictions encountered and imposed within the planar lipid bilayer.

1.6.3 Lipid bilayers

Artificial lipid bilayers are the next step from micellar systems towards native membranes. Lipid bilayers created in the lab have the benefit of capturing the planar nature of native membranes and may be composed of a single lipid or any combination of lipid molecules, providing control over net charge, lipid tail packing, and fluidity [130]. Lipids vary from detergent molecules ultimately in their cylindrical vs. detergent conical shape imparted by the presence of two hydrophobic alkyl chains (Figure 1.7). Like detergents, lipids self-associate in
solution with their hydrophobic tails sequestered together, leaving the polar head groups exposed to the environment [131]. However, unlike detergents, lipids self-assemble into planar bilayers with their hydrophobic tails sandwiched in the middle [35]. In this manner, artificial lipid bilayers closely mimic the structures of native membranes, capable of imparting lateral pressure and mimicking the anisotropic hydrophobicity gradient across the width of the membrane [130].

While more technically demanding to prepare than detergents, lipid bilayers can more accurately capture the restrictive environment of native bilayers, while accounting for impacts of specific lipid-protein and lipid-lipid interactions (Figure 1.7C). Lipid bilayers (or liposomes) have been used to study native like oligomeric states [132], membrane protein functions [133], and insertion propensities successfully [7]. However, lipid bilayers are much larger than detergent-protein complexes, limiting their use in some biophysical assays.

1.7 Techniques for measuring membrane insertion

A variety of techniques exist to measure and infer membrane insertion and TM topology [30]. Described briefly below are some of the common biophysical and biological methods for establishing membrane vs. soluble states.
1.7.1 Biophysical techniques

1.7.1.1 Circular dichroism (CD)

CD provides a rapid assessment of protein secondary structure, allowing for structural comparisons in the presence and absence of membrane mimetics [134]. A change from unstructured to an α-helix is often inferred as a favorable association and folding within the membrane mimetic [135]. However, CD alone cannot distinguish between a surface bound and TM topology within lipid bilayers. Oriented CD (OCD), utilizing oriented lipid bilayers can provide information on helix tilt relative to the bilayer normal and may therefore be used as a direct measure of membrane insertion [136].

1.7.1.2 Tryptophan fluorescence and quenching

The fluorescent properties of tryptophan (Trp) make it an excellent probe of local environment. The emission maxima of Trp fluorescence spectra can reveal information on the polarity of the surrounding environment, with a strong blue shift indicating localization in a highly hydrophobic environment [137]. In the presence of membrane mimetics, a blue shift in Trp emission maxima can imply favorable association of a protein segment with the membrane environment. Aqueous buffer exposure and burial depth into lipid bilayers can be assessed through the addition of soluble quenchers (e.g. acrylamide) or membrane permeable/embedded quenchers (e.g. 10-doxylnonadecene, dibrominated lipids) [138–141].
1.7.2 Biochemical/biological techniques

1.7.2.1 Accessibility to chemical modifications

The introduction of non-native Lys or Cys residues into a sequence of study can be used to probe accessibility to chemical modifiers (e.g. sulfhydryl reagents, maleimides) [30]. Variation in membrane permeability of chemical modifiers can provide information on a protein’s insertion/translocated status, as well as topology of specific domains. While requiring modifications to the native sequence (removal of native Lys/Cys and introduction of specifically positioned non-native Lys/Cys residues), the alterations are often minimally disruptive and avoid addition of larger protein domains. Addition of chemical modifiers can be carried out on whole cells or proteoliposomes.

1.7.2.2 Proteolysis

A similar technique used to assess membrane protein insertion and topology involves the accessibility of soluble protein regions to protease degradation [30]. This technique relies on in vitro transcription/translation or isolation of native membranes followed by exposure to a protease. Subsequent protein migration rates on SDS-PAGE provide information as to whether a protein is accessible (i.e. translocated/soluble state) or inaccessible (i.e. membrane inserted) to the externally added protease. Membrane embedded sequences will present as protease resistant, with only translocated domains susceptible to proteases. The technique, however, is not highly sensitive and often requires the addition of a soluble protein domain onto the potential TM sequences for detection [30].
1.7.2.3 Localization of extra-membranous tags

The correct cellular localization of extra-membranous domains or enzymes can be used to assess the membrane inserted state of a neighboring potential TM domain. Common examples include the addition of alkaline phosphatase, β-lactamase, or β-galactosidase up/downstream of a sequence of study [30]. Both alkaline phosphatase and β-lactamase fail to fold and function if not localized to the periplasm of bacteria, while β-galactosidase only functions within the cytosol [30]. Placement of these enzymes up/downstream of a potential TM domain, followed by a test of their functionality, can therefore provide information on the insertion state and topology of the connected TM domain. However, such studies must be conducted in a complementary manner, assessing insertion in two separate constructs with enzymes of differing preference (i.e. periplasm vs. cytoplasm) [30]. Further, the assay assumes the neighboring soluble domains do not affect the membrane integration of potential TM sequences.

1.7.2.4 Glycosylation

Differential glycosylation is a well-established technique to assess the membrane insertion and topology of potential TM domains [30]. Experiments often involve the introduction of a sequence of study into an existing membrane protein. As described above in section 1.1.1.4, a commonly used system is the Lep construct which utilizes a modified version of the membrane protein leader peptidase (Figure 1.2), with engineered glycosylation sites flanking a sequence of
interest [11,29]. Examination of glycosylation patterns provide information on the fraction of membrane inserted (singly glycosylated) /translocated (doubly glycosylated) protein.

Studies utilizing differential glycosylation can be performed in whole cells or through transcription/translation systems, and detection is relatively simple (migration on SDS-PAGE) (Figure 1.2B). However, the technique has received some criticism for the assumption that neighboring protein regions and introduced glycosylation sites do not interfere with membrane integration or translocation [142–144].

1.8 Thesis hypothesis and outline

While great advances have been made in understanding the ‘molecular code’ for membrane protein insertion into native membranes [11,29], much remains unknown about the specific role(s) of the incipient TM sequence and the receiving environment of the membrane. ‘Marginally hydrophobic’ TM helices can lead to ambiguous TM domain prediction, representing potential ‘hot spots’ for membrane protein misfolding. How the cell detects these non-traditional TM sequences for membrane integration is unclear. As such, the degree to which the lipid bilayer can accommodate non-compatible components, such as polar and charged amino acids, and what qualifies as ‘sufficiently’ hydrophobic to reside within the bilayer, are influential factors. This situation allows us to address questions not only about what constitutes a TM domain, but more broadly, what is hydrophobicity in a membrane?

In this thesis, we use a peptide-based approach to investigate the features that define a TM domain, with emphasis as to how the receiving environment impacts membrane insertion.
We approach this by using several biophysical and biological techniques, in concert with various membrane mimetics, to assess how hydrophobicity varies between compositionally identical TM domains that differ only in hydrophobic patterning. Unhindered by extended protein domains and the inherent complexity of native membranes, studying peptide interactions with membrane mimetics allows us to isolate specific features of protein sequence and environment responsible for observed peptide-membrane interactions. To determine the limits of what can be defined as a TM domain, many of the TM sequences studied herein were designed to reside on the edge of membrane insertion, containing at times several polar residues. Thus, in Chapter 3, we designed a set of ‘extreme’ marginally hydrophobic sequences composed of nine polar Ser residues, nine apolar Leu residues and a single centrally placed Trp [145]. Sequences were varied to produce different patterns of hydrophobic and polar character. Using various biophysical techniques, we examined the highly sequence dependent nature of hydrophobicity.

Chapter 4 examines the boundaries of where hydrophobic patterning influences membrane insertion in a subset of TM helices of increasing polar character [146]. Clear thresholds for membrane ‘insertion’ were found that varied between artificial membranes and native membranes. Results imply the translocon channel lowers the hydrophobicity threshold for insertion compared to in vitro partitioning into artificial membranes.

In Chapter 5, we extended our studies to assess the roles of the isomers Leu and Ile in TM domains. The two amino acids, varying only in the placement of a methyl group within the side chain, can alter the surface topology of a TM domain in a manner to induce drastic changes in TM domain behavior [119,147–149]. The preference for one isomer over the other appears to be both sequence and lipid dependent.
2 Materials and Methods
2.1 Peptide synthesis

Peptides were synthesized as previously described [145,146]. Briefly, an automated PS3 peptide synthesizer (Protein Technologies Inc., AZ, USA) was used, with standard solid state Fmoc (N-(9-fluorenlyl)methoxycarbonyl) and HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (Novabiochem, ON, Canada) chemistry on a low-load PAL−PEG resin (Applied Biosystems, CA, USA) that produced an amidated C-terminus after cleavage. Coupling times were 30–45 minutes and were repeated twice. The initial Lys residue, all β-branched amino acids (Ile), and those that followed a β-branched residue in the sequence, were double coupled.

Peptides were cleaved using a standard TFA (trifluoroacetic acid) cleavage mix (88% TFA, 5% water, 5% phenol, 2% TIPS (triisopropylsilane) and reacting for 2 hours. Cleaved peptide was precipitated using ether, washed, and lyophilized prior to purification.

2.2 Peptide purification and quantification

All peptides were purified using reverse-phase high-performance liquid chromatography (RP-HPLC) with a C4 semipreparative column (250 x 21.20 mm, 300 Å pore size, Phenomenex, CA, USA). All peptides were purified to a single peak by RP-HPLC, masses confirmed using mass spectrometry.
2.2.1 Ser-Leu peptide series

Lyophilized ether-precipitated Ser-Leu peptides were initially dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), sonicated 5 minutes, then allowed to dry into a thin film. HFIP films were subsequently dissolved in 100% acetonitrile and then diluted with water until the solution became clear (generally ~ 30% acetonitrile). Solubilized samples were sonicated 5 minutes prior to injection on the column.

Typically, linear acetonitrile/water gradients were employed with initial conditions of 80% solvent A (95% water, 5% acetonitrile, and 0.1% TFA) and 20% solvent B (95% acetonitrile, 5% water, and 0.1% TFA) with a flow rate of 10 mL/min. Eluted peptide was lyophilized, HFIP treated, and stored as HFIP films at -20°C until further use.

HFIP films were dissolved in 2,2,2-trifluoroethanol (TFE) and sonicated 5 minutes prior to quantification. Peptides were quantified using tryptophan absorbance at 280 nm and a molar extinction coefficient of 4806 M⁻¹ cm⁻¹ as calculated under section 2.2.3.

2.2.2 Ala-Leu-Ser and Ala-Ile-Ser peptide series

Lyophilized ether-precipitated peptides were dissolved in water, sonicated 5 minutes prior to injection on the column. Typically, linear acetonitrile/water gradients were employed with initial conditions of 80% solvent A (95% water, 5% acetonitrile, and 0.1% TFA) and 20% solvent B (95% acetonitrile, 5% water, and 0.1% TFA) with a flow rate of 10 mL/min. Eluted peptide was lyophilized and stored as powder at -20°C until further use. Lyophilized peptide was
dissolved in water and diluted for quantification. Peptides were quantitated using tryptophan absorbance at 280 nm and an estimated molar extinction coefficient (5500 M\(^{-1}\) cm\(^{-1}\)) [150].

### 2.2.3 Calculation of tryptophan absorbance extinction coefficient in TFE

N-Acetyl-L-tryptophanamide (Sigma-Aldrich, MO, USA) (50 mg) was weighed out on an analytical scale and dissolved in 1 mL TFE and bath sonicated for 10 minutes. Aliquots of varying concentrations were created and absorbance at 280 nm detected using a quartz cuvette with a pathlength of 1 cm and an Ultrospec 3000 UV/Vis spectrophotometer (Pharmacia Biotech, Sweden). An absorbance over concentration curve was generated and data fitted with a linear regression function (Figure 2.1). Each data point was repeated 3 times. The slope of the line was taken as the extinction coefficient: 4806 M\(^{-1}\) cm\(^{-1}\).

![Graph](image)

**Figure 2.1. Absorbance vs. concentration curve of N-Acetyl-L-tryptophanamide in TFE.** Absorbance of N-Acetyl-L-tryptophanamide in TFE was recorded at 280 nm. Data was fit using a linear regression. The equation of the line and R\(^2\) value is reported in the figure. Each data point represents 3 independently prepared samples.
2.3 Detergent solubilization

Ser-Leu peptides were reconstituted into micellar sodium dodecyl sulfate (SDS) solutions using a protocol adapted from Duarte et al., [151]. Briefly, TFE-solubilized peptides were added to solubilized detergent in water and shaken for 15 min. Samples were lyophilized, and the resulting peptide-detergent powder was resuspended in water. It was determined empirically that a peptide to detergent ratio of 1:7000 was adequate to enable solubilization. Detergent samples were equilibrated for 1 hour prior to measurements.

Ala-Leu-Ser and Ala-Ile-Ser peptides, being soluble in water, were added directly to solutions of SDS (1:7000 peptide to detergent ratio) and allowed to equilibrate overnight.

2.4 Liposome preparation

Lipids in chloroform (Avanti Lipids Inc., AB, USA) were mixed with pre-dissolved peptides in TFE, vortexed, and dried into thin films under N₂ gas. Lipid-peptide films are reconstituted into water, frozen, and lyophilized again to remove residual chloroform. The remaining lipid-peptide powder is dissolved in aqueous buffer (10 mM Tris- HCl and 10 mM NaCl, pH 7.4) and freeze thawed 5 times by alternating between dry ice and a 50°C water bath. Freeze thawed samples are extruded by passing through a 0.2 micron sized polycarbonate low binding membrane 7 times. Extruded samples are left to equilibrate overnight prior to measurements. Pure and mixed lipids with transition temperatures higher than room temperature are heated during extrusion and maintained at a temperature above the transition temperature. Specifically, 1,2-dipalmitoyl-sn-glycero-3 phosphocholine (DPPC; Tm = 41°C) samples were
extruded at 50˚C and maintained at 45˚C. 1,2-dimyristoyl-\(sn\)-glycero-3-phosphocholine (DMPC; Tm = 24˚C) sample were extruded at 45˚C and maintained at 37˚C. 1-palmitoyl-2-oleoyl-\(sn\)-glycero-3-phosphocholine (POPC; Tm = -2˚C) and 1,2-dioleoyl-\(sn\)-glycero-3-phosphocholine (DOPC; Tm = -17˚C) were both extruded and maintained at room temperature.

2.5 Secondary structure assessment

Circular Dichroism (CD) was used to assess peptide secondary structure in various membrane mimetics. CD spectra were recorded on a Jasco J-720 CD spectropolarimeter (Jasco Inc., MD, USA) using a 0.1 cm pathlength quartz cuvette. Generally, samples were read using a 50 nm/sec scanning speed, measurements recorded between 190-250 nm. Detergent solubilized peptides (25 µM) in 175 mM SDS (1:7000 peptide to detergent ratio) were scanned 3 times. Lipid solubilized samples (2.5 mM POPC, DOPC, DPPC, DMPC) were scanned 7 times. Spectra represent the average of at least 3 replicates. All spectra were background subtracted and converted to mean residue molar ellipticity (MRE) using the formula below where \(mdeg\) is raw millidegree signal from the CD spectra, \(c\) is concentration in µM, \(l\) is cuvette pathlength in cm, and \(n\) is the number of amino acids.

\[
MRE = \frac{100 \times mdeg}{c \times l \times n}
\]
2.6 Tryptophan fluorescence

Fluorescence spectra were recorded on a Photon Technology International spectropolarimeter (Photon Technology International, NJ, USA) using a 1 cm path length quartz cuvette. Excitation slit widths were set to 2 nm, emission slit widths to 4 nm. Tryptophan was excited at 280 nm, and emission spectra were recorded between 300-400 nm with a step size of 2 nm and integration time of 1 sec. Detergent solubilized peptide (5 μM peptide, 35 mM SDS) were scanned 3 times. Peptides reconstituted into lipid bilayer samples (10 μM peptide, 2.5 mM POPC/DOPC/DPPC/DMPC) were scanned 3-7 times. All samples were background subtracted, and the wavelength of maximal fluorescence emission intensity recorded.

2.7 Tryptophan fluorescence quenching

2.7.1 Dibrominated lipid quenching

Dibrominated lipids, 1-palmitoyl-2-(6,7-dibromo)stearoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-(9,10-dibromo)stearoyl-sn-glycero-3-phosphocholine, or 1-palmitoyl-2-(11,12-dibromo)stearoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids Inc., AL, USA) were mixed with POPC at various mol % amounts (0, 10, 20, 30, 40). Peptides were reconstituted into membranes containing various amounts of quencher lipids, tryptophan excited at 280 nm, and emission spectra recorded between 310-400 nm. The area under the fluorescence curves was used to assess the extent of Trp quenching. Stern-Volmer plots were obtained by plotting initial fluorescence values / fluorescence in presence of quencher ($F_0/F$) vs. mol % brominated lipid. The slope values of the corresponding curves provide information on the degree of Trp quenching.
quenching and therefore burial within the membrane. The larger the slope, the more quenching, the deeper the peptides reside within the membrane [138,152]. Three different peptide/protein controls were used to represent i) a membrane inserted state (sequence: KKK-AAAAAALLLWLAAAAAAA-KKK, [146]), ii) a surface bound state (sequence: KKK-ASASASLLLWLLASASASA-KKK, [146]), and iii) a non-membrane interacting, soluble state (bovine serum albumin (BSA)).

2.7.2 Acrylamide quenching

Acrylamide was dissolved in water to a final concentration of 5 M and titrated into peptide samples reconstituted into membranes (0.05 M acrylamide per addition). Tryptophan was excited at 295 nm rather than 280 nm to decrease absorbance by acrylamide. Emission spectra were recorded between 310-400 nm using a quartz cuvette with a micro stir bar. Samples were equilibrated with stirring prior to recording emission spectra. The area under the fluorescence curves was used to assess the extent of Trp quenching. Stern-Volmer plots were obtained by plotting initial fluorescence values / fluorescence in presence of quencher ($F_0/F$) vs. concentration of acrylamide (M). The slope values of the corresponding curves provide information on the degree of Trp quenching and therefore availability to the aqueous quencher [139,140].
2.8 SDS-PAGE and gel shifts

Ser-Leu peptides were prepared for SDS-PAGE by dissolving peptide-detergent samples (see section 2.3 on Detergent reconstitution) in 1X sample loading buffer prepared without detergent. All samples were equilibrated for 1 hour at room temperature prior to loading. Aqueous soluble peptides (Ala-Leu-Ser and Ala-Ile-Ser peptides) were prepared by addition of NuPAGE LDS sample buffer (4X, Invitrogen, CA, USA) and diluted appropriately. Samples were heated for 10 minutes (50˚C) and centrifuged for 10 minutes (12 000 rpm) prior to loading. All samples were run on 12% NuPAGE Bis-Tris protein gels (Invitrogen, CA, USA) in MES running buffer at 200 V for approximately 30 minutes and stained with GelCode Blue Stain Reagent (Pierce Biotechnology, MA, USA) or Coomassie Blue dye. Migration rates were calculated as percent peptide gel shifts using the equation below [153].

\[
% \text{Gel shift} = \frac{\text{Actual molecular weight} - \text{Theoretical molecular weight}}{\text{Theoretical molecular weight}} \times 100\%
\]

2.9 RP-HPLC retention time

Reverse phase HPLC on a Zorbax StableBond C-18 analytical column (Agilent Technologies, CA, USA) was performed using 20 μg of peptide dissolved in 1 mL of mobile phase solvent. The retention time of each peptide sample was normalized to the retention time of an internal standard (uracil, 5 μg in 50 μL of water) injected prior to the addition of the sample to the column. Retention time values are therefore reported as unitless. Ser-Leu peptides were eluted using a mobile phase composition of 60% solvent A (95% water, 5% acetonitrile, and
0.1% TFA) and 40% solvent B (95% acetonitrile, 5% water, and 0.1% TFA). Ala-Leu-Ser peptides with 5-Leu were eluted using a mobile phase composition of 60% solvent A and 40% solvent B and 65% solvent A and 35% solvent B for samples with 7-Leu.

### 2.10 Peptide models

Peptide core sequences (lacking Lys-tags) were modelled as ideal helices using the Chimera1.12 software [154]. Ser, Trp, Leu, and Ile were modelled with the most probable \( \chi \) (Chi) angles as observed in soluble [155] and TM helical domains [156] (Table 2.1). All peptide models were tested and adjusted (if necessary) for steric clashes between side chains.

#### Table 2.1 Prevalent rotamer angles in soluble and transmembrane helices

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Soluble helix( ^a )</th>
<th>Transmembrane helix( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser</td>
<td>( \chi_1 , -66^\circ , (48%) )</td>
<td>( \chi_1 , -60^\circ , (65%) )</td>
</tr>
<tr>
<td>Trp</td>
<td>( \chi_1 , -180^\circ , \chi_2 , 85^\circ , (36%) )</td>
<td>( \chi_1 , -60^\circ , \chi_2 , 120^\circ , (28%) )</td>
</tr>
<tr>
<td>Leu</td>
<td>( \chi_1 , 180^\circ , \chi_2 , 62^\circ , (47%) )</td>
<td>( \chi_1 , -60^\circ , \chi_2 , 180^\circ , (52%) )</td>
</tr>
<tr>
<td>Ile</td>
<td>( \chi_1 , -67^\circ , \chi_2 , 170^\circ , (85%) )</td>
<td>( \chi_1 , -60^\circ , \chi_2 , 180^\circ , (72%) )</td>
</tr>
</tbody>
</table>

\( ^a \)\( \chi_1 \) and \( \chi_2 \) angles observed in soluble \( \alpha \)-helices are reported [155]. \( ^b \) \( \chi_1 \) and \( \chi_2 \) angles observed in transmembrane \( \alpha \)-helices are reported [156]. The probability of the specified \( \chi \) angle occurring in a dataset of known protein structures is listed in parentheses.
2.11 Lipid accessible surface area (LASA) determination

LASA was calculated using the QUILT software [157,158] and peptide models generated in Chimera1.12 (see section 2.10). A probe of 1.88 Å radius was used to mimic the size of a methylene group and therefore to provide a measure of how accessible a peptide’s surface area is to a lipid tail.

2.12 Förster resonance energy transfer (FRET)

Peptide oligomeric states were assessed through FRET assays in both SDS micelles and POPC lipid bilayers. Peptides were labeled with FRET fluorophores on resin. Briefly, 100 mg of peptide bound resin was reacted with 8 mg of either dansyl or dabsyl chloride dissolved in DMF supplemented with DIEA. Peptide resins were N₂ blanketed, protected from light, and nutated for 1 hour. Resins were then drained of labelling solution and washed with DMF. The labeling reaction was then repeated and allowed to run overnight. Peptide resins were washed with DMF and methanol the following day and subsequently cleaved from the resin and purified as described in section 2.2. Purified FRET labeled peptides were dissolved in TFE and dabsyl-labeled peptides quantitated by absorbance at 470 nm using the extinction coefficient 29 600 cm⁻¹ M⁻¹. Corresponding dansyl-labeled peptides were quantitated by first taking CD spectra of dabsyl-peptides to calculate accurate MRE values from which raw millidegree values from CD spectra of the appropriate dansyl-labeled peptide may be used to back calculate concentration (see MRE calculation in section 2.5). Once quantitated, FRET labeled peptides and unlabeled peptide were mixed to the appropriate ratios in TFE and either added to concentrated aqueous SDS or to chloroform dissolved lipids. Detergent and lipid samples were prepared in the same
manner as described in section 2.3 and 2.4, respectively. Detergent and lipid samples were brought up in aqueous buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.4) and allowed to equilibrate overnight prior to reading. Fluorescence spectra were recorded using a Spectramax i3x plate reader (Molecular Devices, CA, USA) in corning opaque plates. Dansyl was excited at 341 nm and emission spectra recorded between 350-400 nm. All samples were background subtracted and values for total area under the curve used to calculate the degree of FRET.

2.13 Detergent titration studies

Procedures were adapted from Alvares et al., [159]. Increments of 0.2 mM SDS from a concentrated stock supplemented with peptide (to avoid changes in total peptide concentration over time) were added to peptide samples (15 μM in 10 mM Tris-HCl, 10 mM NaCl, pH 7.4) and allowed to equilibrate for eight minutes prior to recording a spectra. Titrations were carried out until the detergent CMC was surpassed (3.46 mM SDS, see section 2.13.1). Data points were fit with inhibitory dose-response curves using GraphPad Prism 7.0.

2.13.1 Critical Micelle Concentration (CMC) determination

The CMC of SDS in the buffer of choice (10 mM Tris-HCl, 10 mM NaCl, pH 7.4) was calculated through assessment of fluorescence change of 8-anilinonaphthalene-1-sulfonic acid (ANS) upon subsequent additions of detergent. Fluorescence of ANS was recorded on a Photon Technology International spectropolarimeter (Photon Technology International, NJ, USA) using a 1 cm path length quartz cuvette. Excitation and emission slit widths were set to 3 nm. ANS was
excited at 370 nm, and emission spectra were recorded between 450-550 nm with a step size of 1 nm and integration time of 1 second. Spectra were background subtracted and emission maxima recorded. SDS was added to a solution of ANS in 1.0 mM increments from a concentrated stock. The concentration of ANS was adjusted for volume changes upon SDS addition and fluorescence emission maxima plotted against detergent concentration. Linear regressions were fit to the fluorescence curves and with the intersection taken as the CMC value.

![Graph showing CMC determination for SDS](image)

**Figure 2.2. Determination of critical micelle concentration for SDS under conditions of study.** Arbitrary fluorescence units (AFU) are plotted vs. SDS concentration (mM) in buffer (10 mM Tris-HCl and 10 mM NaCl, pH 7.4). Linear regression was used to find the optimal fit for values below and above the CMC. Precise measurements of the CMC were determined using the equation for the two linear fits (below CMC, \(Y = 318.7 \times X + 7343\); above CMC, \(Y = 3212 \times X - 2670\)). Values are obtained from three independent experiments; error is reported as standard deviation.

### 2.14 Protease protection assay

Lipid-reconstituted peptides (10 µM) were treated with proteinase K (100 µg/mL, BioShop Canada Inc., Canada) in aqueous buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.4) supplemented with 5 µM CaCl₂. Samples were incubated at 37°C and 30 µL removed after each time point and protease activity blocked by the addition of 10 mM PMSF (200 mM stock dissolved in isopropanol). Protease inactivated samples were frozen at -20°C until further use.
Digestions were repeated 3 times. Frozen samples were supplemented with 8 µL of 4X NuPAGE, sample loading buffer (Invitrogen, CA, USA), heated (50°C) for 10 minutes, and centrifuged for 10 minutes (12 000 rpm) prior to loading the gel (12% NuPAGE Bis-Tris protein gel with MES running buffer). Gels were run at 200 V for ~35 minutes and stained using Coomassie Blue dye. Densitometry of bands was determined using ImageJ software with background subtraction [160]. All samples were normalized to an untreated sample.

2.15 Prediction of translocon-mediated free energy of insertion

Insertion energies were predicted for peptide sequences using the online ΔG_{app} predictor (http://dgpred.cbr.su.se) [11,29]. Options for default settings (helix length of 15-30 amino acids) and subsequence allowance, to produce the lowest ΔG_{app} (minimum helix length of 15 amino acids), were available. ΔG_{app} values for the peptide sequences within this thesis were predicted using the subsequence allowance applied, to account for their marginal average hydrophobic. The core of the peptide sequences (Lys-tags omitted) with added GGPG--GPGG flanks was run through the online server, and the corresponding ΔG_{app} values and predicted TM segments were recorded. The more negative a ΔG_{app} value, the more favorable the free energy of membrane insertion.
2.16 Experimentally derived translocon-mediated free energy of membrane insertion

All LepB constructs used carried two acceptor sites for N-linked glycosylation (Asn-Ser-Thr) and (Asn-Ala-Thr), as indicated in Figure 1.2. LepB constructs encoding different peptide sequences were generated by modifying the lepB gene in the pGEM-1 vector containing SpeI and KpnI restriction sites. Double-stranded oligonucleotides encoding peptide sequences with flanking GGPG…GPGG segments including an N-terminal SpeI and a C-terminal KpnI restriction site were created by annealing two pairs of complementary oligonucleotides with overlapping overhangs. All sequences encoding different peptide sequences and the pGEM-1 vector were digested with SpeI and KpnI, followed by insertion of the peptide sequences into the cut vector by ligation. Oligonucleotides were diluted to a concentration of 20 mM. 20 µL of each forward and reverse complementary segments were mixed with 4.4 ml annealing oligonucleotide buffer (20 mM Tris-HCl pH 7.4, 20 mM MgCl2 and 500 mM NaCl) and heated at 85°C for 10 min. Samples were kept in the heating block until the temperature was 30°C. 20 µL each of the annealed samples were mixed and heated at 65°C for 5 min. Samples were kept in the heating block until the temperature was 30°C. The annealed segment was ligated into the cut vector overnight at 16°C followed by transformation into bacteria. All inserts and mutagenesis were confirmed by sequencing of plasmid DNA at Eurofins MWG Operon (Ebersberg, Germany).

The TNT Quick coupled transcription/translation system (Promega, WI, USA) was used to transcribe and translate cloned constructs cloned in pGEM-1. 1 µl of DNA template (150-200 ng/µl), 1 µl of [35S]Met (10 mCi; 1 Ci/437 GBq), and 1 µl of dog pancreas rough microsomes were mixed with 10 µl of TNT lysate mix. Samples were incubated for 90 min at 30°C. The full
sequence of the expressed LepB gene, with an introduced sequence of interest (5L0S₀), is shown in Figure 2.3.

MANMFALILVIATLVTGILWCVDKFFFFAPKRERRRQAADAQAAAGDSLKDKatLKKVAPKFWLETGASVFVPVLAIIVLIVRSFIYEFPQIPSGSMMPTLNSTDILVEKFAYGIKDPIYQKTLIETGHPKRGDIVVFKYEDPKLDYIKRAVGLPVDKVTYDPVSCTRLITIQPGCSSQACENALPVYNSVEPSDFVQTFSSRNGGEATSGFFEVPKQETKENGIRSETSGGPGAAAAALLWLAAAAAAAAAGPGGVPGQQNATWIVPPGQYFMMGDNDNSADSRYWGFVPEANLVGRATAGGSSDKQEGEWPTGLRLSRRGGIH

Figure 2.3. Full sequence of the LepB 5L0S₀ construct. The two N-terminal transmembrane segments in LepB are underlined, the 5L0S₀ segment is in red with flanking GGPG…GPGG residues in italics, and the two glycosylation sites are in blue.

Samples were mixed with SDS sample buffer and incubated at 30°C for 15 min before loading on a 10% SDS/polyacrylamide gel. Proteins bands were visualized in a Fuji FLA-3000 phosphoimager (Fujifilm, Tokyo, Japan). The Image Gauge V 4.23 software (Fujifilm, Tokyo, Japan) was used to generate a two-dimensional intensity profile of each gel lane, and the multi-Gaussian fit program from the Qtiplot software package (www.qtiplot.ro) was used to calculate the peak areas of the protein bands. An apparent equilibrium constant between the membrane-integrated and non-integrated forms was calculated as $K_{app} = f_{1g}/f_{2g}$, where $f_{1g}$ is the fraction of singly glycosylated LepB molecules and $f_{2g}$ is the fraction of doubly glycosylated LepB molecules. The results were then converted to apparent free energies of membrane insertion via the equation below. All experiments were conducted at 30°C.

$$\Delta G_{app} = -RT\ln K_{app}$$

Cloning of the LepB constructs and experimental calculations of free energy of translocon-mediated membrane insertion into rough microsomes was carried out by Dr. Nina Schiller in Dr. Gunnar von Heijne’s laboratory at Stockholm University.
3 Hydrophobic blocks facilitate lipid compatibility and translocon recognition of transmembrane protein sequences


Author contributions: TAS and CMD designed research. TAS performed research on peptides in membrane mimetics. NS carried out translocon-mediated membrane insertion of sequences. TAS analyzed the data. TAS, CMD, NS, and GvH wrote the paper.
3.1 Introduction

The hydrophobicity of a potential TM segment can, in principle, be evaluated by simply averaging the hydrophobicity of its component amino acids along the TM domain’s length. However, the prediction of marginally hydrophobic TM segments remains difficult. While studies by Hessa et al. using an ER-insertion assay have led to a deeper understanding of sequence and position-specific influences on membrane insertion [11,29], the specific roles of hydrophobic and helical patterning remain less explored.

The hydrophobicity of a TM segment can be influenced concomitantly by residue patterning of local sequence, secondary structure propensity, and/or environment (water vs. membrane) [1]. These overall considerations raise the question as to how a segment is identified as belonging in an integral membrane protein, and more broadly, given that hydrophobicity is a water-driven phenomenon, what is the role of a TM segment’s lipid compatibility with the target bilayer in determining the candidacy of a protein segment for prospective membrane insertion? To address these issues systematically, here we have synthesized a library of peptides of identical composition but varied sequence, with average segmental hydrophobicity suitable for membrane insertion (≥0.4 by the Liu-Deber hydropathy scale [8]), and undertaken an in vitro biophysical analysis of their membrane compatibility, complemented by an in vivo translocon-dependent ER-insertion assay for the corresponding sequences. The results reveal the importance of local residue patterning, particularly with respect to the presence and positioning of hydrophobic blocks, on the suitability of a given peptide segment for transmembrane insertion.
3.2 Results

3.2.1 Design of Ser-Leu peptide sequences with varied patterns of hydrophobic and polar residues

We hypothesized that detecting subtle differences in peptide partitioning among micellar or isotropic apolar media might best be achieved by initially imposing ‘extremes’ of TM sequence polarity and/or amphipathicity. We therefore designed 19-residue TM sequences that contain an equal number of polar and hydrophobic residues – nine Ser and nine Leu residues – each with a centrally-positioned Trp residue as a fluorescent probe. As the most commonly occurring amino acid in native TM helices, Leu was a natural choice [43], while Ser is the most commonly occurring polar residue (Thr being a close second) and can participate in both side chain-side chain and side chain-backbone H-bonds [43]. As well, synthesis requirements of β-branch-rich peptides rendered Thr as a less feasible choice. When averaged over the full 19-residue core segment, this ‘9 x 9 x 1’ residue composition exceeds the hydrophobicity threshold (0.4 by the Liu-Deber hydropathy scale) required for peptide partitioning into apolar phases with an averaged hydrophobicity of 1.16 [8], yet remains similar to that of ‘marginally hydrophobic’ TM helices (predicted ΔG_{app} around 1.4 kcal/mol) as measured by the ‘biological’ hydrophobicity scale of Hessa et al. [11,29].

Broadly, the peptides we synthesized may be categorized into two groups, i.e., Leu-block vs. non-Leu-block (Table 3.1) in which a ‘Leu-block’ peptide was defined as a sequence containing a contiguous stretch of more than four Leu residues in the primary sequence. Sequences failing to meet these criteria were categorized as ‘non-Leu-block’ peptides. The
peptide LSL was categorized as a non-Leu-block peptide due to the presence of a Ser-block that is larger than the present Leu-block and the lack of overlap of the present Leu-block with the center of the peptide. For sequences where the central Trp is placed within a Leu-block, the Trp residue is considered non-interrupting to the Leu-block character. While numerous permutations may be envisaged, here sequences were varied to create patterns of hydrophobic and helical character in the form of (i) continuous stretches of hydrophobic residues in the primary sequence (Leu-blocks); (ii) an amphipathic sequence (Leu-face and Ser-face) when folded into a helix; and (iii) examples of equally or randomly distributed Leu and Ser residues (Table 3.1; Figure 3.1A).

Peptide nomenclature is as follows: Leu-block peptides have the number of Ser on the N-terminus designated (i.e., S9L9 indicates nine Ser residues on the N-terminus followed by a single Trp residue and nine Leu on the C-terminal side). Non-Leu-block peptides are named according to the specific Ser-Leu patterning (i.e., SL alternates Ser-Leu residues, while SL\textsuperscript{amp} indicates Ser-Leu residues positioned to create an amphipathic helix). Top-down views of the Ser-Leu peptides (Figure 3.1B) illustrate how variations in hydrophobic patterning lead to varying exposure of Leu residues on the surface of the peptides (i.e., hydrophobic character that extends around the circumference of the peptide, vs. hydrophobicity that is concentrated on a single face of the helix).
Table 3.1. Sequences of designed Ser-Leu peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Leu-block</td>
<td></td>
</tr>
<tr>
<td>SLn</td>
<td>KKK–<strong>S</strong>LSLS<strong>S</strong>LSWLSLSLSL–KKK</td>
</tr>
<tr>
<td>LSL</td>
<td>KKK–<strong>L</strong>LLLLSSSSWSSSSSLLLL–KKK</td>
</tr>
<tr>
<td>SLscr1</td>
<td>KKK–<strong>S</strong>LLSLS<strong>S</strong>WLLSLLSLLS–KKK</td>
</tr>
<tr>
<td>SLscr2</td>
<td>KKK–<strong>S</strong>LLSL<strong>S</strong>LSWLSLLSSLL–KKK</td>
</tr>
<tr>
<td>SLamp</td>
<td>KKK–<strong>S</strong>LLS<strong>S</strong>LLSSWLLSSLLSLL–KKK</td>
</tr>
<tr>
<td>Leu-block</td>
<td></td>
</tr>
<tr>
<td>S9L9</td>
<td>KKK–<strong>S</strong>SSSSSSWSLLLLLLLLL–KKK</td>
</tr>
<tr>
<td>S5L9</td>
<td>KKK–<strong>S</strong>SSSSLLLLWLLLLSSSS–KKK</td>
</tr>
<tr>
<td>S3L9</td>
<td>KKK–<strong>S</strong>SSSSLLLLWLLLLSSSS–KKK</td>
</tr>
<tr>
<td>S2L9</td>
<td>KKK–<strong>S</strong>SSSSLLLLWLLLLSSSS–KKK</td>
</tr>
<tr>
<td>S1L9</td>
<td>KKK–<strong>S</strong>SSSSSSWSLLLLSSSS–KKK</td>
</tr>
<tr>
<td>S0L9</td>
<td>KKK–<strong>S</strong>SSSSSSWSLLLLSSSS–KKK</td>
</tr>
</tbody>
</table>

Ser, Leu, and Trp residues are shaded blue, yellow, and black, respectively. Peptides are tagged with three Lys residues at each of the N- and C-termini (black) [161]. Sequences are categorized as either non-Leu-block or Leu-block as described in section 3.2.1.

Figure 3.1. Helical models of Ser-Leu peptides. Peptides (excluding Lys-tags) were modeled as α-helix monomers (see section 2.10) and are shown with the van der Waals radii of Ser (blue), Leu (yellow), and Trp (gray) side chains, respectively. (A) View perpendicular to the helix axis, the N-terminus is at the top and the Trp residue oriented into the plane of the page. (B) Top-down view parallel to the helix axis from the peptide N-terminus. Peptide sequences are listed in Table 3.1.
3.2.2 SDS-PAGE migration rates and Trp blue shifts of the Ser-Leu peptide library

Interactions between membrane proteins and detergents are complex and have been classically shown to occur in a sequence-specific manner, leading to variations in detergent coating, SDS-PAGE migration rates, and Trp-fluorescence [124,127,129,153,162]. These effects are manifested in the wide variations of migration rates for the Ser-Leu peptide library we observed on SDS-PAGE, with Leu-block peptides tending to migrate more slowly on the gel than the compositionally-identical non-Leu-block peptides (Figure 3.2). Clustered Leu residues may increase a peptide’s local hydrophobicity, resulting in increased interaction with the hydrophobic detergent and thus an increase in the observed peptide-detergent complex size. The peptides SL_{scr1} and SL_{scr2} migrate the fastest, consistent with the absence of a Leu-rich locus in the scrambled peptides that would promote favorable burial into a hydrophobic micelle. The similarly fast migration of the amphipathic SL_{amp} peptide is likely a result of lengthwise exposure of both hydrophobic (Leu-face) and polar (Ser-face) residues. A combination of favorable interactions of the Leu-face with the interior of the micelle and favorable interactions of the Ser-face with water, position the peptide on the micelle surface rather than buried in the interior, decreasing the observed peptide-detergent size. Interestingly, significant variations are found in the SDS-PAGE migration rates (% peptide gel-shift) among the Leu-block peptides themselves, with S3L9 traveling the slowest. The limited solubility of the non-Leu-block sequences SL_{n} and LSL led to their exclusion from further experiments in detergent and liposomes.
Ser-Leu peptide SDS-PAGE gel migration. SDS-PAGE gel of SDS-soluble Ser-Leu peptides. Ser-Leu peptides have identical molecular weights (2774 Da) and composition but display significant differences in % peptide gel-shift (p < 0.05, one-way ANOVA). Peptide samples were run on a single gel and then arranged according to Leu-block properties. Lane 1 is the molecular weight marker (Mark12™). Lanes 2-4 are the non-Leu-block Ser-Leu sequences. Lanes 5-10 are the Leu-block sequences. Peptides SL$\alpha$ and LSL are omitted from the gel due to their low solubility.

In conjunction with SDS-PAGE experiments, the Ser-Leu peptides were examined for the occurrence and extent of blue shifts in Trp fluorescence spectra – ostensibly a measure of the ‘degree of burial’ of the Trp moiety in the hydrophobic region of the SDS micelles. We found that all Ser-Leu sequences exhibit Trp blue shifts in the presence of SDS micelles, varying between 320-335 nm vs. the typical aqueous position near 350 nm (Figure 3.3), indicating their overall micelle compatibility and detergent coating of the peptides, albeit no pattern could be discerned between the extent of the Trp blue shift and either the peptide migration position on SDS-PAGE, or of the presence/absence of Leu-blocks.
Figure 3.3. Ser-Leu peptide tryptophan emission spectra in detergent micelles. Trp fluorescence spectra show that all Ser-Leu peptides exhibit blue shifts in the presence of SDS detergent micelles. Spectra represent the average of three independent experiments. Peptides SL$_n$ and LSL were excluded as they formed aggregates on SDS-PAGE (Figure 3.2). A dashed horizontal line represents aqueous exposed Trp fluorescence emission maxima (350 nm).

3.2.3 Structural assessment of Ser-Leu peptides in detergent micelles by circular dichroism spectroscopy

CD spectra of the Ser-Leu peptide series in SDS micelles established that peptide secondary structure is highly sensitive to sequence and patterning (Figure 3.4A, B). Peptides exhibited helical CD patterns, with ellipticities ranging from \( \text{ca. } -5000^\circ \) (non-Leu-block peptides) to \(-13,000^\circ \) (SL$_{amp}$ and Leu-block peptides) at 222 nm. The only peptide not to display at least a partial helical conformation was SL$_n$ (a classic ‘silk-like’ sequence for $\beta$-sheet structures) that perhaps unsurprisingly exhibited a CD spectrum consistent with $\beta$-sheet features in SDS micelles (Figure 3.4A), and could not be observed on SDS-PAGE, suggesting poor solubility and/or aggregation.
Figure 3.4. Ser-Leu peptide secondary structure in SDS micelles. CD spectra of Ser-Leu peptides in SDS micelles (1:7000 peptide to SDS ratio). (A) Non-Leu-block peptides. SL_n notably adopts a β-sheet structure. (B) Leu-block peptides. Significant differences in helicities exist between the different categories of Ser-Leu peptides (p < 0.05, one-way ANOVA). Spectra shown are an average of at least 3 independent experiments.

3.2.4 Assessment of peptide apparent hydrophobicity by reverse phase HPLC

Consistent with the trends observed above in SDS-PAGE migration positions and helicities in CD spectra, measurements of apparent peptide hydrophobicity through HPLC retention times on a C-18 column indicated an increased hydrophobicity for peptides with continuous stretches or ‘blocks’ of hydrophobic character. Thus, as shown in Table 3.2, the overall range in retention times (normalized to the retention of uracil and therefore unitless) for this series of compositionally identical peptides varied from 0.31 to 4.14, with values that could qualitatively be sub-divided into earlier-eluting non-Leu-block sequences (0.31-1.05) and later-eluting Leu-block sequences (1.72-4.14). An important exception was the SL_amp peptide, which
eluted at 2.10, essentially within the center of the Leu-block range (Table 3.2). This result indicates that in the relatively isotropic environment of the solvent-column interface, the hydrophobic ‘Leu-face’ of this amphipathic peptide, similar to a Leu-block, exhibits a strong interaction with the alkyl chains of the C-18 column. In this regard, the amphipathic peptide with a Leu-face is found to display similar hydrophobic character as a Leu-block peptide. Assessment of peptide helical character in the HPLC solvents (60% solvent A, 40% solvent B), revealed most of the Ser-Leu peptides (SLₙ, SLscr₂, SLamp, and the Leu-block peptides) to be of similar helicity (Figure 3.5). LSL notably adopted a mixed random coil/α-helix conformation, while SLscr₁ failed to adopt any helical character. No correlation was observed between Ser-Leu peptide helicity in HPLC solvents and retention time within a C-18 column.

Table 3.2. Ser-Leu peptide retention on a C-18 column

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Normalized retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Leu-block</td>
<td></td>
</tr>
<tr>
<td>SLₙ</td>
<td>0.53</td>
</tr>
<tr>
<td>LSL</td>
<td>0.31</td>
</tr>
<tr>
<td>SLscr₁</td>
<td>0.63</td>
</tr>
<tr>
<td>SLscr₂</td>
<td>1.05</td>
</tr>
<tr>
<td>SLamp</td>
<td>2.10</td>
</tr>
<tr>
<td>Leu-block</td>
<td></td>
</tr>
<tr>
<td>S9L9</td>
<td>1.73</td>
</tr>
<tr>
<td>S5L9</td>
<td>1.90</td>
</tr>
<tr>
<td>S3L9</td>
<td>3.48</td>
</tr>
<tr>
<td>S2L9</td>
<td>4.14</td>
</tr>
<tr>
<td>S1L9</td>
<td>2.57</td>
</tr>
<tr>
<td>S0L9</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Normalized retention time values in a C-18 column under isocratic mobile phase conditions. Peptide retention time is normalized to that of uracil and therefore is a unitless value. Non-Leu-block peptides (exception of SLamp) elute significantly faster than Leu-block peptides (p <0.01, one-way ANOVA). Values are an average of at least three independent experiments. Error values were no larger than ± 0.2 standard deviation for each point.
Figure 3.5. Ser-Leu peptide helicities in HPLC solvents. CD spectra of Ser-Leu peptides in HPLC solvents (60% solvent A, 40% solvent B). (A) Non-Leu block peptides. (B) Leu-block peptides. Ser-Leu peptides exhibit similar helicity with the exception of LSL and SL_{scr1} which are notably less helical. Spectra shown are an average of at least two independent experiments.

3.2.5 Ser-Leu peptides partition into POPC liposomes

To assess whether the Ser-Leu peptides could satisfactorily interact not only with micellar detergent, but also with phospholipid bilayer phases, we reconstituted each of the peptides into POPC liposomes, and measured their propensity to adopt helical structures, accompanied by observation of the anticipated blue shifts of their central Trp residue fluorescence. CD spectra confirmed helical conformations of the Leu-block peptides in the presence of POPC liposomes (Figure 3.6A), with ellipticity values comparable to those observed in SDS. Of the non-Leu-block peptides, only SL_{amp} was soluble, similarly adopting a helical conformation (Figure 3.6A). SL_{scr1} visibly aggregated and SL_{scr2} adopted a β-sheet-like conformation. All helical peptides exhibited strong blue shifts in a manner expected for a Trp
residue buried in the interior of the bilayer, with values clustered around 320-328 nm (Figure 3.6B). SL<sub>amp</sub> exhibited the weakest blue shift (to 332 nm) (Figure 3.6B).

![Graphs showing CD and Trp fluorescence spectra](image)

**Figure 3.6. Ser-Leu peptide secondary structure and Trp fluorescence in POPC liposomes.** (A) CD spectra of Ser-Leu peptides in POPC liposomes (1:250 peptide to lipid ratio). SL<sub>scr1</sub> was insoluble in POPC while SL<sub>scr2</sub> adopts a β-sheet like conformation. SL<sub>amp</sub> and the Leu-block sequences all adopt helical conformations. Spectra shown represent an average of three independent experiments. (B) Trp fluorescence spectra show that all the Leu-block peptides and SL<sub>amp</sub> exhibit blue shifts in the presence of POPC lipid bilayers. A dashed horizontal line represents aqueous exposed Trp fluorescence emission maximum (350 nm).

3.2.6 Translocon-mediated insertion of Ser-Leu sequences into the ER membrane

The data from liposome partitioning confirm that the Ser-Leu sequences containing a Leu-block are lipid compatible, and therefore good candidates for membrane insertion. Thus, in these instances, the high hydrophobicity imparted by nine Leu residues is apparently capable of masking the polarity of some of the Ser residues. To examine this extreme scenario in the context of translocon-mediated membrane insertion, the corresponding Ser-Leu sequences were incorporated into the Lep construct [11] and translated *in vivo* in the presence of ER-derived dog
pancreas rough microsomes (Figure 3.7). In this assay, the sequence of interest (red) is placed downstream of two native TM segments (black) and flanked by glycosylation sites (G1, G2). If the translocon inserts the Ser-Leu sequence (red) into the membrane, only one glycosylation site (G1) will be exposed to the oligosaccharide transferase enzyme within the ER lumen and become glycosylated (mono-glycosylation). If the Ser-Leu sequence fails to insert and is translocated across the membrane into the ER lumen, both glycosylation sites (G1 and G2) will be exposed to oligosaccharide transferase and glycosylated (di-glycosylation). The degree of glycosylation may be differentiated by size on SDS-PAGE with di-glycosylated proteins running slower than mono-glycosylated proteins. In this manner, the relative extents of mono- and/or di-glycosylation provide a direct measurement of the extent of translocon-mediated insertion of a given Ser-Leu segment ($K_{\text{app}}$; see Chapter 2 for details). We found that Ser-Leu sequences containing a Leu-block generally inserted well ($K_{\text{app}} > 1$), while non-Leu-block sequences failed to insert ($K_{\text{app}} < 1$) (Figure 3.7). Perhaps most notably, the amphipathic SL\text{amp} sequence failed to insert into the microsomal membrane, despite its strong interaction with the membrane-mimetics reported above. Corresponding experimental $\Delta G_{\text{app}}$ values derived from the $K_{\text{app}}$ values are listed in Table 3.3 along with the % inserted protein. A $K_{\text{app}}$ value $> 1$ produces a favorable negative $\Delta G_{\text{app}}$ values, and a higher degree of membrane insertion.

We further observed that the sequence with the most centrally located Leu-block (S5L9) does not exhibit the most favorable insertion energy (Table 3.3). Instead, the sequence with the most N-terminally located Leu-block (S0L9) displayed the most favorable experimental $\Delta G_{\text{app}}$ value (-0.8 kcal/mol) (Table 3.3). Indeed, insertion efficiency overall increased as the Leu-block was moved closer to the N-terminus (i.e., the luminal side); this trend is emphasized in the comparison of S0L9 to S9L9 ($\Delta G_{\text{app}} \exp. = -0.8 \text{ kcal/mol vs. } \Delta G_{\text{app}} \exp. = +0.4 \text{ kcal/mol}$) (Table 3.3; Figure 3.7).
Figure 3.7. SDS–PAGE gel of Lep constructs containing Ser-Leu sequences. (A) Protein samples were run on two separate gels and then arranged according to Leu-block properties. Lane 1 (-RM) contained the Lep construct run in the absence of rough microsomes. Lanes 2-6 contained the non-Leu-block Ser-Leu sequences. Lanes 7-12 contained the Leu-block sequences. Translocated, doubly glycosylated sequences are indicated by the upper band (●●), inserted, singly glycosylated sequences by the middle band (●), and un-glycosylated sequences by the lower band (○). (B) Fraction of inserted segment over translocated (K<sub>app</sub>). Values represent an average of three independent experiments. Error bars are reported as the standard deviation.

Experiments were performed by Nina Schiller in Dr. Gunnar von Heijne’s lab, Stockholm University.

The experimental ΔG<sub>app</sub> values are in good correspondence with their predicted values (Table 3.3, Figure 3.8D), except that the trend towards lower ΔG<sub>app</sub> experimental values when the Leu-block is moved towards the luminal, N-terminal end of the segment is not captured by the ΔG<sub>app</sub> predictor. This result is as expected, since the underlying model assumes symmetric effects of N- and C-terminally located residues [29]. It is further interesting to note that the TM
stretches predicted to insert are considerably shorter than 19 residues for the Leu-block segments, leaving some terminal Ser residues outside the membrane, a behavior that has been seen in molecular dynamics simulations of short hydrophobic peptides [112]. The good correlation between the measured and predicted ΔG_{app} values largely disappears if the entire 19-residue long Ser-Leu stretch is confined to the membrane in the calculation of ΔG_{app} for the Leu-block segments (Figure 3.8D).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>ΔG_{app pred.} (kcal/mol)</th>
<th>ΔG_{app exp.} (kcal/mol)</th>
<th>% inserted protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Leu-block</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLn</td>
<td>1.8</td>
<td>1.3</td>
<td>10.9</td>
</tr>
<tr>
<td>LSL</td>
<td>2.9</td>
<td>1.4</td>
<td>8.6</td>
</tr>
<tr>
<td>SL_{scr1}</td>
<td>1.8</td>
<td>1.4</td>
<td>9.7</td>
</tr>
<tr>
<td>SL_{scr2}</td>
<td>1.4</td>
<td>1.3</td>
<td>13.2</td>
</tr>
<tr>
<td>SL_{amp}</td>
<td>1.8</td>
<td>1.2</td>
<td>11.8</td>
</tr>
<tr>
<td>Leu-block</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9L9</td>
<td>-0.4</td>
<td>-0.4</td>
<td>33.0</td>
</tr>
<tr>
<td>S5L9</td>
<td>0.3</td>
<td>0.0</td>
<td>48.7</td>
</tr>
<tr>
<td>S3L9</td>
<td>0.0</td>
<td>-0.3</td>
<td>62.2</td>
</tr>
<tr>
<td>S2L9</td>
<td>-0.3</td>
<td>-0.6</td>
<td>72.3</td>
</tr>
<tr>
<td>S1L9</td>
<td>-0.3</td>
<td>-0.8</td>
<td>78.0</td>
</tr>
<tr>
<td>S0L9</td>
<td>-0.4</td>
<td>-0.8</td>
<td>79.5</td>
</tr>
</tbody>
</table>

\(^a\)Predicted free energies of insertion (ΔG_{app pred.}) of Ser-Leu peptides according to the ΔG predictor (http://dgpred.cbr.su.se) with subsequence allowance turned on (see section 2.15) [11,29]. \(^b\)Experimentally determined free energies of insertion (ΔG_{app exp.}). Values calculated using the fraction of mono- and di-glycosylated bands in Figure 3.7. ΔG_{app exp.} values represent an average of 3 independent experiments. Error values were no larger than ± 0.3 standard deviation for each point. Significant differences are found between the ΔG_{app exp.} of all Leu-block peptides (p < 0.001, one-way ANOVA), except between S0L9 and S1L9. \(^c\)% inserted protein = fraction of singly glycosylated protein over total protein.
Figure 3.8. Comparison of biophysical measurements of hydropathy and translocon-mediated membrane insertion. (A) Comparison of normalized retention time within a C-18 column to ΔG_{app} exp. (R^2 = 0.65, p < 0.01). (B) Comparison of % gel-shift in SDS-PAGE to ΔG_{app} exp. (R^2 = 0.80, p < 0.01). (C) Comparison of % gel-shift in SDS-PAGE to normalized retention time within a C-18 column (R^2 = 0.62, p < 0.05). (D) Comparison of ΔG_{app} experimental values to ΔG_{app} predicted values (default conditions; black circles) and ΔG_{app} predicted values with subsequence allowance (red squares) (see Chapter 2 for details). A significant correlation is only found when shortened subsequences are predicted (R^2 = 0.81, p < 0.001, in red).
3.3 Discussion

*In vitro* hydrophobicity measurements of a Ser-Leu library of peptides with identical composition but varying sequence patterning, using helicity in SDS micelles, % peptide gel-shift in SDS-PAGE, retention times on a C-18 column, and partitioning in helical form into POPC bilayers, are shown to be relatively accurate predictors of candidacy for membrane insertion. In combination with these observations, insertion studies with the mammalian Sec61 translocon demonstrate that the relatively high apparent hydrophobicity of the peptides containing Leu-block sequences is biologically relevant, as this feature clearly promotes membrane insertion (Table 3.3). Significant correlations are found between the experimentally determined free energy of insertion of the Ser-Leu sequences with both peptide HPLC retention times (Figure 3.8A; $R^2 = 0.65$) and with % peptide gel-shifts on SDS-PAGE (Figure 3.8B; $R^2 = 0.80$).

3.3.1 SDS-PAGE migration of Ser-Leu peptides

When run on SDS-PAGE, the Ser-Leu peptides migrate at a range of positions despite having identical molecular weights (Figure 3.2), with the Leu-block peptides traveling the slowest at molecular weights corresponding to *ca.* double their actual monomeric molecular weight (2.7 kDa). In concert with previous analyses of membrane protein migration rates on SDS-PAGE, we suggest that the relatively slow migration of the Leu-block peptides is a consequence of a greater local binding of SDS to these hydrophobic blocks, resulting in a relatively larger peptide-detergent complex size [153]. Interestingly the amphipathic sequence (SL_{amp}) traveled similar to the scrambled sequences (SL_{scr1} and SL_{scr2}), implying that despite similar hydrophobicity readings, a Leu-face does not bind as much detergent as a Leu-block.
Fürster Resonance Energy Transfer (FRET) experiments to assess oligomeric state(s) (viz., monomeric vs. dimeric) were inconclusive, as the covalent addition of dansyl/dabsyl probes tended to render the peptides insoluble in the media required. While the possibility of higher order oligomers in detergent cannot be excluded, we believe it to be unlikely, given that the Leu-block peptides lack a specific interface able to contribute to oligomer formation, and the previously shown inability of poly-Leu sequences to self-associate [163,164]. Further, comparison of peptide retention times in the isotropic environment of the C-18 column – a medium non-conducive to oligomer formation – to % peptide gel-shift on SDS-PAGE, reveals a moderate correlation (Figure 3.8C; $R^2 = 0.62$), supporting the idea that the observed slow migration rates in SDS-PAGE arise predominantly from the relatively high local hydrophobic character of Leu-block peptides. As well, since TM segments enter the translocon as monomers, the strong correlation between translocon-mediated insertion ($\Delta G_{\text{app exp.}}$) and % peptide gel-shift (Figure 3.8B; $R^2 = 0.80$) implies the observed increased migration rates of Leu-block peptides in SDS-PAGE are due to increased hydrophobic character of these peptides rather than self-association. Nevertheless, the possibility of peptide self-association in detergent remains and could be responsible for the observed lower helicity values in detergent of the Ser-Leu series, as in such circumstances, the peptide molecular weight may be confounded by the existence of larger peptide complexes.

3.3.2 Translocon-mediated insertion of Ser-Leu sequences into native bilayers

As predicted by measurements of translocon-mediated membrane insertion candidacy, the Leu-block peptides all produced favorable insertion values, establishing that they are capable of becoming membrane embedded (Table 3.3). The significant difference in insertion energy
between S0L9 and S9L9, which vary only in the extreme positioning of the Leu-block on the peptide N- vs. the C-terminus, has implications for the role of the translocon in insertion. As these two peptides display essentially parallel \textit{in vitro} properties in detergent, on the C-18 column, and in POPC liposomes, it is likely that the difference observed in translocon-mediated insertion is imparted by the translocon itself rather than the membrane. Further, the inner and outer leaflets of the ER membrane are believed to be symmetrical, supporting the idea that the position-dependent differences arising in insertion efficiency of TM segments are not dictated by the lipid bilayer [36].

Similar biases that favor the positioning of hydrophobic residues at one helix terminus over the other have been previously observed [29,94,165]. Thus, in yeast, a cluster of three Leu residues within a poly-Ala segment similarly experienced increased insertion efficiency when positioned closer to the luminally disposed N-terminus of the segment, away from the hydrophobic constriction ring within the translocon channel [94]. Further, based on cryo-EM structures of the mammalian translocon, the lateral gate is presumed to open when highly hydrophobic membrane sequences enter the channel [76], implying an early recognition of incipient TM sequences. Thus, if the translocon ‘senses’ an area of high hydrophobic character, such as a Leu-block, perhaps the gate is opened, enabling exposure of the threading segment to the lipid bilayer, allowing favorable partitioning to occur. In this scenario, the Leu-block is recognized as membrane-competent, leading to opening of the lateral gate, with insertion efficiency decreasing as the position of the Leu-block is shifted toward the cytosolically disposed C-terminus of the TM segment.
3.3.3 Leu-faces vs. Leu-blocks within the translocon channel

A striking difference is seen between the \textit{in vitro} biophysical techniques and the biological ER-insertion assay in their relative ability to identify an amphipathic segment as a candidate for insertion. In the majority of the \textit{in vitro} studies using detergent and a C-18 column, SL\textsubscript{amp} behaves very similarly to the Leu-block peptides, displaying comparable helicity, Trp burial, and HPLC retention time. It is not until SL\textsubscript{amp} is confronted by the translocon that the Leu-face becomes clearly distinguishable from the Leu-block sequences (Table 3.3; Figure 3.7). While the Leu-block sequences insert efficiently, SL\textsubscript{amp} fails to insert into the ER membrane. Previously, an increase in amphipathicity has been seen to decrease insertion efficiency \cite{11}. As well, the SL\textsubscript{n} and the scrambled Ser-Leu sequences fail to insert (Table 3.3; Figure 3.7). These results are consistent with the notion that the translocon requires a hydrophobic surface that extends around the entire circumference of a nascent TM segment to open its lateral gate (Figure 3.9A); in the present Leu-block peptides, nine consecutive Leu residues would comprise two to three turns of a helical peptide. As such, the translocon constriction ring, itself replete with a circle of hydrophobic residues, may be acting as a surrogate for recognition of the destination environment, wherein phospholipids would similarly surround the inserted segment. Thus, lacking both circumferential hydrophobicity and compatibility with the bilayer, the SL\textsubscript{amp} peptide is directed by the translocon into the lumen along with the remaining non-Leu-block sequences (Figure 3.9B). From this perspective, the poly-Ala/Leu segments (\textit{e.g.,} ALLLAAAAAAAAAAAAAAAA) studied by Demirci \textit{et al.} \cite{94} would satisfy this ‘hydrophobic ring’ requirement at all points along their sequence, rendering the gate open regardless of Leu positioning. Insertion efficiency of these poly-Ala/Leu sequences would then be dependent solely on interaction of the Leu-block with the constriction ring.
Figure 3.9 Schematic translocon model for insertion of Leu-block sequences vs. translocation of amphipathic and scrambled sequences of Ser-Leu peptides. (A) Top-down view (from the C-terminus) of the Leu-block sequence S9L9 (SSSSSSSSWLLLLLLLLL) as part of the Lep construct (not shown), in contact with the translocon channel (colored gray). The hydrophobic constriction ring is colored yellow and the translocon lateral gate red (closed) and then green (open). The circumferential hydrophobic surface area of the passing peptide is signified by the continuous yellow circle encompassing the peptide. Below is a lateral view of the translocon with S9L9 within the channel with the lateral gate open toward the viewer. (B) Top-down view of non-Leu-block sequences, shown for the amphipathic sequence SLamp (SLLSSLSSWLLSSLSSL) and the scrambled sequence SLscr1 (SLSLSLSWSLLSLSSLSL), passing through the channel. The discontinuous hydrophobic surface area common to both peptides is represented by the yellow (hydrophobic) and blue (polar) circle encompassing each peptide. The translocon remains closed during passage of the non-Leu-block sequences. Below are lateral views of the translocon translocating the amphipathic and non-Leu-block sequences. Figure adapted with permission from Stone et al. (2015) [145].

Whether the observed positional bias for Leu-block proximity to the luminally disposed N-terminus is due to an earlier opening of the lateral gate and therefore immediate exposure to lipid, or to the unfavorable positioning of numerous bulky Leu residues near the narrow
hydrophobic constriction ring, or to a combination of both, remains uncertain. Nevertheless, we
find that the clustering of nine Leu residues raises the perceived hydrophobicity of these
marginal TM segments to allow the insertion of some or all of the nine Ser residues as ‘cargo’.

3.4 Conclusion

*In vitro* biophysical analysis by several biochemical and biophysical techniques in
membrane-mimetic environments established the sequence-dependent lipid compatibility of a
series of synthetic peptides of identical Leu/Ser/Trp composition. Most prominently, an overall
increase in a TM segment’s apparent hydrophobicity is observed when the peptides present an
extensive continuous hydrophobic face (Leu-block peptides; SLamp, Leu-face). The present work
further demonstrates that simply averaging the hydrophobicity of a segment is not an adequate
measure of the segment’s ‘actual hydrophobicity’ or likelihood for membrane insertion.

Intriguingly, only two of the six biophysical techniques (SDS-PAGE migration and
tryptophan fluorescence in liposomes) were able to distinguish between an amphipathic helix and
the Leu-block sequences. The more complex ΔG_{app} predictor that takes both positional variation
in residue hydrophobicity as well as amphiphilicity and overall length of the membrane-
embedded segment into account works better in this regard [29]. The overall results thus suggest
that while current *in vitro* partitioning techniques are generally excellent predictors of potential
TM segments, they may not completely capture the subtleties of patterning of polar and apolar
residues along a protein segment that the translocon can discern.
Hydrophobic clusters raise the threshold hydrophilicity for insertion of transmembrane sequences \textit{in vivo}.


Author contributions: TAS designed research. TAS and NW performed research on peptides in membrane mimetics. NS carried out translocon-mediated membrane insertion of sequences. TAS and NW analyzed the data. TAS, CMD, NS, and GvH wrote the paper.
4.1 Introduction

While our understanding of the roles of hydrophobic patterning has been expanded through our studies using a series of TM peptides with intentionally exaggerated polar character (50/50 polar/apolar design) (Chapter 3; [145]), the subtle effects of hydrophobic and helical patterning within more-native-like TM domains remain to be elucidated. In the work presented in this Chapter, we have extended our studies of hydrophobic patterning in marginally hydrophobic TM domains, by applying the concepts learned in Chapter 3, to a new set of peptides ranging in overall polar content from 0 to 30%. In this manner we can assess the impact of hydrophobic patterning on the membrane integration of increasingly polar sequences, to address the question of how or when does hydrophobic patterning affect the hydrophobicity threshold for membrane insertion. Through the assessment of peptide properties across different membrane mimetics, utilizing a combination of biophysical and biological techniques, we were able to isolate the features of protein sequence and environment(s) that influence the polar threshold for peptide partitioning into membranes.
4.2 Results

4.2.1 Peptide design

To define the sequence context in which clustering hydrophobic amino acids together impacts the overall properties of a TM domain, we went on to design compositionally identical sequences with hydrophobic amino acids either clustered together into a central, contiguous sequence (termed “block” (bl) peptides) or dispersed along the length of the TM (termed “scrambled” (scr) peptides), amid increasing average polar character (Table 4.1). The core sequences are each 19 residues long, composed of a poly-Ala background supplemented with five or seven Leu residues and a single centrally-positioned Trp to enable quantification and fluorescent studies. Polar character was increased through the stepwise substitution of two Ala with Ser residues (2S peptides), up to a total of six Ser residues total (6S peptides). Ala was chosen for its ‘neutral’ behavior towards membrane insertion [8]; Leu, as described in Chapter 3, is the most common amino acid found in natural TM helices, while Ser is the most common polar amino acid [43]. All peptides were Lys-tagged (three on each terminus) to increase their aqueous solubility and ease of handling [161]. Addition of Lys-tags to TM peptides has been shown to have no effect on core sequence properties [166]. Each peptide was designed with an average segmental hydrophobicity value above that required for spontaneous membrane insertion (≥ 0.4 on the Liu-Deber scale [8]). Peptides are named according to how many Leu and Ser residues are present with subscripts indicating the patterning of the Leu residues (i.e., 5L2Sbl indicates there are five Leu residues, two Ser, and the five Leu are clustered together into a hydrophobic block).
### Table 4.1. Sequences and segmental average hydrophobicity of Ala-Leu-Ser peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average hydrophobicity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5-Leucine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5L0S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-AAAAAALLLLLLLAAAAAA-KKK</td>
<td>1.6</td>
</tr>
<tr>
<td>5L0S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>KKK-LAAAAALAAAAAALAAAAA-KKK</td>
<td></td>
</tr>
<tr>
<td>5L2S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-AAASSAAALLLLLAAAAASAA-KKK</td>
<td>1.3</td>
</tr>
<tr>
<td>5L2S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>KKK-LAAASALAAWLALALSAAA-KKK</td>
<td></td>
</tr>
<tr>
<td>5L4S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-ASASALASLLLLLAAAAASASA-KKK</td>
<td>1.0</td>
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<tr>
<td>5L6S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-ASASALASWLALASASAASSA-KKK</td>
<td>0.7</td>
</tr>
<tr>
<td>5L6S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>KKK-LSAASALASWLALASASAASSA-KKK</td>
<td></td>
</tr>
<tr>
<td><strong>7-Leucine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7L2S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-AAAASAAALLLLLLLAAAAASAA-KKK</td>
<td>1.8</td>
</tr>
<tr>
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<td>KKK-LASALAAALAAAAAALASAA-KKK</td>
<td></td>
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<tr>
<td>7L4S&lt;sub&gt;bl&lt;/sub&gt;</td>
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<tr>
<td>7L4S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>KKK-LSAALASALWALASALASAA-KKK</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Ala, Leu, Trp and Ser are colored red, yellow, black, and blue, respectively. Peptides contain three Lys residues on each terminus (black). The subscript bl denotes Leu-block peptides; scr denotes peptides where Leu residues have been “scrambled”. Note that Leu positions in the 5L0S<sub>scr</sub> peptide are maintained in subsequent scrambled sequences, and similarly for 7L2S<sub>scr</sub> and 7L4S<sub>scr</sub> peptides. <sup>b</sup>Peptide average hydrophobicity is reported using values calculated from the Liu-Deber scale [8].

#### 4.2.2 Peptide HPLC retention times

Peptide retention times on a C-18 column were measured under isocratic mobile phase conditions to determine peptide effective hydrophobicity, viz., peptides that preferentially interact with the column alkyl chains relative to the mobile phase will be retained longer. Not unexpectedly, peptides of relatively high average hydrophobicity displayed the longest retention times (Table 4.2). Yet, large differences were observed in retention times of compositionally identical sequences when no or few polar amino acids were present (0, 2S), a difference that diminished as polar character is increased. Thus, while 5-Leu block peptides with 0, 2 or 4 Ser
eluted later than their corresponding scrambled peptides, the elution times of the 5-Leu 6S block and scrambled peptides were indistinguishable from one another.

Comparison of the CD spectra of the 5-Leu and 7-Leu peptides in the isocratic solvent ratios used in the HPLC retention time experiments to peptide retention time revealed no correlation between peptide helicity and time of elution. All the peptides (except 5L6S<sub>scr</sub>) exhibit identical helical spectra in HPLC isocratic solvents (Figure 4.1), confirming that the wide variations in retention times measured for the 5-Leu and 7-Leu peptide series (3-10 minutes; Table 4.2) reflect the perceived hydrophobicity of each segment as it partitions from the mobile phase into the acyl chain stationary phase of the column.

### Table 4.2. Ala-Leu-Ser peptide retention on a C-18 column

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Normalized retention time</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>5-Leucine</strong></td>
<td></td>
</tr>
<tr>
<td>5L0S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>10</td>
</tr>
<tr>
<td>5L0S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>5.3</td>
</tr>
<tr>
<td>5L2S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>4.9</td>
</tr>
<tr>
<td>5L2S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>5L4S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>3.7</td>
</tr>
<tr>
<td>5L4S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>3.2</td>
</tr>
<tr>
<td>5L6S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>3.4</td>
</tr>
<tr>
<td>5L6S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>7-Leucine</strong></td>
<td></td>
</tr>
<tr>
<td>7L2S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>4.9</td>
</tr>
<tr>
<td>7L2S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>4.7</td>
</tr>
<tr>
<td>7L4S&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>3.2</td>
</tr>
<tr>
<td>7L4S&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Normalized retention time values within a C-18 HPLC column under isocratic mobile phase conditions (see Chapter 2 for details). Peptide retention time is normalized to that of uracil and therefore is a unitless value. Values are an average of at least three independent experiments. Error values were no larger than ± 0.2 standard deviation for each point.
Figure 4.1. CD spectra of 5-Leu and 7-Leu peptides in column conditions. (A) 5-Leu peptides in 60% solvent A and 40% solvent B (see Chapter 2 for details) (25 μM peptide). (B) 7-Leu peptides in 65% solvent A and 35% solvent B (25 μM peptide). All peptides adopted helical conformations. Spectra shown represent an average of three independent experiments.

4.2.3 Helicity and Trp burial in detergent micelles

Secondary structure content of each peptide was assessed in sodium dodecyl sulfate (SDS) detergent micelles using CD spectroscopy. All peptides adopted helical conformations in the presence of detergent micelles, with sequences of higher average hydrophobicity exhibiting more helical character than those with higher polarity (Figure 4.2A). Except for the 5-Leu 2S peptides, the block peptide was always more helical than the corresponding scrambled peptide when comparing sequences of identical composition.
Figure 4.2. Helicity and tryptophan emission spectra in detergent micelles. (A) Mean Residue Ellipticity (MRE) values at 222 nm, taken from CD spectra of the indicated peptides in SDS micelles (25 µM peptide, 175 mM SDS). Data represent the average of three independent experiments. Error bars are reported as the standard deviation; significant differences are denoted by *, p < 0.05; ***, p < 0.001; ****, p < 0.0001 (one-way ANOVA). (B) 5-Leu peptides in SDS micelles (5 µM peptide, 35 mM SDS). Peptides are listed in the legend in order of decreasing fluorescence intensity. The fluorescence emission maxima of free Trp in water is denoted by a vertical dotted black line (350 nm). Spectra shown represent an average of three independent experiments.

We used Trp fluorescence as a measure of how ‘buried’ the centrally placed Trp residue was within the hydrophobic micelle core. While increases in blue shift were correlated with higher average hydrophobicity, variations were found between block and scrambled sequences when polar residues were present (Figure 4.2). 5-Leu peptides lacking polar amino acids (0S), exhibited identical strong blue shifts for both peptide variants, while sequences of intermediate polarity (2S and 4S), showed relatively greater blue shifts for block sequences. The 5-Leu peptides with the highest polarity (6S) had the most solvent-exposed block sequence.
In control experiments with dabsyl and dansyl-labeled peptides, Förster Resonance Energy Transfer (FRET) experiments established that the 5L0S\textsubscript{bl} and 5L0S\textsubscript{scr} peptides were monomeric species in SDS micelles (Figure 4.3A); the remaining peptides travel at similar molecular weights as the 0S peptides on SDS-PAGE gels, similarly implying monomeric states (Figure 4.3B, C).

**Figure 4.3. FRET titration and gel shifts of peptides in detergent micelles.** (A) Acceptor titration curve of 5L0S ‘block’ (solid black line) and ‘scrambled’ (dashed black line) dansyl and dabsyl labeled peptides in SDS micelles. No significant FRET was observed. For comparison, the acceptor titration curve of a previously homodimer (human tetherin peptide) is shown in red [167]. Data points represent the average of three independent experiments. (B) SDS-PAGE of 5-Leu peptides (0-6 Ser). (C) SDS-PAGE of the 7-Leu 2S and 4S peptides. The corresponding 5-Leu 2S and 4S peptides are repeated for comparison of migration rates. While variations arise in band migration, all peptides migrate similar to the monomeric 5L0S\textsubscript{bl/scr} peptides.
4.2.4 Peptide interactions with detergent molecules below the critical micelle concentration

CD spectroscopy was used to measure increases in peptide helicity as detergent monomers (concentration below CMC) were titrated into peptide samples. Using the concentration of detergent (mM) at which a peptide had reached half of its full helicity, noted as the helical mid-point, we obtained a measure of how sub-micellar detergent monomers (or finite aggregates) initially interact with exposed hydrophobic loci. Initial charge pairing between positively charged peptide Lys-tags and negatively charged sulfate head groups of SDS caused an interim loss in peptide helical structure at low concentrations of detergent [159]. Subsequent small increments (0.2 mM) of detergent then induced folding of the peptides into helical conformations. In almost all cases, the peptides adopted full helical character prior to SDS concentrations surpassing the CMC. We found that peptides with relatively high average hydrophobicity reached their helical mid-point at lower concentrations of detergent (Figure 4.4). Interestingly, the largest differences in helical mid-point values between pairs of block and scrambled sequences are observed when no polar amino acids are present (0S) and decrease as polarity is increased. At high polarity (5-Leu 4S and 6S), little to no difference in helical mid-point is observed between sequence pairs. The data are summarized in Table 4.3.
Figure 4.4. Peptide interaction with detergent monomers. Detergent monomers were added (0.2 mM increments) to the indicated peptides (15 μM), and helical character was recorded using CD spectroscopy. Data points were fit with inhibitory-dose response curves (see Chapter 2 for details). Detergent CMC is represented by a vertical, dotted line (3.46 mM, 0.54 = log(3.46); see Figure 2.2). Peptides containing block sequences are depicted as solid lines with data points as squares, and corresponding scrambled sequences as dashed lines and circles. Data points represent the averaged results of at least two independent experiments.

Table 4.3. Ala-Leu-Ser peptide helical mid-points upon detergent titration

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Helical mid-point (mM SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Leucine</td>
<td></td>
</tr>
<tr>
<td>5L0S_{bl}</td>
<td>0.6</td>
</tr>
<tr>
<td>5L0S_{scr}</td>
<td>1.1</td>
</tr>
<tr>
<td>5L2S_{bl}</td>
<td>1.0</td>
</tr>
<tr>
<td>5L2S_{scr}</td>
<td>1.3</td>
</tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>5L6S_{scr}</td>
<td>2.4</td>
</tr>
<tr>
<td>7-Leucine</td>
<td></td>
</tr>
<tr>
<td>7L2S_{bl}</td>
<td>1.7</td>
</tr>
<tr>
<td>7L2S_{scr}</td>
<td>2.0</td>
</tr>
<tr>
<td>7L4S_{bl}</td>
<td>1.6</td>
</tr>
<tr>
<td>7L4S_{scr}</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Peptide helical mid-points, as calculated from Figure 4.4, represent the concentration of SDS at which half of full helical character is regained during a detergent titration below the CMC (see Chapter 2 for details). Error values were no larger than ± 0.05 standard deviation for each point.
4.2.5 Helicity and Trp burial in liposomes

The secondary structures of the peptides in the presence of POPC liposomes were determined using CD spectroscopy. All peptides, with the exception of the 5-Leu 6S peptides, were helical, with sequences of higher average hydrophobicity displaying relatively greater helical content (Figure 4.5A). The two 5-Leu 6S sequences did not adopt helical conformations, but rather exhibited CD spectra representative of unstructured peptides.

Figure 4.5. Circular dichroism and Trp fluorescence of 5-Leu peptides in lipid bilayers. (A) Peptides in POPC liposomes (10 μM peptide, 2.5 mM SDS). 5-Leu 0S, 2S, and 4S peptides each adopted helical conformations. 5-Leu 6S peptides were unstructured. Peptides are listed in the legend in decreasing order of MRE at 222 nm. Spectra shown represent an average of three independent experiments. (B) Tryptophan fluorescence spectra of the peptides in POPC liposomes. 0S and 2S peptides exhibit strong blue shifts (black and blue lines, respectively). 4S and 6S have spectra similar to fully solvent exposed Trp (red and green lines, respectively). The fluorescence emission maximum of free Trp in water is denoted by a vertical, dashed black line (350 nm). Leu-block peptides are depicted by solid lines, and scrambled peptides as dashed lines. Spectra shown represent an average from three independently prepared samples.

Observation of significant blue shifts in the presence of liposomes, in conjunction with adoption of helical structure, is a positive indication of favorable lipid interaction. We found that
the 5-Leu 0S and 2S sequences exhibit strong blue shifts, while the 4S and 6S sequences have fluorescence spectra indicative of solvent exposed Trp residues (Figure 4.5B). Notably, no difference is observed between the blue shifts within each pair of compositionally identical sequences at all polarity levels.

4.2.6 Translocon-mediated membrane insertion assay

The ability of the translocon to identify and insert the sequences of the present peptide library into native membranes was tested using the Lep construct and rough microsomes, as described previously [11]. Differential glycosylation of the Lep construct reveals the relative inserted/translocated state(s) of the sequence of interest, from which a $K_{app}$ for insertion may be derived and the apparent free energy of insertion ($\Delta G_{app}$) calculated (see Chapter 2 for details). When polar character is absent (5L0S$_{bl}$ and 5L0S$_{scr}$; Figure 4.6A, black bars) or high (5L6S$_{bl}$ and 5L6S$_{scr}$; Figure 4.6A, green bars), no difference was observed in the membrane insertion efficiency of paired block and scrambled sequences, with the two inserting equally well (0S) or equally poorly (6S). However, with the incorporation of two Ser residues, the block sequence inserts significantly better than the scrambled sequence, a trend that continues for the 4S peptide pair (Figure 4.6B; blue and red bars, respectively). The $\Delta G_{app}$ values calculated from the data in Figure 4.6B are summarized in Table 4.4. Comparison of the $\Delta G_{app}$ experimental values to values predicted using the online $\Delta G_{app}$ prediction server (http://dgpred.cbr.su.se/) yields a strong correlation ($R^2 = 0.9$, Figure 4.6B). For a given sequence composition, predicted $\Delta G_{app}$ values are always lower for the block sequence compared to the scrambled sequence because Leu residues are concentrated near the middle of the block sequences where they contribute more to the overall $\Delta G_{app}$ value [29].
Figure 4.6. Experimental free energies of translocon-mediated membrane insertion. (A) Differential glycosylation patterns were used to derive $K_{app}$ and $\Delta G_{app\ exp.}$ values (see Chapter 2 for details). $\Delta G_{app\ exp.}$ values represent an average of three independent experiments. Significant differences are denoted by ****, $p < 0.0001$ (one-way ANOVA). Error bars are reported as the standard deviation. Experiments were performed by Nina Schiller in Dr. Gunnar von Heijne’s lab, Stockholm University. (B) Comparison of experimental $\Delta G_{app}$ values to free energies of membrane insertion predicted from the online server (http://dgpred.cbr.su.se/) with the subsequence allowance (see Chapter 2 for details). A strong correlation is observed ($R^2 = 0.9$, $p < 0.0001$). Experimental values are ~ 0.5 kcal/mol more favorable than predicted values (equation of the line: $y = 0.8x - 0.5$).
Table 4.4. Predicted and experimentally derived free energy of insertion values ($\Delta G_{app}$) for Ala-Ser-Leu sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\Delta G_{app}$ pred. (kcal/mol)$^a$</th>
<th>$\Delta G_{app}$ exp. (kcal/mol)$^b$</th>
<th>% insertion$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5L0S$_{bl}$</td>
<td>-0.79</td>
<td>-0.80</td>
<td>79.0</td>
</tr>
<tr>
<td>5L0S$_{scr}$</td>
<td>-0.74</td>
<td>-0.95</td>
<td>82.9</td>
</tr>
<tr>
<td>5L2S$_{bl}$</td>
<td>0.15</td>
<td>-1.11</td>
<td>86.0</td>
</tr>
<tr>
<td>5L2S$_{scr}$</td>
<td>0.20</td>
<td>-0.22</td>
<td>58.9</td>
</tr>
<tr>
<td>5L4S$_{bl}$</td>
<td>0.87</td>
<td>0.23</td>
<td>40.5</td>
</tr>
<tr>
<td>5L4S$_{scr}$</td>
<td>1.30</td>
<td>0.80</td>
<td>20.8</td>
</tr>
<tr>
<td>5L6S$_{bl}$</td>
<td>2.13</td>
<td>1.15</td>
<td>12.9</td>
</tr>
<tr>
<td>5L6S$_{scr}$</td>
<td>2.31</td>
<td>1.16</td>
<td>12.8</td>
</tr>
<tr>
<td>7L2S$_{bl}$</td>
<td>-0.91</td>
<td>-1.01</td>
<td>84.3</td>
</tr>
<tr>
<td>7L2S$_{scr}$</td>
<td>-0.48</td>
<td>-0.97</td>
<td>83.3</td>
</tr>
<tr>
<td>7L4S$_{bl}$</td>
<td>-0.30</td>
<td>-0.65</td>
<td>74.7</td>
</tr>
<tr>
<td>7L4S$_{scr}$</td>
<td>0.44</td>
<td>-0.29</td>
<td>61.6</td>
</tr>
</tbody>
</table>

$^a$Predicted free energies of insertion ($\Delta G_{app}$ pred.) according to the $\Delta G$ predictor (http://dgpred.cbr.su.se) with subsequence allowance turned on (see section 2.15) [11,29].

$^b$Experimentally determined free energies of insertion ($\Delta G_{app}$ exp.). $\Delta G_{app}$ exp. values represent an average of 3 independent experiments. Error values were no larger than ± 0.2 standard deviation for each point. Significant differences are found between the $\Delta G_{app}$ exp. of 5L2S, 5L4S, and 7L4S block and scrambled sequences (p < 0.0001, one-way ANOVA). $^c$% inserted protein = fraction of singly glycosylated protein over total protein.

4.2.7 Increasing the number of hydrophobic amino acids

Experiments were repeated using sequences with an increased number of Leu residues (seven), in combination with 2- and 4-Ser residues (Table 4.1). We found that the trend persists of minimizing differences between compositionally-identical block and scrambled sequences as polarity increases. A large difference in HPLC elution time was observed between block and scrambled 7-Leu 2S peptides, while the retention times of the 7-Leu 4S pair of peptides were equivalent in a manner reminiscent of the 5-Leu 6S peptides (Table 4.2).
In SDS micelles, the 7-Leu block peptides were more helical than the corresponding scrambled peptides (Figure 4.7A). For all polarity levels, the block peptide was more blue-shifted than its scrambled counterpart (Figure 4.7B). In detergent titration assays, the block versions of the 2S and 4S 7-Leu peptides reached helical mid-point at lower concentrations of detergent than the corresponding scrambled peptides (Table 4.3). Similar to the 5-Leu peptides, little to no differences are observed between the peptide pairs with respect to liposome interactions (Figure 4.7C, D). However, translocon-mediated membrane insertion was found to favor the block sequence when 4 Ser residues are present (Figure 4.6A; brown bars), while the 7-Leu 2S block and scrambled peptides insert into the membrane equally well (Figure 4.6A; grey bars), analogous to the results with the 5-Leu 0S sequences. $\Delta G_{\text{app}}$ exp. values are summarized in Table 4.4.
Figure 4.7. Circular dichroism and Trp fluorescence spectra of 7-Leu peptides in detergent micelles and in lipid bilayers. (A) CD spectra of 7-Leu peptides and (B) Trp fluorescence spectra in SDS micelles. (C) CD spectra of 7-Leu peptides and (D) Trp fluorescence spectra in POPC bilayers. Emission maximum of Trp in water is denoted by a vertical dotted line (350 nm). Spectra shown represent an average of three independent experiments.

4.3 Discussion

4.3.1 Effective hydrophobicity depends on sequence, composition, and environment

We have explored the role(s) of protein sequence and environment on the membrane insertion and folding of TM domains using a variety of biophysical and biological techniques, in
conjunction with systematic comparisons of Leu-block vs. scrambled sequences, and from comparisons of peptides with low vs. higher polar (Ser) content. When we evaluated peptide interactions with a C-18 HPLC column, and SDS detergent micelles and monomers (sub-micellar concentrations) – environments that enable peptide polar residues to ‘escape’ to water (viz., the column/solvent interface or the micelle surface) – block peptides tended to have longer retention times and be more helical and buried into the micelle’s effective interior than corresponding scrambled peptides. In these water-accessible membrane environments, local areas of high hydrophobic character, rather than an averaged value, tended to dictate peptide behavior. As well, peptides containing a strong hydrophobic locus read out as more hydrophobic, reinforcing the ‘compatibility’ of the Leu cluster for acyl chains, and are therefore more likely to undergo membrane integration both in vitro and in vivo.

In liposomes, a notably more restrictive environment where embedded segments have little contact with water, no difference was seen in the helicity or Trp burial of each pair of compositionally-identical block and scrambled peptides at all polarity levels (Figure 4.5B). Regarding prospective insertion into the bilayers of POPC liposomes, sequences containing 0 or 2 Ser interacted favorably with bilayers (highly helical, solvent shielded Trp), while sequences containing 4 or 6 Ser did not. Thus, the 0S and 2S block and scrambled peptides were found to be equally lipid compatible, while the 4S and 6S peptides were equally lipid ‘incompatible’, a result pointing to an inevitable ‘toleration limit’ of bilayer membranes for polar content of sequences. Thus, interaction with lipid bilayers was found to be an ‘all-or-nothing’ event: sequences with core hydrophobicity below a threshold may not become membrane-integrated without the intervention of the insertion machinery or neighboring proteins within the bilayer [71].
Here, the comparison of direct incorporation of peptides into POPC liposomes (Figure 4.5B) to their translocon-mediated membrane insertion (Table 4.4; Figure 4.6A) is particularly apt. We found that sequences with high lipid compatibility and average hydrophobicity (Figure 4.6A; 5L0S\textsubscript{bl} and 5L0S\textsubscript{scr}), readily underwent translocon-mediated membrane insertion, independent of hydrophobic patterning. However, upon addition of polar character, sequences lacking a strong hydrophobic locus did not readily undergo membrane integration (e.g., 5L2S\textsubscript{scr}, 5L4S\textsubscript{scr}, Figure 4.6A). Thus, within the water-filled translocon channel, favorable solvation of the Ser residues may compete with the strong hydrophobic loci driven towards the membrane interior by the unfavorable entropy associated with ordering of water around a hydrophobic surface, as depicted schematically in Figure 4.8. The energetic sum of these favorable and opposing interactions determines the final translocation/insertion state of the sequence. The presence of polar residues within an incipient TM strand can therefore impede partitioning of sequences lacking a strong hydrophobic locus, until ultimately, sequences that are deemed to be incompatible with the lipid bilayer do not undergo translocon-mediated membrane insertion even when a strong hydrophobic center is present (e.g., 5L6S\textsubscript{bl}, Figure 4.6A).
Figure 4.8. The hydrophobic effect drives membrane integration of Leu-block sequences with moderate average hydrophobicity. Schematic of the comparative insertion/translocation of Leu block peptide 5L4S_{bl} with its scrambled counterpart 5L4S_{scr}. Three perspectives are provided: the interior of the translocon channel (grey), the channel-lipid interface, and the lipid bilayer. Phospholipid molecules are colored white (hydrogen), red (oxygen) and grey (carbon). 5L4S_{bl} and 5L4S_{scr} sequences are depicted as helices with Ala in red, Leu in yellow, and Ser in blue. The 5L4S_{bl} sequence has a ‘cylinder’ of oriented water molecules surrounding the Leu block (highlighted in light blue). In the water-filled translocon channel, the unfavorable loss in water entropy may drive membrane insertion of the Leu-block upon which the 5L4S_{bl} sequence becomes fully embedded within the POPC bilayer. The 5L4S_{scr} peptide, having a significant amount of solvated polar amino acids and lacking a strong hydrophobic locus along its length, is translocated rather than membrane integrated. Figure adapted with permission from Stone et al. (2016) [146].
4.3.2 Hydrophobic blocks promote membrane integration when segmental hydrophobicity is moderate

We further undertook to assess the limiting influence of hydrophobic content in the presence of increasing polar content through design and synthesis of a second set of peptides with higher average segmental hydrophobicity (Table 4.1; 7-Leu peptides). Indeed, we found that the increased Leu content resulted in a corresponding shift in the number of polar amino acids required to observe a difference between pairs of block and scrambled sequences in biophysical and biological assays. Largely, the 7-Leu 2S sequences (average hydrophobicity above 5-Leu 0S) behave like the 5-Leu 0S peptides, with block and scrambled sequences undergoing translocon-mediated membrane insertion equally well (Table 4.4; Figure 4.6A, grey bars). The 7-Leu 4S sequences (average hydrophobicity in-between 5-Leu 0S and 2S) behave in a manner paralleling the 5-Leu 2S sequences, with block sequences preferentially inserting into native membranes over corresponding scrambled sequences (Table 4.4; Figure 4.6A, brown bars).

We thus find that the extents of insertion determined experimentally in the Lep construct assay favor the block vs. scrambled sequences for the 5L2S, 5L4S, and 7L4S peptide pairs (viz., those of moderate hydrophobicity) (Figure 4.6A). This trend is expected from previous observations that hydrophobic residues located centrally in a TM segment promotes membrane insertion more strongly than peripherally located hydrophobic residue [29], and is captured by the ΔG_{app} online prediction server based on these data (Figure 4.6B).

Our combined biological and biophysical results thus identify an overall hydrophobicity window within which the hydrophobic content and patterning play the decisive role in
determining whether a given protein segment will be suitable for insertion into a lipid bilayer. We further find that when average segmental hydrophobicity exceeds or falls below given threshold values, hydrophobic patterning has little effect on the efficiency of membrane integration (Figure 4.6; 5-Leu 0S, 6S and 7-Leu 2S). In protein-free lipid bilayers, a distinct “threshold hydrophilicity” is observed, in which sequences of higher polar character are deemed incompatible, independent of sequence hydrophobic patterning (Figure 4.5B; 4S and 6S). However, sequences partitioning from the translocon channel into the lipid bilayer experience an increase in the “threshold hydrophilicity”, as lipid-incompatible sequences of higher polar content readily undergo membrane integration when a hydrophobic locus is present (Figure 4.6; 5L4Sbl).

4.4 Conclusion

By defining a range of average hydrophobicity and polar residue content within which a hydrophobic block promotes membrane integration, our work provides insight into potential mechanisms for the membrane integration of membrane protein sequences. As models for partitioning of potential TM sequences from water to lipid, detergent-peptide and column-peptide interactions present to the peptide chain the types of interactions similar to those experienced during translocon-mediated membrane insertion, while liposome studies reveal information about the integration of protein sequences into the receiving environment, the lipid bilayer. Significantly, our results suggest that the change of a single amino acid within a protein sequence, altering average hydrophobicity and hydrophobic patterning, may easily tip the balance in the wrong direction, resulting in anomalous translocation/insertion.
A comparison of Leu/Ile in hydrophobic peptides

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Author contributions: TAS designed and performed research on peptides in membrane mimetics. NS carried out translocon-mediated membrane insertion of sequences (*unpublished*). TAS analyzed the data. TAS and CMD wrote the paper.
5.1 Introduction

Inherent differences in secondary structure preference of Leu vs. Ile in soluble proteins commonly places these amino acids in either α-helical domains, or β-sheets, respectively [168]; the interchange of one isomer for the other can therefore have a direct functional consequence on the folding and function of soluble proteins, at times inducing a disease phenotype (Table 5.1). However, within membrane proteins, both Leu and Ile exert similar propensities for helical structures, the hydrophobic nature of the Ile side chain overcoming the β-sheet promoting nature through the burial of this residue within the membrane as an α-helix [169]. The relative impact of Leu/Ile mutations within membrane proteins and membrane interactive peptides remains less documented, as the two amino acids are believed to generally provide similar functions in the membrane environment, with many hydrophobicity scales reporting the two isomers as very similar in membrane insertion propensity (Table 1.1; Chapter 1). Some notable examples highlight how the subtle change in positioning of a single methyl group can greatly alter how proteins/peptides interact with and associate within the lipid bilayer. For example, the well-described antimicrobial peptides aurein 2.2 and 2.3 – varying only in a single Leu/Ile – have differing activity against various bacterial species [170]. Studies using peptides derived from δ-lysin, an amphipathic peptide known to bind membrane surfaces, have provided insight that suggests peptides rich in Ile rather than Leu may bind lipid bilayers more efficiently [171]. The DiMaio group, through a series of elegant experiments, have shown how Leu-to-Ile mutations and vice versa in synthetic TM domains can greatly impact receptor binding and alter receptor specificity [148,149,172]. The subtle but distinct differences imparted on a TM domain surface through the movement of just one methyl group has been shown to be responsible for these observed variations and specificity in target binding [149]. Previously in our lab, the relative
roles of Leu, Ile, Val were considered in glycophorin A (GpA) dimerization [119]. Using TOXCAT to assess dimerization strength within the bacterial membrane, the native two Val residues were replaced by two Leu or two Ile residues, with dramatic changes in dimerization explained by the varying surface areas the two mutants presented to the membrane and neighboring helices within the bilayer [119]. All of these results highlight the subtle interplay between protein surfaces and the lipid bilayer that can be modulated with slight changes in amino acid composition.

Previous work in this thesis has focused on the use of designed hydrophobic peptides to analyze the dependence of hydrophobic patterning in lipid environments (Chapters 3 and 4). In those studies, we had predominantly used Leu to represent typical hydrophobic amino acids within TM domains. In this Chapter, we extend the biophysical aspects of these approaches to include peptides where Leu is compared directly to its corresponding isomer Ile, and we explore the potential roles and differences Leu and Ile play in mediating peptide-membrane interactions and TM domain membrane insertion.

In Chapters 3 and 4, we have worked with three different sequence patterns:

- ‘Scrambled’ peptides, which contain bulky hydrophobic residues (e.g. Leu, Ile) dispersed along the TM helix length in a manner to avoid the creation of a single hydrophobic block or face and therefore possessing the smallest hydrophobic moment.
- ‘Block (or clustered)’ peptides, which have hydrophobic residues positioned in a continuous stretch in the primary sequence, to produce a ring of hydrophobicity around the circumference of the folded helix.
• ‘Amphipathic’ peptides, which have hydrophobic and polar residues segregated to opposite faces of the helix, generating one hydrophobic and one polar face and thus a strong hydrophobic moment.

We now introduce an additional sequence pattern, which we have termed ‘lipopathic’ [127], referring to the differential preference for lipid of one helical face over the other in a helix that is highly hydrophobic along its full length:

• ‘Lipopathic’ peptides, which have large, hydrophobic residues located on a single face of the helix, resulting in a dual-faced helix containing two non-polar faces of unequal hydrophobicity.

In this Chapter we use a panel of biophysical and biochemical methods to further our understanding of how the re-positioning of a single methylene group can modulate peptide-lipid interactions. Placing peptide sequences in the Lep construct then allow us to assess translocon-mediated membrane insertion, as a measure of how Leu/Ile differ during membrane insertion in vivo.
Table 5.1. Examples of Leu/Ile mutations in soluble domains and their manifestation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutation</th>
<th>Manifestation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosomal membrane protein (LIMP II)</td>
<td>L475I (signal sequence)</td>
<td>Decreased lysosome targeting; implicated in lysosomal storage diseases</td>
<td>Sandoval et al., <em>JBC</em> (1994) [173]</td>
</tr>
<tr>
<td>HIV-1 Reverse transcriptase</td>
<td>L74V/I</td>
<td>Increased drug resistance</td>
<td>Kleim et al., <em>PNAS</em> (1996) [174]</td>
</tr>
<tr>
<td>Cystic fibrosis transmembrane conductance regulator (CFTR)</td>
<td>I506L (nucleotide binding domain 1)</td>
<td>Mild lung disease; Recurrent respiratory infections</td>
<td>Zielenski et al., <em>Cystic Fibrosis Genetic Analysis Consortium</em> (1998) [175]</td>
</tr>
<tr>
<td>Carboxyl-transferase (ACCase CT)</td>
<td>I1781L</td>
<td>Resistance to all ACCase herbicides</td>
<td>Zagnitko et al., <em>PNAS</em> (2001) [176]</td>
</tr>
<tr>
<td>Dihydrofolate reductase (DHFR)</td>
<td>I164L</td>
<td>Increased sulfadoxine/pyrimethamine resistance</td>
<td>Ochong et al., <em>Malar J.</em> (2003) [177]</td>
</tr>
<tr>
<td>Hepatocyte nuclear factor-1 alpha (HNF-1alpha)</td>
<td>I27L</td>
<td>Maturity-onset diabetes</td>
<td>Yang et al., <em>Cell Biosci.</em> (2016) [179]</td>
</tr>
</tbody>
</table>

*Protein names, including soluble proteins and membrane proteins where the mutation occurs in a soluble domain. *Mutation with corresponding position in primary sequence. For mutations that occur in a membrane protein, the soluble domain is indicated underneath in parenthesis. *Reported manifestation of the mutation.
5.2 Results

5.2.1 Design of Leu- and Ile-containing hydrophobic sequences

Using peptides of identical compositions, we systematically studied the consequence of replacing Leu residues in designed TM peptides with the β-branched isomer Ile. Sequences were varied to produce three different patterns of local hydrophobicity, including ‘scrambled’, ‘block/clustered’, and ‘lipopathic’ as described in detail in section 5.1 and shown in Table 5.2. All peptides studied have core sequences of 19 amino acids in length, and are composed primarily of Ala residues, supplemented with five Leu/Ile, one centrally positioned Trp, and two polar Ser residues. As identified in our previous work [146], Ala-Leu sequences containing two Ser produced maximal differences in translocon-mediated membrane insertion between closely hydrophobic sequences varying only in hydrophobic patterning. Finally, each peptide contains three-residue ‘Lys-tags’ at both N- and C-termini, which facilitate synthesis, and subsequent solubility/workup in aqueous media [161].

Calculations of peptide average hydrophobicity (Lys-tags omitted) using the Liu-Deber hydrophobicity scale [8] indicate that all peptides have an average hydrophobicity reading that surpasses the threshold for spontaneous membrane insertion (≥ 0.4), but reveals little to no difference between Leu- and Ile-containing peptides. The amphipathic/lipopathic nature of each peptide sequence pattern is expressed as a hydrophobic moment, representing the vector sum of hydrophobicity along the length of the peptide (Lys-tags omitted) [180]. Calculations of peptide hydrophobic moments were done using the online ΔG_{app} predictor software (http://dgpred.cbr.su.se/) [11,29].
### Table 5.2. Sequences and average hydrophobicity of Leu- and Ile-containing peptides

<table>
<thead>
<tr>
<th>Peptide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Average hydrophobicity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Hydrophobic moment&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leucine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>KKK-&lt;sub&gt;LA&lt;/sub&gt;SA&lt;sub&gt;LA&lt;/sub&gt;AAW&lt;sub&gt;LA&lt;/sub&gt;AL&lt;sub&gt;LA&lt;/sub&gt;SSA-KKK</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Leu&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-&lt;sub&gt;AA&lt;/sub&gt;SA&lt;sub&gt;AL&lt;/sub&gt;LLW&lt;sub&gt;LA&lt;/sub&gt;AAAASSA-KKK</td>
<td>1.3</td>
<td>0.34</td>
</tr>
<tr>
<td>Leu&lt;sub&gt;lipo&lt;/sub&gt;</td>
<td>KKK-&lt;sub&gt;AA&lt;/sub&gt;SA&lt;sub&gt;LA&lt;/sub&gt;LWLLAAAASSA-KKK</td>
<td>1.0</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Isoleucine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile&lt;sub&gt;scr&lt;/sub&gt;</td>
<td>KKK-&lt;sub&gt;IA&lt;/sub&gt;SA&lt;sub&gt;IA&lt;/sub&gt;AAW&lt;sub&gt;IA&lt;/sub&gt;AI&lt;sub&gt;IA&lt;/sub&gt;SSA-KKK</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Ile&lt;sub&gt;bl&lt;/sub&gt;</td>
<td>KKK-&lt;sub&gt;AA&lt;/sub&gt;SA&lt;sub&gt;II&lt;/sub&gt;IIW&lt;sub&gt;II&lt;/sub&gt;AAAASSA-KKK</td>
<td>1.2</td>
<td>0.35</td>
</tr>
<tr>
<td>Ile&lt;sub&gt;lipo&lt;/sub&gt;</td>
<td>KKK-&lt;sub&gt;AA&lt;/sub&gt;SA&lt;sub&gt;AI&lt;/sub&gt;IIW&lt;sub&gt;II&lt;/sub&gt;AAAASSA-KKK</td>
<td>1.2</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<sup>a</sup>scr – scrambled hydrophobic character; bl – block (clustered) hydrophobic character; lipo – lipopathic character (i.e., one face of the helix is more hydrophobic [Leu/Ile face] relative to the other face [Ala face]).

<sup>b</sup>Peptide sequences with Leu in yellow, Ile in red, Ser in blue, Ala, Trp, and Lys in black. Each peptide contains three Lys on each terminus.

<sup>c</sup>Average hydrophobicity was measured using the Liu-Deber hydrophobicity scale [8].

<sup>d</sup>Hydrophobic moments, representing the vector sum of hydrophobic character along the length of the helix [180], were determined using the online ΔG<sub>app</sub> predictor software (http://dgpred.cbr.su.se/) [11,29].

5.2.2 Translocon-mediated membrane insertion of Leu/Ile sequences

Comparing experimental apparent free energies of membrane insertion (ΔG<sub>app exp.</sub>) for the Leu- and Ile-containing peptide sequences in Table 5.2, revealed some broad differences (Table 5.3; Figure 5.1). While no significant differences were observed for ΔG<sub>app</sub> experimental values between Leu/Ile scrambled and lipopathic sequences, a large difference was seen when Leu and Ile were clustered together, with Leu<sub>bl</sub> exhibiting a highly favorable insertion free energy (Figure 5.1). Lipopathic sequences overall were the least favorable for membrane insertion with positive ΔG<sub>app</sub> experimental values (~ 0.3 kcal/mol, ~30% inserted), a feature likely explained by the increased hydrophobic moment of these two peptides (Table 5.2), which has previously been shown to hinder translocon-assisted membrane insertion [11,29,145]. The two scrambled sequences (Leu<sub>scr</sub> and Ile<sub>scr</sub>) and Ile<sub>bl</sub> exhibited similar ΔG<sub>app exp.</sub> values (~ -0.2
kcal/mol, ~60% inserted). Leu\textsubscript{bl} however displayed a relatively much more favorable $\Delta G_{\text{app exp.}}$ value of -1.1 kcal/mol (86% inserted). As reference, a purely hydrophobic sequence containing 14 Ala and five Leu (total length of 19 residues) gives a $\Delta G_{\text{app exp.}}$ value close to -1.0 kcal/mol, corresponding to > 80% of the sequence in the membrane embedded state [11]. Notably, all sequences inserted better than a poly-Ala sequence (~ 1.4 kcal/mol predicted), often used as reference for a sequence impartial to membrane integration/translocation [8,11].

![Graph](image)

**Figure 5.1. Experimental free energies of translocon-mediated membrane insertion.** Differential glycosylation patterns were used to derive experimental apparent free energy of membrane insertion ($\Delta G_{\text{app exp.}}$; see Chapter 2 for details). $\Delta G_{\text{app exp.}}$ values represent an average of three independent experiments. In comparisons of Leu- vs. Ile-containing sequences, a large difference is found between $\Delta G_{\text{app exp.}}$ of Leu\textsubscript{bl} and Ile\textsubscript{bl}. Significant differences are denoted by ****, p < 0.0001 (unpaired t-test between Leu/Ile pairs). Error bars are reported as the standard error of the mean. Experiments were performed by Nina Schiller in Dr. Gunnar von Heijne’s lab, Stockholm University.
Table 5.3. Predicted and experimentally derived free energy of insertion values ($\Delta G_{\text{app}}$) for Leu/Ile sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\Delta G_{\text{app}}$ predicted (kcal/mol)$^a$</th>
<th>$\Delta G_{\text{app}}$ experimental (kcal/mol)$^b$</th>
<th>% insertion$c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu$_{\text{scr}}$</td>
<td>0.20</td>
<td>-0.22</td>
<td>58.9</td>
</tr>
<tr>
<td>Leu$_{\text{bl}}$</td>
<td>0.15</td>
<td>-1.1</td>
<td>86.0</td>
</tr>
<tr>
<td>Leu$_{\text{lipo}}$</td>
<td>0.47</td>
<td>0.34</td>
<td>36.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile$_{\text{scr}}$</td>
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<td>-0.28</td>
<td>61.5</td>
</tr>
<tr>
<td>Ile$_{\text{bl}}$</td>
<td>0.13</td>
<td>-0.23</td>
<td>59.6</td>
</tr>
<tr>
<td>Ile$_{\text{lipo}}$</td>
<td>0.55</td>
<td>0.27</td>
<td>38.9</td>
</tr>
</tbody>
</table>

$^a$Predicted free energies of insertion ($\Delta G_{\text{app}}$ pred.) according to the $\Delta G$ predictor (http://dgpred.cbr.su.se) with subsequence allowance turned on (see section 2.15) [11,29].

$^b$Experimentally determined free energies of insertion ($\Delta G_{\text{app}}$ exp.). $\Delta G_{\text{app}}$ exp. values represent an average of 3 independent experiments. Error values were no larger than ± 0.2 standard deviation for each point. Significant differences are found between the $\Delta G_{\text{app}}$ exp. of Leu$_{\text{bl}}$ and Ile$_{\text{bl}}$ ($p < 0.0001$, unpaired t-test).

$^c$% inserted protein = fraction of singly glycosylated protein over total protein.

5.2.3 Biophysical assessment of Leu/Ile sequences in lipid bilayers

Considering the translocon insertion data, we assessed the ability of Leu and Ile peptides to preferentially interact with membranes by measuring their secondary structure and Trp burial upon reconstitution into lipid bilayers.

CD spectra of the peptides in aqueous buffer show a mix of secondary structures. While the Leu-containing peptides adopt helical structures, the Ile-containing peptides (apart from Ile$_{\text{lipo}}$, which adopted a helical structure similar to Leu$_{\text{bl}}$ and Leu$_{\text{scr}}$), adopted primarily random coil structures (Figure 5.2A). The broader range in secondary structure for Ile-containing peptides speaks toward the lower inherent helical nature of Ile over Leu and a greater sensitivity
to sequence changes. To assess sequence specific interactions within lipid bilayers, peptides were reconstituted into POPC lipid bilayers, where they displayed helical spectra close to the Leu peptides in aqueous buffer, with values in the range of \( \text{ca.} -10,000^\circ \) at 222 nm (Figure 5.2B).

![Figure 5.2. CD spectra of Leu/Ile peptides in aqueous buffer and POPC lipid bilayers.](image)

Tryptophan fluorescence blue shifts in the presence of POPC lipid bilayers revealed that despite displaying helical character close to peptides under aqueous conditions, all peptides significantly associate with POPC lipid bilayers (Figure 5.3), with subtle differences arising among isomeric peptides (Figure 5.3). Scrambled and block peptides showed no variation between Leu/Ile peptides with peptides exhibiting strong blue shifts (15-18 nm), indicative of their Trp residues residing within a hydrophobic environment (Figure 5.3B). The lipopathic peptides displayed relatively weaker blue shifts (10-13 nm), implying that the environment immediately surrounding their corresponding Trp residues does not embed these residues in a
comparably non-polar environment (Figure 5.3B). Interestingly, it is only within the lipopathic pair that differences between Leu and Ile emerge, with Ile_{lipo} displaying a significantly larger blue shift than the Leu_{lipo} peptide.

Figure 5.3. Peptide tryptophan fluorescence spectra and blue shift in the presence of lipid bilayers. (A) Trp fluorescence spectra of Leu (black lines) and Ile (red lines) peptides reconstituted into POPC lipid bilayers (10 μM peptide, 2.5 mM POPC). Scrambled peptides are denoted by dashed lines, block peptides as solid lines, and lipopathic peptides as dotted lines. The typical emission maximum value for the peptides in aqueous buffer is reported as a dotted vertical line at 350 nm. (B) Trp blue shift calculated using the Trp emission maxima of individual peptides in aqueous buffer. Data represent the average of at least three independent experiments. Error is reported as standard error of the mean. Significant differences are reported as *, p < 0.05 (unpaired t-test between Leu/Ile pairs).

Results with POPC lipid bilayers imply the scrambled and block peptides show little to no difference between Leu/Ile-containing sequences, unlike in the translocon insertion assay. Differences do appear to arise between Leu/Ile peptides when the bulky hydrophobic residues are positioned on a single face of the helix.
5.2.4 Protease susceptibility of Leu/Ile sequences in lipid bilayers

To test the aqueous exposure of peptides reconstituted into POPC lipid bilayers, samples were treated with a protease and the degree of peptide degradation determined. Proteinase K (ProtK), a well-established and highly active general protease that cleaves peptide bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids [181,182], was added to proteoliposomes containing Leu/Ile peptides. As shown in Figure 5.4A, experiments evaluating the protease susceptibility of the peptides revealed that Leu- and Ile-containing peptides were equally susceptible to degradation in aqueous buffer alone. However, we found that Ile peptides were more protected from ProtK than Leu peptides when in the presence of lipid bilayers (Figure 5.4B). Increased protection from protease degradation should, in principle, position the Ile peptides more deeply into the lipid bilayer where access to the protease is diminished.

![Figure 5.4 Protease degradation of Leu and Ile peptides in aqueous and lipid environments.](image)

(A) Leu (black) and Ile (red) peptides were incubated with ProtK (10 µM peptide to 100 µg/mL protease) in aqueous buffer. Remaining peptide was quantitated via densitometry on SDS-PAGE. Leu and Ile peptides show equivalent susceptibility to protease degradation. (B) Proteoliposomes prepared from POPC reconstituted with Leu/Ile peptides (10 µM peptide, 2.5 mM lipid) were incubated with ProtK (100 µg/mL) and peptide degradation monitored using SDS-PAGE. The peptide legends to the right of each diagram correspond to the order from top to bottom of the 300 min. points for each peptide. Error bars report standard error of the mean. Data represent the average of three independent experiments.
5.2.5 Lipid accessible surface area of Leu/Ile lipopathic peptides

Despite the fact that Leu and Ile side chains are isosteres of one another, they present different surface areas when positioned on a helical face (Table 5.4). To quantify the differences in surface area between the isomeric peptides, models were generated using the most commonly occurring chi angles ($\chi$) found in helical domains of soluble proteins and TM domains (see Chapter 2 for details), as most peptides exhibit similar helical character to one another under aqueous conditions and in the presence of POPC liposomes (Figure 5.2) (exception of Ile$\text{bl}$ and Ile$\text{sc}$, which show a significant loss in helical character under aqueous conditions). Further, ambiguity over the insertion state of the peptides, particularly considering the relatively more red shifted Trp fluorescence of the lipopathic sequences, renders assessment of both soluble and TM rotamers feasible. The accessibility of peptide surfaces to a lipid acyl chain, mimicked by a methylene sized probe (1.88 Å radius), was calculated and lipid accessible surface area (LASA) determined (Table 5.4).

Notable differences are found in LASA values between peptides, with those modelling rotamer angles commonly found in TM helical domains having consistently larger accessible surface areas. Additionally, Ile-containing peptides generally exhibit smaller LASA values than Leu counterparts (Table 5.4). Ile, being $\beta$-branched, has the bulk of its side chain density positioned closer than Leu to the core of the helix. In this manner, Ile provides the peptides with a relatively more uniform, albeit reduced surface area for contact with lipids (Table 5.4). In contrast, the presence in Leu of a more extended side chain results in a more protruding surface, capable of increasing van der Waals-like contacts with surrounding lipids, yet potentially contributing to disruption of membrane acyl chain packing. Together, the biological membrane insertion data (section 5.2.2) and biophysical partitioning data (section 5.2.3) suggest that
differences, although subtle, arise between Leu/Ile peptides in a sequence dependent manner that we evaluate in further detail below.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>LASA values for soluble helix (Å²)</th>
<th>LASA values for TM helix (Å²)</th>
<th>Change in LASA (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrambled</td>
<td>1798.16</td>
<td>1848.63</td>
<td>55.7</td>
</tr>
<tr>
<td>Blocked</td>
<td>1769.21</td>
<td>1830.81</td>
<td>45.48</td>
</tr>
<tr>
<td>Lipopathic</td>
<td>1775.47</td>
<td>1806.52</td>
<td>65.67</td>
</tr>
</tbody>
</table>

Table 5.4. Lipid Accessible Surface Area (Å²) of Leu/Ile peptides

aLASA values are given for Leu and Ile peptides modelled as α-helices with rotamer $\chi$ angles commonly found in soluble helices [155]. bLASA values are given for Leu and Ile peptides modelled as α-helices with rotamer $\chi$ angles commonly found in TM helices [156]. Rotamer angles are provided in section 2.10, Table 2.1. *Ile_{scr} and Ile_{b} exhibit significantly less helical character in aqueous buffer relative to the other peptides (Figure 5.2). The change in LASA values between Leu/Ile isomers is provided (Leu peptide LASA value - Ile peptide LASA value). A positive value indicated that Leu peptide models have a larger LASA.

5.3 Comparisons of Leu/Ile-block peptide sequences

To uncover a biophysical explanation for the observed differences in translocon-mediated membrane insertion of Leu/Ile block sequences (Figure 5.1), peptide interactions with various membrane mimetics were assessed.
5.3.1 Leu/Ile-block peptide interactions with detergent micelles

As shown initially in Figure 5.2, the CD spectra of Leu\textsubscript{bl} and Ile\textsubscript{bl} peptides in aqueous buffer show a large difference in helical character. The Leu-containing peptide adopted a weak helical structure while the Ile peptide displayed a spectrum more characteristic of a random coil. Upon addition of detergent micelles (SDS or DPC), both peptides adopted helical conformations with no significant variation between Leu and Ile peptides (Fig. 5.6A; solid and dotted lines). Assessment of Trp blue shifts revealed Leu and Ile peptides are similarly buried within the either micelle core (Fig. 5.6B). Notably the blue shift values for the zwitterionic DPC micelles are much smaller than those for the anionic SDS micelles, likely a result of the attractive electrostatic forces between positively charged peptide Lys-tags and the negatively charged SDS headgroups.

![Figure 5.5](image)

**Figure 5.5. CD spectra and tryptophan blue shift of Leu/Ile peptides in detergent micelles.** (A) Leu (black) and Ile (red) peptides in SDS micelles (solid lines; 10 µM peptide, 175 mM) and DPC micelles (dotted lines; 25 mM). Spectra represent the average of at least three independent samples. See Chapter 2 for details of sample preparation. (B) Trp blue shift in the presence of DPC (2.5 mM) and SDS (35 mM) detergent micelles. Leu\textsubscript{bl} is depicted by black bars, Ile\textsubscript{bl} by white bars. Blue shifts were calculated using the Trp emission maxima of peptides in aqueous buffer (348, 349 nm for Leu\textsubscript{bl} and Ile\textsubscript{bl} respectively). Data represent the average of three independent experiments. Error is reported as standard error of the mean.
5.3.2 Leu/Ile-block peptide interactions with lipid bilayers

Expanding on the experiments performed using POPC as a standard lipid bilayer, peptides were reconstituted into 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipid bilayers. The higher transition temperature of DMPC bilayers (Tm = 24°C) allows ready control over membrane fluidity, unlike POPC bilayers which have a much lower transition temperature (Tm = -2°C). At temperatures below the transition temperature, bilayers exist in a non-fluid gel phase, and at higher temperatures, in a fluid liquid-crystalline phase reminiscent of native bilayers. Secondary structure and the Trp burial of Leu/Ile block peptides were assessed for DMPC bilayers in both the gel and liquid-crystalline phase.

Under gel phase conditions (DMPC at 15°C), Leu_{b1} adopts a helical structure, like that observed for the peptide in aqueous buffer and POPC lipid bilayers (Figure 5.6; solid lines). The Ile_{b1} peptide, while showing a more noticeable increase in helical character from aqueous buffer to the gel phase lipid bilayer (Figure 5.6; dashed lines), retained overall less helical character than Leu_{b1}. Membranes heated to the liquid-crystalline phase (DMPC at 35°C) showed a much larger increase in helical character for both peptides, with helicity closely matching that observed in the presence of detergent micelles (Figure 5.6). No significant differences were observed in helical character between the Leu and Ile peptides in DMPC at 35°C, while Ile_{b1} was significantly less helical in aqueous buffer and DMPC bilayers at 15°C.
Figure 5.6. CD spectra of Leu/Ile peptides in aqueous buffer and lipid bilayers. Leu (solid line) and Ile (dashed line) peptides in aqueous buffer (10 µM peptide, 10 mM Tris 10 mM NaCl pH 7.4), DMPC lipid bilayers (10 µM peptide, 2.5 mM lipid) at 15°C and 35°C. Spectra represent the average of at least three independent samples. See Chapter 2 for details of sample preparation.

To assess if the peptides were differentially buried within the core of the lipid bilayer, Trp fluorescence and quenching were measured in the presence of DMPC lipid bilayers in both the gel and liquid-crystalline phase. Both Leu\(_{bl}\) and Ile\(_{bl}\) exhibited favorable blue shifts (~ 20 nm) in the presence of fluid DMPC lipid bilayers and minimal blue shifts (~2 nm) for DMPC bilayers in the gel phase (Figure 5.7). Heating gel phase membranes resulted in an increase in Trp blue shift (Figure 5.7B), while cooling liquid-crystalline membranes resulted in a red shift in the Trp fluorescence (Figure 5.7A). Thus, the fluidity of the membrane greatly impacts the degree of Trp burial for both Leu and Ile-containing peptides, with more fluid bilayers being more amenable to peptide penetration. Regardless of differences arising from starting conditions (gel phase vs. liquid-crystalline), Leu\(_{bl}\) and Ile\(_{bl}\) peptides exhibited identical Trp burial in DMPC membranes of varying fluidity. Addition of acrylamide, a soluble Trp fluorescent quencher, revealed that both Leu/Ile block peptides were equally susceptible to quenching (Figure 5.8), reinforcing the idea of both peptides having similar degrees of Trp burial.
Figure 5.7. Tryptophan emission maxima in gel and liquid-crystalline phase lipid bilayers. (A) DMPC lipid bilayers were heated from 15°C to 35°C and peptide Trp emission maxima recorded. (B) DMPC lipid bilayers were cooled from 35°C to 15°C. Leu<sub>bl</sub> peptides are depicted as black circles, Ile<sub>bl</sub> peptides as red circles. The Trp emission maxima for the peptides in aqueous buffer was experimentally determined (348 nm) and is shown as a horizontal dotted line, labeled Aq. The transition temperature of the DMPC is noted by a vertical dotted line at 24°C. Error is reported as standard deviation. Each data point represents the average of at least three independent experiments.

Figure 5.8. Stern-Volmer plot of tryptophan quenching in the presence/absence of fluid DMPC lipid bilayers. Leu (black) and Ile (red) block peptides were dissolved in aqueous buffer (dashed line) or reconstituted into DMPC lipid bilayers (solid line). Trp fluorescence quenching was recorded measured while bilayers were in the liquid-crystalline phase (35°C). Initial fluorescence in the absence of quencher ($F_0$) divided by fluorescence in the presence of quencher ($F$) was plotted against acrylamide concentration (M). Minimal quenching is observed for peptides in the presence of liquid-crystalline bilayers (solid lines). Stern-Volmer slopes are nearly identical for Leu<sub>bl</sub> and Ile<sub>bl</sub> peptides in the presence of lipid bilayers (2.2 vs. 2.17 for Leu<sub>bl</sub> and Ile<sub>bl</sub>, respectively).
5.4 Comparisons Leu/Ile lipopathic peptide sequences

We then explored the impact of positioning Leu/Ile on a single face of a helix, generating ‘lipopathic’ sequences as detailed in section 5.2.1 (Peptide design). Initial studies reconstituting the peptides into POPC lipid bilayers revealed Ile\textsubscript{lipo} to have its Trp residue more buried than the corresponding Leu-containing peptide. To determine the origin of this apparent preference for Ile over Leu in a lipopathic face, Trp quenching experiments and peptide interactions with lipid bilayers of altered lipid acyl chain packing were conducted.

5.4.1 Tryptophan quenching within the core of the lipid bilayer

To further evaluate the apparent increased Trp burial of the Ile-lipopathic peptide over the Leu-lipopathic peptide in POPC lipid bilayers, membrane-embedded Trp quenchers were used to assess the degree of Trp burial. Thus, dibromo-lipid quenchers (i.e., 6,7-dibromoPC, 9,10-dibromoPC, and 11,12-dibromoPC) containing two Trp-quenching bromine atoms located at positions (6,7), (9, 10), and (11, 12) within its stearoyl-acyl chain, positioning them ~ 11, 8.3, and 6.5 Å, respectively, from the core of the bilayer. Significant quenching of the centrally placed Trp residue was found at all dibromoPC quencher depths relative to the negative control for membrane association (Figure 5.9; BSA vs. Leu/Ile\textsubscript{lipo}). However, the degree of quenching between the two peptides was observed to vary, with Ile\textsubscript{lipo} experiencing more quenching than Leu\textsubscript{lipo} near the core of the bilayer (Figure 5.9C; 11,12-dibromoPC, 6.5 Å from the bilayer center), an indication that the Ile\textsubscript{lipo} peptide is relatively more buried into the quencher-substituted POPC lipid bilayer. When compared to two control peptides available in the lab,
including a highly hydrophobic peptide and a more polar peptide (see Chapter 2 for details), the Ile\textsubscript{lipo} peptide exhibits quenching closer to the hydrophobic insertion control, with Leu\textsubscript{lipo} exhibiting quenching closer to the polar surface bound control (Figure 5.9C).

![Figure 5.9](image)

**Figure 5.9. Stern-Volmer plots of Trp fluorescence quenching within the lipid bilayer.** Peptides were reconstituted into POPC bilayers substituted with increasing amounts of (A) 6,7-dibromoPC or (B) 9,10-dibromoPC or (C) 11,12-dibromoPC each corresponding to different membrane depths (11, 8.3, 6.5 Å from the bilayer core respectively). \(F_0/F\) is plotted vs. mol % dibrominated POPC. A highly hydrophobic insertion control peptide (sequence: KKK-AAAAAALLLWLLAAAAAAA-KKK, [146]) and a polar surface bound control peptide (sequence: KKK-ASASASLLLWLLASASASA-KKK, [146]) are as indicated on the diagram. Bovine serum albumin (BSA) was used as a negative control for membrane interaction. Data points represent the average of at least three independent experiments. Slope values as measured by linear regression analysis are used to determine and compare Trp quenching.

5.4.2 Impact of varied lipid composition on peptide interactions with lipid bilayers

In view of the Trp quenching experiments, we undertook to assess how the lipid environment *per se* impacts peptide interaction and burial into membranes by determining whether altered lipid composition would impact peptide-lipid interactions relative to POPC bilayers. To perform these experiments, peptides were reconstituted in lipid bilayers with varying acyl chain saturation. Two additional lipids beyond POPC were employed, including 1,2-
dioleoyl-\(sn\)-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-\(sn\)-glycero-3-phosphocholine (DPPC). DOPC, having two unsaturated oleoyl-acyl chains (18:1 cis), decreases lipid packing while DPPC, containing two saturated palmitoyl-acyl chains (16:0 cis), increases lipid packing relative to POPC (single palmitoyl and oleoyl-acyl chain; 16:0, 18:1). Trp blue shifts generally decreased (relative to 100% POPC) with increasing amounts of unsaturated acyl chains (DOPC) (Figure 5.10A). In comparisons of Leu/Ile_{lipo} peptides, the Ile-containing peptide initially displayed the larger blue shift relative to Leu_{lipo} in bilayers composed of 100% POPC. However, upon substitution of POPC bilayers with 25% and further 50% of DOPC, the peptide displaying the larger blue shift became the Leu_{lipo} (Figure 5.10A).

To increase lipid packing relative to POPC, membranes were substituted with the doubly saturated DPPC (16:0 cis). Due to the high transition point of DPPC, all samples, including a POPC repeat control, were heated during extrusion and Trp fluorescence spectra were recorded at elevated temperatures (see Chapter 2 for details). Under heated conditions, Ile_{lipo} and Leu_{lipo} gave similar strong blue shifted values for 100% POPC (Figure 5.10B). Trp blue shifts generally decreased (relative to 100% POPC) with increasing amounts saturated acyl chains (DPPC). Upon substitution of 25% and further 50% of the bilayer with DPPC, the peptide displaying the larger blue shift became Ile_{lipo} (Figure 5.10B). Peptides displayed minimal changes in helical character across membranes of different compositions (Figure 5.10C, D).
Figure 5.10. **Trp fluorescence blue shifts and helical character in bilayers of varying acyl chain saturation.** Leu (solid circles) and Ile (open circles) peptides reconstituted into POPC lipid bilayers (10 µM peptide to 2.5 mM lipid) with increasing amount of DOPC or DPPC. (A) Trp blue shifts across POPC/DOPC and (B) POPC/DPPC membranes. Trp was excited at 280 nm and emission spectra recorded between 300-400 nm. (C) Peptide helicity as measured by MRE at 222 nm across POPC/DOPC and (D) POPC/DPPC membranes. Samples in panel B and D (POPC/DPPC mixtures) were extruded and measured under elevated temperatures (see Chapter 2 for details). Error bars report standard error of the mean. Leu and Ile peptides that displayed significantly different blue shifts and helicity were denoted with (*), p <0.05 (unpaired t-test between Leu/Ile pairs). Data represent the average of at least two independent experiments.

5.5 Discussion

Where there is a requirement for hydrophobic character, such as in the core of the tertiary structure of soluble proteins to promote folding, or in nascent TM segments of membrane
proteins to promote their insertion into lipid environments, it is broadly accepted that Leu and Ile, can interchangeably serve these functions. In the present work, we undertook to dissect, in part, the role(s) of these amino acids, focusing on the similarities and/or distinctions we can identify between the contributions of Leu vs. Ile to membrane protein structure and folding.

In comparisons of experimental values to predicted $\Delta G_{\text{app}}$ values derived from the online sever (http://dgpred.cbr.su.se/) (Table 5.3), no significant correlation is found (with or without the allowance for shorter subsequences, see Chapter 2 for details), indicating an inability for the server to accurately differentiate between these sequences of close hydrophobicity but varied hydrophobic patterning (Table 5.3; Figure 5.11). While the online server (with shorter subsequences allowed) was able to more closely predict the favorable insertion of the scrambled and Leu$_{bl}$ sequences, prediction of both lipopathic sequences and Ile$_{bl}$ remains difficult (Table 5.3; Figure 5.11).

Increased hydrophobic moments of the lipopathic peptides (Table 5.2) can likely explain the observed less than favorable insertion free energies, as large hydrophobic moments have been previously shown to decrease membrane insertion efficiency [11]. However, Leu$_{bl}$ and Ile$_{bl}$ have nearly identical hydrophobic moments (Table 5.2), and yet Ile$_{bl}$ exhibits a significantly less favorable $\Delta G_{\text{app}}$ experimental value. What it is about an Ile-block vs. a Leu-block that decreases membrane integration in vivo remains to be resolved.
Figure 5.11. **Comparison of experimentally derived free energies of insertion to predicted values.** Experimental apparent free energies of membrane insertion were compared to predicted values listed in Table 5.3. No significant correlation is observed between experimental and predicted values ($R^2 = 0.31$, $p = 0.25$). Generally, sequences exhibited more favorable insertion energies than predicted. Leu peptides are depicted as solid circles, Ile peptides as open circles. Data represents the average of three independent experiments. Error is reported as standard error of the mean.

5.5.1 **Leu/Ile-block differentiation during translocon-mediated membrane insertion**

Despite a large difference between the free energies of insertion for Leu-block and Ile-block sequences in the Lep construct, biophysical techniques measuring peptide partitioning into detergent micelles and lipid bilayers show no difference between the two peptides. Detergent studies have shown $\text{Leu}_{\text{bl}}$ and $\text{Ile}_{\text{bl}}$ peptides interact with micelles in a similar manner, displaying identical CD spectra (Figure 5.5A) and Trp blue shifts (Figure 5.5B). Studies with DMPC lipid bilayers have shown that both peptides interact with lipid bilayers in a comparable manner independent of bilayer phase (Figure 5.6, Figure 5.7). The data herein suggest that a contiguous
stretch of Leu or Ile is analogous in biophysical properties for the sequences and conditions tested. The failure of the biophysical techniques to recapitulate the differences observed between a Leu-block and Ile-block during translocon-assisted membrane insertion therefore requires a more detailed explanation.

The answer may reside in the inherent structure of both sequences under aqueous conditions. $\text{Leu}_{bl}$ exhibits more helical character than $\text{Ile}_{bl}$ in aqueous buffer (Figure 5.5A), as predicted by traditional Chou-Fasman secondary structure propensities [168]. When transiting through the aqueous translocon channel the adoption of a helical structure would present the contiguous stretch of hydrophobic Leu residues in the primary sequence as a strong hydrophobic locus extending around the circumference of the sequence. $\text{Ile}_{bl}$, while containing a contiguous stretch of hydrophobic amino acids, may lack adequate helical character under aqueous conditions to present the stretch of Ile as the strong hydrophobic locus needed to drive membrane insertion.

The effects of the greater helical character under aqueous conditions of the $\text{Leu}_{bl}$ sequence do not appear to manifest in the biophysical assays described above perhaps because they are conducted after the systems have come to an equilibrium state. The peptides when exposed to a membrane mimetic can minimally associate with the surface of the micelle or lipid bilayer as well as the micellar or bilayer interior. During translocon-mediated membrane insertion the sequence does not have the opportunity to remain associated with the translocon-membrane interface but must become membrane integrated or translocated.
5.5.2 Lipopathic peptide interactions with artificial membranes

The lipopathic design, placing bulky hydrophobic Leu/Ile residues opposite the relatively ‘benign’ Ala face, pits the Trp-proximal Leu vs. Ile residues directly against each other, allowing subtle differences between the isomeric peptides to be discovered. Trp fluorescence experiments with POPC lipid bilayers revealed a greater blue shift for the Ile\textsubscript{lipo} peptide over Leu\textsubscript{lipo} (Figure 5.3B). Quenching experiments using dibromo-phosphatidylcholine lipids confirm the increased penetration depth of Ile\textsubscript{lipo} over Leu\textsubscript{lipo} (Figure 5.9).

To gain an understanding of the impact the lipid bilayer environment has on peptide-lipid interactions, we systematically increased and decreased the degree of acyl chain saturation within the bilayer and measured peptide helicity and Trp blue shift. Results suggested that burial of the Trp, proximal to the Leu/Ile face, is dependent on the relative degree of lipid packing as evidenced by the fluctuations in Trp blue shift values (Figure 5.10A, B). Bilayers with less lipid packing – higher content of unsaturated acyl chains – are initially more amenable to the burial of peptides with larger surface areas, \textit{viz}., the bulkier Leu peptides (Figure 5.10A). The Leu face becomes more buried relative to the Ile face as the more ‘fluid’ bilayer favors increased van der Waals/hydrophobic contacts between peptide and lipid over lipid-lipid packing (Fig. 5.11A). On the other hand, bilayers with more lipid packing – higher content of saturated acyl chains – are initially more amenable to the burial of the peptides with the smaller surface area, \textit{i.e}., the ‘smoother’ Ile peptides (Figure 5.10B). In more-well-packed bilayers, lipid-lipid packing is favored over increased contacts between peptide and lipid, favoring the least lipid packing-disruptive peptides.

While there are distinct differences in LASA values across this peptide series (Table 5.4), the essential similarity of the patterns in the CD spectra in POPC liposomes of all six peptides
establishes their uniform ability to adopt helical structures and interact with bilayers, where they assume predominantly helical structures (Figure 5.2B). However, the CD spectra provide no direct information as to whether the peptides are embedded in the bilayer surface or take TM orientations. The Trp fluorescence blue shift values do provide a measure of the local environment surrounding the centrally located Trp. In one scenario, we can regard the lipopathic peptides as rotating and sampling within the water/membrane interface, with the consequence that the Leu peptides will exert their membrane-disruptive potential, potentially rendering the local environment more susceptible to ProtK than their Ile counterparts. Our findings with the ProtK degradation experiments (Figure 5.4B) broadly support these considerations. Thus, the Ile peptides appear more protected from the protease than their Leu counterparts, again implying that the Ile peptides either penetrate further into the membrane, and/or impart less local disruption to the bilayer.

The overall results suggest that two main interactions beyond the initial hydrophobic effect appear to dominate peptide folding and interactions within the membrane: (1) hydrophobic interactions between amino acid side chains and the surrounding lipid; and (2) degree of disruption of lipid-lipid packing. This ‘battle of giants’ is likely responsible for observations that Leu and Ile will accordingly play varying roles in membrane protein folding. The Leu peptides, with their greater surface area, may increase overall hydrophobic interactions via van der Waals packing with surrounding lipids but at the cost of disrupting packing between lipid tails. The Ile peptides, with a smaller exposed surface area, may decrease the disruption to lipid-lipid packing; however, this may be at the cost of decreasing favorable van der Waals contacts that ostensibly could maximize their availability for favorable contacts in interfacial interactions with neighboring helices.
5.6 Conclusion

Given the subtleties in assessing the ‘differences’ between Leu- vs. Ile-containing peptides, it is likely that the largest impact of their relative structure/function roles will manifest in membrane protein folding, where helix-helix vs. helix-lipid packing interactions are the crucial determinants. Thus, for a given membrane protein, whether Leu/Ile-containing TM segments will increase interactions with lipids, drive membrane insertion, or promote protein-protein interactions, will be highly dependent on the nature of the surrounding bilayer and – going beyond the local effects of Leu vs. Ile residues described here – ultimately on the longer-range set of interactive features of the specific protein sequence(s) involved.
6 Discussion
6.1 Defining a transmembrane domain

Throughout our studies we have designed ‘TM’ sequences to test the boundaries of what is considered acceptable for membrane integration. Every sequence studied herein had an average hydrophobicity above that required for spontaneous membrane insertion [8], and yet displayed vastly different insertion propensities in both biophysical and biological experiments. Despite a substantial content of polar residues, many sequences tested in Chapter 3 were capable of favorably interacting with membrane mimetics and adopting TM configurations, notably if a contiguous hydrophobic block was present (nine consecutive Leu) [145]. In this manner, sequences with higher polar content can become ‘membrane embedded’ if a strong and sufficiently long hydrophobic core is present to overcome the unfavorable barrier of burying some polar side chains within the bilayer [112].

The increased burial of polar residues when found alongside a hydrophobic block is observed again in Chapter 4, in which block sequences (five or seven contiguous Leu) containing polar Ser residues underwent membrane integration more readily in vivo than sequences lacking such a hydrophobic locus [146]. Partitioning between an aqueous phase and lipid bilayers identified a distinct polar threshold for favorable peptide-lipid interactions that is indifferent to hydrophobic patterning. Therefore, the ability of the hydrophobic block to drive translocon-mediated membrane insertion is dependent on the unique environment the insertion machinery provides (i.e., channel-bilayer interface). Thus, the ability of a hydrophobic block to ‘carry’ polar residues into the bilayer may rely heavily on the hydrophobic core to be long and/or hydrophobic enough to span the bilayer alone (Ser-Leu series) or require passage through the translocon channel to lower the energy barrier to membrane insertion (Ala-Leu-Ser series).
The failure of some sequences to undergo membrane integration via the translocon, despite favorable biophysical properties, identifies additional ‘checkpoints’ that must be satisfied for selection of TM domains in vivo. Notable examples include the amphipathic $SL_{amp}$ sequence and the positional dependence of hydrophobic blocks on insertion efficiency. As previously described, high amphipathicity decreases insertion potential, the polar face of sequences remaining favorably associated with the channel, lowering the driving force for membrane integration [11]. Positional biases for hydrophobic blocks along the length of a TM helix are only apparent during translocon-mediated membrane insertion, in which a hydrophobic block located on the C-terminal half of the helix, and consequently closer to the hydrophobic constriction ring of the translocon, results in decreased membrane integration [94].

A lack in inherent helical character can also impact the ability to undergo membrane integration, as seen in Chapter 5. While early studies showed helicity within the aqueous channel was not a requirement for insertion efficiency, these studies used predominantly hydrophobic sequences (i.e., poly-Val, poly-Leu) [183]. The overwhelming need to bury these highly hydrophobic sequences within the bilayer core could outweigh any negative effects from an inherently less helical sequence. Additional studies have suggested the early adoption of a helical structure in either the ribosome exit tunnel [184,185] or the translocon channel [76] can pre-dispose a sequence for insertion, promoting entry into the bilayer. Moreover, as suggested by previous studies in our lab, “delta helices” found in secreted proteins with higher inherent helical character are more readily converted into membrane embedded domains [53].

The Leu/Ile-block sequences described in Chapter 5 allowed us to uncouple helical propensity from hydrophobicity. Peptides were similar in average hydrophobicity, but displayed very different secondary structure propensities under aqueous conditions [168]. This variation in
secondary structure may be responsible for the observed disparity in membrane insertion efficiency between the Leu and Ile-block sequences. The increased helical propensity for the Leu-containing sequence under aqueous conditions – and perhaps within the translocon channel – may promote membrane integration over the predominantly unstructured Ile-containing sequence.

Overall, the data gathered within this thesis suggest that the definition of a TM domain can be quite broad and extend beyond traditional expectations, particularly for marginally hydrophobic TM helices. Local hydrophobic and helical patterning within a sequence, amphipathicity/lipopatricity, and helical propensity in both aqueous and membranous phases, can play equally important roles in defining a TM domain (Figure 6.1). Further, the initial starting phase, the final ‘receiving’ environment, and the pathway into the membrane, are all critical in how hydrophobicity is perceived along a nascent TM domain. Deviations between biophysical and biological experiments have revealed the importance of the unique environment imparted by the translocon channel itself, and how it can greatly influence membrane integration (i.e., restrictions imparted by the constriction ring). In the prediction of native TM domains and design of new synthetic sequences, the culmination of features above that define a membrane embedded segment must be taken into consideration.
Figure 6.1. Summary of the forces contributing to the membrane insertion of marginally hydrophobic TM domains. High inherent helical character, low amphipathicity/lipopathicity, and the presence of a strong hydrophobic locus (i.e., a contiguous stretch of hydrophobic amino acids that extends around the circumference of the helix) can all promote the translocon-mediated membrane insertion of marginally hydrophobic TM domains. Protein sequences are modelled as either α-helical or unfolded segments. Depicted are Ile\textsubscript{bl} (unfolded and translocated), 5L2S\textsubscript{scf} (helical but translocated), and Leu\textsubscript{bl} (helical and membrane embedded).

6.2 Relative role(s) of Leu and Ile in membrane proteins

In Chapter 5 we undertook a systematic study of how Leu and Ile affected peptide-lipid interactions. These two amino acids occur with relatively high frequency within TM domains (Leu, 15.8 % and Ile, 9.8%) \cite{43} and are often reported as having similar hydrophobicity and membrane insertion propensity (Table 1.1). Varying only in the placement of a single methyl group (Figure 1.1), these two amino acids provide us with a unique scenario in which we can assess how a modest variation in peptide surface area, but not in hydrophobicity, can alter peptide-lipid interactions.

As discussed above, Leu/Ile-block peptides appear to differ very little in how they interact with membranes and mimetics. The differential roles of Leu and Ile in transmembrane
domains may therefore manifest primarily in protein-protein interactions. Most reports in the literature that comment on functional differences between Leu and Ile in TM domains, involve the interchange of these residues within protein-protein interfaces [119,147–149,186]. Our Leu/Ile sequences, lacking discrete oligomeric states (Appendix 1), therefore present comparable peptide-membrane interactions.

The Leu/Ile lipopathic peptides did reveal variations in interactions with lipid bilayers; however, their interactions were more reminiscent of surface bound, membrane interactive peptides rather than membrane spanning TM domains. Leu- and Ile-containing lipopathic peptides displayed varying preferences for lipid bilayers, dependent on bilayer acyl chain packing; Ile-containing peptides, having less accessible surface area, interacted more favorably with more-well-packed lipid bilayers, potentially minimizing disruption of lipid-lipid packing. Leu-containing peptides, having increased accessible surface area, interacted most favorably with less-well-packing bilayers. These results have implications for how membrane interactive peptides, such as antimicrobial and cell penetrating peptides, contact and bind membranes. Studies with naturally occurring antimicrobial peptides have previously suggested a potential role for Ile/Leu in modulating binding to membranes [171]. The relative role(s) of Leu and Ile within membrane proteins is thus dependent on the specific role/state of the protein sequence itself (i.e., TM or surface bound; monomeric or oligomeric) and the lipid bilayer itself (i.e., tightly or loosely packed acyl chains).

6.3 What is hydrophobicity in the membrane?

The role of hydrophobicity in soluble protein folding is well described [2]. However, when we consider a polypeptide chain folding within a predominantly apolar environment, we
must re-think how we view hydrophobicity and protein folding. Throughout the present work, we have endeavored to capture the dynamic and environment dependent nature of hydrophobicity in the folding of TM domains. Through our studies of compositionally identical, sequentially different peptides and the application of various membrane mimetics, we have shown how sensitive hydrophobicity measurements are to protein sequence patterning and the receiving environment.

While hydrophobicity in the traditional sense is apparent during initial membrane interactions and insertion (i.e., during partitioning from the aqueous translocon channel, or aqueous phase into the membrane), compatibility of the incoming protein segment with the receiving environment then becomes an important factor. In the broadest context, the fact that there is no water in the membrane does not render a Leu/Ile/Val-rich sequence any less “hydrophobic”; rather, the accessibility to lipid that each residue contributes to a protein’s irregular surface becomes a measure of “lipophilicity/lipopathy” – love/hate of lipid. The accumulation of favorable protein-lipid and protein-protein interactions contrasted against the favorable packing of the lipid acyl chains to themselves is responsible for adoption of a membrane protein’s final biologically-functional structure. The present findings are consistent with earlier work from our laboratory, which indicates that the acyl chains in a lipid bilayer are in fact a non-ideal solvent for proteins, with the consequence that the lipid as a solvent acts, in part, to promote membrane protein folding [1,118]. If lipid were a good solvent, each TM helix would simply be surrounded and solvated by lipid, and multi-spanning helices might not be driven to contact one another. In the longer range, a further understanding of how lipids interact with the various irregular surfaces presented by transmembrane domains is particularly apt when elucidating how subtle changes to a protein segment can drastically alter proper membrane protein folding and function.
6.4 Future directions and implications

While current understandings of what constitutes a TM segment enable accurate prediction of most TM domains, the prediction of marginally hydrophobic or atypical TM domains remains a challenge [52]. Restrictions on membrane insertion imparted by the translocon insertion machinery itself, and cumulative effects from hydrophobic/helical patterning of the nascent protein sequence, both remain areas for further exploration. The current ‘biological hydrophobicity scale’ as developed by Hessa et al. [11,29], may be used to systematically probe the translocon channel for further TM sequence motifs that induce deviations from typical membrane insertion profiles. Pairing *in vivo* membrane insertion data with biophysical studies of membrane partitioning will enable direct comparisons of the role(s) of the translocon and the bilayer in membrane integration.

Our findings identified hydrophobic ‘blocks’, within model protein sequences, capable of increasing membrane interactions and bilayer insertion [145,146]. The limit for which clustering hydrophobic residues together could drive increasingly polar sequences into the bilayer was tested with polar residues placed external to the hydrophobic block [146]. To determine whether a hydrophobic cluster could truly ‘carry’ a polar/charged residue into the bilayer, hydrophobic block sequences with polar/charged residues placed central to the block itself could be completed. Further, a search of native TM sequences rich in polar/charged residues may reveal common sequence elements (*i.e.* clustering of hydrophobic residues in a continuous block or face) in nature that may promote the insertion of marginally hydrophobic TM domains *in vivo*.

Increasing our knowledge as to how hydrophobic and helical patterning impact protein-lipid interactions would, in principle, provide insight into how the careful balance between
protein-protein and protein-lipid interactions is maintained to ensure the correct fold and function of membrane proteins. Subtle differences in protein surface area have been implicated in the specificity of TM helix-helix interactions [119,148]. Long-range impacts of protein-lipid interactions on protein-protein interactions could be further delineated by assessing the self-association of model peptides containing common oligomerization motifs amid a background sequence varying in hydrophobic patterning. The role(s) of the bilayer, in conjunction with hydrophobic sequence patterning, on TM-TM oligomerization could be expanded through studies involving bilayers of varied properties, altering protein-lipid packing.

Ultimately, an understanding of how hydrophobicity is ‘read’ within the membrane and further how changes to both protein sequence (i.e. patterning of residues) and membrane properties (i.e. lipid bilayer fluidity, thickness, packing, etc.) mediate protein-protein interactions and protein-membrane interactions will increase the accuracy of membrane protein prediction, further our understanding of how membrane proteins fold, and aid in de novo design of membrane proteins and membrane interactive peptides.
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Figure 7.1. Competition FRET experiments of Leu/Ile-block peptides in detergent micelles and lipid bilayers. (A) Leu\textsubscript{bl} homo-FRET in SDS micelles. (B) Ile\textsubscript{bl} homo-FRET in SDS micelles. (C) Leu\textsubscript{bl} homo-FRET in POPC liposomes. (D) Ile\textsubscript{bl} homo-FRET in POPC liposomes. Peptides were co-dissolved in TFE prior to addition of detergent or chloroform dissolved lipids (see Chapter 2 for details). Donor (Dansyl chloride) labeled peptide is denoted by D, Acceptor (Dabsyl chloride) labeled peptide by A, and unlabeled peptide (U). Donor and Acceptor peptides were mixed in a 1:1 ratio (DA). Donor, Acceptor, Unlabeled peptides were mixed in a 1:1:2 ratio (DAU). Significance is denoted by **, p < 0.01, ****, p < 0.001 (one-way ANOVA). No significant, specific FRET is observed for Leu/Ile peptides in SDS micelles or POPC liposomes.
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