Chapter 1: Introduction

Table of Contents

[1.1 Introduction to membrane proteins 3](#_Toc167876587)

[1.2 The two-stage model of membrane protein folding 5](#_Toc167876588)

[1.3 Determining driving forces in membrane protein folding 7](#_Toc167876589)

[1.3.1 Hydrogen bonding and polar interactions 7](#_Toc167876590)

[1.3.2 Electrostatics and weak hydrogen bonding 9](#_Toc167876591)

[1.4 Methods to study transmembrane helix oligomerization 13](#_Toc167876592)

[1.4.1 *In vitro* techniques 13](#_Toc167876593)

[1.4.2 *In vivo* assays 14](#_Toc167876594)

[1.4.3 *In silico* tools 16](#_Toc167876595)

[1.5 Understanding van der Waals as a driving force 17](#_Toc167876596)

[1.6 Thesis overview 22](#_Toc167876597)

[1.7 References 24](#_Toc167876598)

* 1. ****Introduction to membrane proteins****

A close-up of a string

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The cell membrane is a bilayer that separates internal cellular components from the outside environment. The membrane bilayer is composed of phospholipids, amphipathic molecules made of two distinct components: hydrophilic (water-loving) heads and hydrophobic (water-fearing) tails. To form the bilayer, hydrophobic tails are sandwiched between hydrophilic heads exposed to the soluble cell cytoplasm and outer environment. Despite this separation, it is critical to communicate outside of the cell to sense external stimuli and maintain cell survival. This process is regulated by a class of proteins tethered to the membrane: membrane proteins (MPs).

MPs can be broken down into two groups: peripheral MPs and integral MPs. Peripheral MPs are composed of both hydrophilic and hydrophobic components, allowing them to localize to the edges of the cell membrane while still exposed to the soluble environment. Unlike peripheral MPs, integral MPs are primarily hydrophobic: they are embedded within the membrane with little exposure outside of it. Insertion of integral MPs into the membrane bilayer results from the structural makeup. β-sheets often form open pores through the membrane, functioning as channels and transporters that allow ions and molecules to enter or exit the cell through the bilayer. Conversely, transmembrane (TM) α-helices are crammed into the membrane between lipids and assemble into complex multi-domain structures. Multiple TM α-helices (TMH) can associate in response to environmental stimuli, signaling activation and deactivation of the appropriate genes.

The sequestering of hydrophobic tails into the center of the membrane yields an hydrophobic environment: The core of the bilayer is devoid of polar interactions, which are responsible for the hydrophobic effect that drives soluble protein folding (Tanford, 1980; Yang et al., 1992). For MPs to fold within this hydrophobic environment, they must strike a delicate balance of interactions while surrounded by lipids. Mutations within MPs can drastically affect these interactions, preventing them from folding properly. Misfolding of TMH has been found to be involved in several human diseases such as Parkinson’s, cystic fibrosis, and cancer (Sanders and Myers, 2004; Gregersen et al., 2006). To fully understand how to combat the progression of these diseases, it is necessary to understand the impact of the individual forces that govern the folding process. However, studying MPs is inherently a difficult challenge. MPs are difficult to express in high yields for biophysical experiments, and purification and solubilization of these proteins often lead to aggregation or unfolding (Carpenter et al., 2008). Researchers alternatively have focused on using model systems of MPs to better understand folding.

My research focuses on using a model TMH system to investigate the extent that van der Waals packing can act as a driving force during MP folding and association. I developed an *in silico* protein design algorithm to study the association of single-pass TMH homodimers, complemented with high-throughput experiments to validate my computational models. In this introduction, I review the contributions made to understanding driving forces in MP folding and association. I detail the forces and interactions involved in MP folding, and I highlight the sequence and structural motif GASright, an important control for my research used to juxtapose differences between association by alternate forces outside of van der Waals packing. I then review the tools that have been used to study TMH association and folding before emphasizing the deficiency of research on the contribution of van der Waals packing.

* 1. The two-stage model of membrane protein folding

A diagram of a string and a string

Description automatically generated with medium confidenceEarly MP research focused on identifying membrane embedded regions within proteins using a technique known as hydrophobicity analysis: navigating through the protein amino acid (AA) sequence and scanning for stretches of hydrophobic AAs (Kyte & Doolittle, 1982). Hydrophobicity analysis was successful in predicting the helices in both bacterial photosynthetic reaction centers and bacteriorhodopsin (Engelman et al., 1982; Michel et al., 1986). This method was further developed to determine a charge bias known as the positive-inside rule where charged AAs are likely to be found at outside of the membrane (von Heijne, 1992). Multiple tools are now available, allowing researchers to easily identify TM regions from protein sequences (Wilkins et al., 1999).

In 1990, Popot and Engelman proposed a model for membrane protein folding called the two-stage model: As the protein is threaded into the membrane, TMHs begin to form (stage 1) separate from stabilizing into a folded protein (stage 2). TMHs first reach a thermodynamic equilibrium with the lipid environment before undergoing stage 2, where individual TMHs oligomerize and assemble into the folded protein (Popot & Engelman, 1990, 2000). While stage 1 is driven by the hydrophobic effect to coordinate insertion of hydrophobic protein sequences into the membrane, stage 2 is governed by interactions between individual TM domains. Research on bacteriorhodopsin gives credence to stage 2: denaturing two separate fragments of the protein and resuspending them in lipid vesicles results in an active, folded protein (Popot et al., 1987); two chemically synthesized TMHs of the protein were reconstituted in lipid vesicles with a larger fragment, resulting in the reformation of the bacteriorhodopsin shown by X-ray crystallography (Kahn & Engelman, 1992); and extraction and reconstitution of individual helices of the protein were found to yield activity (Marti, 1998). Additional research on large protein complexes continued to push the field forward, showing that mutating the hydrophobic core of four-helix-bundle protein Rop and five-helix-bundle protein phospholamban decreases the stability of both proteins (Arkin et al., 1994; Munson et al., 1996). With reassembly of MPs being an effective model for studying MP folding, other groups continued to build on this research by exploring model systems of TMHs, aiming to determine how minute changes in sequence and structure influence stability.

A benefit of the simplicity of the two-stage model is that there are unique forces involved in each stage of folding. In the first stage, insertion of the protein into the membrane is driven by the hydrophobic effect, where MPs are more stable found in the membrane than in the soluble environment. When MPs are being translated by the ribosome, a signal sequence on the protein directs translation to the translocon (Dalbey et al., 2011). Together, the translocon and ribosome individually thread hydrophobic segments of the protein into the membrane (Hessa et al., 2005; Rapoport, 2007). In the subsequent stage of folding, van der Waals packing, electrostatics, hydrogen bonding, and weak polar interactions between individual TM domains contribute to guiding the MP to the folded state.

Near the turn of the century, MP studies began to push the field forward to increase understanding of MP folding beyond the simplicity of the two-stage model. As an early advance passed the two-stage model, a third stage was considered taking into account the thermodynamic impact of ligand binding domains, folding of loops outside of the membrane, and inserting other domains into the bilayer (Engelman et al., 2003). Rather than simply focusing on how bulk changes in forces impacts folding, researchers began to characterize the impact of individual AAs and the respective forces that drive MP folding.

1.3 Determining driving forces in membrane protein folding

The elaborate nature of the lipid bilayer makes it difficult to directly study the forces involved in MP folding. As an initial approach, researchers aim to solve the structures of MPs to identify structural features necessary for the folded state. However, solving MP structures is an inherently difficult task due to the need to express and solubilize MPs for experiments (Carpenter et al., 2008). Alternative approaches to study folding utilize a combination of *in vitro* and *in vivo* experimental tools aiming to determine the rules that govern TM folding. Folding of integral TM proteins involves a variety of energetic constraints resulting from the hydrophobic nature of the phospholipid bilayer. The translocon complex assists during translation, inserting TM domains into the proper orientation in the membrane into the membrane (White & von Heijne, 2004). TMs are inherently composed of amide nitrogens and carbonyl oxygens within the protein backbone, groups of atoms amenable to forming hydrogen bonds. However, inserting these hydrogen bond donors and acceptors into the hydrophobic core of the bilayer carries an energetic penalty (Marinko et al., 2019; Popot & Engelman, 1990, 2000). To satisfy the lack of hydrogen bonding within the membrane, TMs adopt standard α-helical and β-sheet structures where hydrogen bonds form along the protein backbone. Experimental tools have been developed to tease out folding interactions after insertion by using model MP systems. I will further detail these tools in section 1.4. In this section, I will summarize advances in understanding driving forces in MP folding, with a particular focus on using single-pass TMHs.

1.3.1 Hydrogen bonding and polar interactions

Hydrogen bonding in MPs plays a key role in regulating protein structure and function, and many mutations on polar residues have been found to promote disease states (Choi et al., 2004; Partridge et al., 2002, 2004; Therien et al., 2001; Wehbi et al., 2008). Research characterizing the impact of polar residues on TMH association suggests that hydrogen bonding and polar interactions can drive TMH association. Using a wild-type like sequence of the GCN4 leucine zipper, a mutation from Asn to Val was found to decrease association on SDS-PAGE (Choma et al., 2000). Synthetic model poly-leucine peptides based on GCN4 were made with three different compositions of AAs at the interface, two being completely hydrophobic and the other hydrophobic with a single Asn. When tested for their ability to associate on SDS-PAGE, only the sequence with Asn was found to have equal amount of monomer and dimer (Zhou et al., 2000). These studies suggest that Asn plays a role in driving TMH association.

Further research began to characterize the impact of other polar amino acids in TMH association systems. Poly-leucine based peptides were made with single AA mutations to a variety of polar residues and tested using the *in vivo* experimental assay TOXCAT. Their results show that larger polar residues (Asn, Asp, Gln, and Glu) capable of being both hydrogen bond donors and acceptors drive association more than poly-leucine alone (Zhou et al., 2001). Another study tested the effect of polar amino acids on the model GCN4 peptide, and again showed that replacing hydrophobic AAs with large polar AAs results in association with higher stabilities (Gratkowski et al., 2001). These studies suggest that large polar AAs drive association. However, large polar AAs are not often found in MP sequences, whereas small polar AAs Thr and Ser are more commonly found due to their ability to more readily form hydrogen bonds with backbone carbonyls on the same helix (Gray & Matthews, 1984; Liu et al., 2002). Further research worked on investigating the possible roles of these small polar AAs on association.

The peptides used in the previous studies were made of bulky hydrophobic amino acids, possibly preventing Ser and Thr from playing the roles in association that they do in naturally occurring sequences. Using TOXCAT, a library of TM sequences that mutated the interface of known dimer glycophorin A (GpA) were screened for their ability to associate. A majority of the proteins found to associate were composed of Thr and Ser at the interface, suggesting that these AAs are important for association (Russ & Engelman, 2000). Additional investigation into the structure of GpA suggests that the Thr at position 87 is able to form interhelical hydrogen bonds at the interface, supporting previous research that mutations at this position disrupt dimerization (Lemmon, Flanagan, Hunt, et al., 1992; Lemmon, Flanagan, Treutlein, et al., 1992; MacKenzie et al., 1997; Smith et al., 2002). Alongside earlier research, this data suggests that hydrogen bonding is a driving force that strongly stabilizes TMH association.

By observing MP structures, researchers have been able to identify and characterize hydrogen bonds between TM helices in multiple solved structures (Adamian & Liang, 2002; Freiberg et al., 2012; MacKenzie et al., 1997; White, 2005). Using double mutant cycle analysis, MPs with multiple TMHs were mutated to determine the contribution of interhelical hydrogen bonding to MP stability. The average contribution for hydrogen bonding in multiple proteins was found to be 0.5kcal/mol +/- 0.7 (Bowie, 2011). Using an SDS unfolding assay, the average contribution of eight hydrogen bonds was found to be 0.6 kcal/mol (Joh et al., 2008). Despite the relatively small contribution in larger protein, hydrogen bond energies estimated in vacuum have been calculated to be around ten times higher (Ben-Tal et al., 1997; Mitchell & Price, 1990; Rose & Wolfenden, 1993; Tsemekhman et al., 2007). Single mutants on a variety of MPs were also tested, determining on average that hydrogen bonding contributes similar stability to water soluble proteins (Bowie, 2011).

Hydrogen bonding and polar interactions are stabilizing forces in MP folding and association. In larger MP complexes, hydrogen bonding contributes stability comparable to the core of soluble proteins. Although the contribution is similar, the hydrophobic nature of the membrane suggests that hydrogen bonding contributes more to stability. Most polar AAs are able to form hydrogen bonds with the backbone of the TM, and this bond must be broken prior to forming a stabilizing interhelical hydrogen bond (Chamberlain & Bowie, 2004). Additionally, mutations on polar AAs have been shown to disrupt association, suggesting that hydrogen bonding can drive association of TMHs.

1.3.2 Electrostatics and weak hydrogen bonding

Electrostatics interactions, or cation-π, are a result of attractive forces between charged AAs Lys and Arg and the electron clouds of aromatic AAs Phe, Tyr, His, and Trp. These interactions are found in a multitude of channels and G protein-coupled receptors, and are equally important for ligand binding of neurotransmitters, metal ions, and toxins (Infield et al., 2021). Charged AAs are not often found in MPs, but MD simulations and potential of mean force calculations supports the thermodynamic stability of Arg in TMs (Ulmschneider et al., 2017). Electrostatic interactions have been studied between a variety of TMH interactions. Johnson et al. mutated a hydrophobic protein with a pair of charged and aromatic AAs. Using TOXCAT, they determined that Lys coupled with Tyr, Trp, and Phe is able to drive these proteins to associate (Johnson et al., 2007). Another study looked at the role of aromatic AAs in the β-barrel outer membrane protein OmpA, and using double mutant cycle analysis found that each side chain contributes more than 1kcal/mol to stability (Hong et al., 2007). Additional SDS-PAGE analysis on helical hairpins demonstrates that TM-TM electrostatic interactions alongside helical turns promote folding (Bañó-Polo et al., 2013).

Similar to hydrogen bonding, electrostatics plays a strong stabilizing role in MP folding and association, able to drive association of TMHs with charged and aromatic interactions. Another class of forces that has been shown to strongly influence association of TMHs is facilitated by the GASright motif, one of the most prevalent sequence and structural motifs found in TM proteins (Walters & DeGrado, 2006). GAS is an acronym for the three AAs typically found in the sequence: Gly, Ala, and Ser. These small residues define the interface of the motif (G/A/S)xxx(G/A/S), resulting in a short interhelical distance between TM helices. Right originates from the structural features in which TM helices associate with a right-handed crossing angle. GASright proteins are frequently found to be involved in a variety of diseases: syndecan-2 overexpression has been found in colorectal cancer cell lines, neuropilin-1 has been shown to intensify symptoms of SARS-CoV-2, and Glycophorin A (GpA) misregulation is involved in sickle cell disease (Benedicto et al., 2021; Marshall et al., 2024; Vicente et al., 2013). Due to the prevalence of GASright proteins in medical applications as well as its well-defined sequence and structural features, many groups have studied these proteins to further understand the forces governing TM association.

GpA is a well-studied protein that associates via the GASright motif. Multiple *in vitro* studies worked to define the interface of GpA, making point mutations along the protein and measuring the changes in dimerization on SDS-PAGE (Lemmon, Flanagan, Hunt, et al., 1992; Lemmon, Flanagan, Treutlein, et al., 1992). Using dimerization as a model system, researchers aimed to further characterize the thermodynamics of dimerization by monitoring changes in stability between the monomer and dimer state. Additional studies using *in vitro* techniques sedimentation equilibrium analytical ultracentrifugation (SE-AUC) and Förster resonance energy transfer (FRET) were able to determine differences in stability, effectively quantifying the thermodynamics of association for GpA (Fisher et al., 1999; Fleming et al., 1997).

After the structure of GpA was solved by solution nuclear magnetic resonance (NMR), groups further characterized their previous thermodynamic data (MacKenzie et al., 1997). Analysis of GpA dimerization in molecular dynamics simulations and mutations on the NMR structure determined that mutations affect association resulting from changes in van der Waals interactions (Fleming et al., 1997; MacKenzie & Engelman, 1998; MacKenzie et al., 1997; Petrache et al., 2000). However, further investigation into the unique sequence and defined structure of the GASright motif has been shown to permit an uncommon structural feature. Small amino acids at the interface allows TM backbones to associate with a short interhelical distance, resulting in the formation of a network of weak hydrogen bonds where donors are Cα carbons and acceptors are carbonyl oxygens on the opposite helix (Cα–H∙∙∙O=C, or Cα–H bonds). Carbon atoms are not commonly associated with hydrogen bond donors because they are less electronegative than typical nitrogen and oxygen donors. However, these carbons are found near electronegative withdrawing groups on the peptide backbone, increasing their electronegativity. Estimates from quantum mechanics calculations suggest that the stabilizing energy of an Cα–H bond may contribute one third to one half of that of an N—H donor in vacuum (Scheiner et al., 2001; Vargas et al., 2000). Measurements of the stretching frequency of these bonds in GpA suggests it could contribute 0.9 kcal/mol of stability to the dimer (Arbely & Arkin, 2004).

Further research on GASright TMs helped to define the geometric structure for the network of hydrogen bonds. This research resulted in CATM, an algorithm that successfully predicted the structures of five known homodimer structures (Mueller et al., 2014). Anderson et al. utilized the CATM algorithm and the experimental assay TOXCAT to determine the influence of this network of Cα–H bonds. By predicting the structures of GASright TMs found in natural sequences and testing their stability using *in vivo* TOXCAT, they showed that structures predicted to have more Cα–H bonds are more thermodynamically stability (Anderson et al., 2017). Additionally, Díaz-Vázquez et al. measured the free energy of association of GASright structures using *in vitro* FRET, concluding that the thermodynamic stability of GASright proteins is well correlated with *in vivo* experiments (Díaz Vázquez et al., 2023). These studies suggest that GASright proteins associate primarily through two forces: weak hydrogen bonding and van der Waals packing. Using a refined version of the CATM algorithm, I designed sequences to associate solely by van der Waals packing as well as GASright sequences as controls. By evaluating sequences designed to associate through van der Waals packing, I was able to differentiate the impact of packing (designs) versus both hydrogen bonding and packing (GASright) on association.

1.4 Methods to study transmembrane helix oligomerization

To investigate MP folding, researchers have developed tools to study oligomerization of TMHs. These tools strive to identify changes in stability between the unfolded and folded states, allowing researchers to uncover MP folding thermodynamics within a variety of systems. The oligomerization process contains biological relevance, as many MP receptors are activated through ligand binding, including epidermal growth factor receptors and other proteins involved in tyrosine kinase signaling cascades that influence cell gene expression (Kumari & Yadav, 2019). Furthermore, this thermodynamic information can be used to assess and validate computational models, which can be further implemented to design and engineer novel proteins. In this section, I detail the tools and techniques that have been implemented to further understand the driving forces in MPs.

1.4.1 *In vitro* techniques

Early tools used to study MP folding monitored the reversible folding of MPs. *In vitro* techniques focus on expressing and solubilizing proteins into suitable membrane mimetics such as detergents. An initial method studied the thermodynamics of TMH association by observing differences in mobility within SDS-PAGE gels. SDS-PAGE studies have been used to tease the effect of point mutations in a variety of TM sequences, including GpA (Choma et al., 2000; Lemmon, Flanagan, Hunt, et al., 1992; Lemmon, Flanagan, Treutlein, et al., 1992; Zhou et al., 2000). Sedimentation equilibrium analytical ultracentrifugation (SE-AUC) is another technique that explores different folding states by varying the concentrations of detergents used to solubilize the protein. These samples are centrifuged at high speeds, resulting in a concentration gradient that can be analyzed to quantitatively determine the transition of the protein between different folding states. SE-AUC has been used to investigate mutations of GpA to better understand its thermodynamics of association (Doura & Fleming, 2004; Fleming et al., 1997; Fleming & Engelman, 2001). These techniques began to push the field forward in understanding the thermodynamics of TMH association, however, they are relatively low throughput and typically used to study MPs solubilized in detergents.

Multiple *in vitro* techniques have been developed to observe MP dynamics within membrane like environments. Disulfide cross-linking has been used to measure TMH oligomerization in both micelles and lipid vesicles to investigate the interfaces of a variety of proteins (Cristian et al., 2003; Hastrup et al., 2001; Kovalenko et al., 2005; Lu et al., 2010). Pulse proteolysis quantitatively measures the thermodynamic stability of MPs by selectively denaturing unfolded MPs and subsequently digesting the unfolded MP (Park & Marqusee, 2005). Bacteriorhodopsin folding was studied using this technique and found to be dependent on changing concentrations of mixed micelles, which was not determined previously (Schlebach et al., 2012; Schlebach et al., 2011). Steric trapping utilizes the streptavidin-biotin binding system to measure the binding affinity of associating TMs in lipid bilayers, and it has been used to further determine the folding energy landscapes of GpA and mutants affecting its association (Blois et al., 2009; Hong & Bowie, 2011; Hong et al., 2013; Howarth et al., 2006; Huang et al., 2022). These methods allow for studying MP folding thermodynamics in lipids and mixed micelles, closer to the environments of the cell membrane compared to previous research. While these techniques approach understanding proteins in native environments, other techniques were developed to further study MPs within cells in higher throughput.

1.4.2 *In vivo* assays

*In vivo* assays have been utilized to investigate the folding and association of MPs in their natural environment. A method that quantitatively measures interaction in protein structures is double mutant cycle analysis. Mutating two non-interacting residues within a protein has allowed researchers to determine the coupling of residues to thermodynamic stability (Carter et al., 1984). Double mutant cycles in bacterial two-hybrid and protein complementation assays allow researchers to determine the strength of protein-protein interactions by changes in cell growth due to mutation, which can be monitored in high-throughput (Horovitz et al., 2019; Salinas & Ranganathan, 2018; Tarassov et al., 2008). Genetic reporter assays allow cells to express MPs of interest fused to a DNA binding domain that can either inhibit or promote transcription of a reporter gene. GALLEX is a two-hybrid system where TMs are fused to DNA binding domain LexA. Association of the TMs results in repression of the β-galactosidase gene (Schneider & Engelman, 2003). Other reporter assay systems have utilized a chimera of the MPs of interest fused to ToxR, dimeric transcription factor, to promote the expression of genes (Gurezka & Langosch, 2001; Russ & Engelman, 1999).

The assay TOXCAT has been used to study TM helix-helix interactions, where the TM of interest is fused to the dimeric transcription factor ToxR. When the TMs associate, ToxR dimerizes and promotes the expression of chloramphenicol acetyltransferase (CAT) which can be measured to determine the strength of association. TOXCAT demonstrated that mutations of polar residues on GpA in the natural membrane environment yield different results than the previous in vitro studies (Russ & Engelman, 1999; Zhou et al., 2000; Zhou et al., 2001). Johnson et al. expanded on these findings, suggesting that electrostatic interactions between charged and ring AAs facilitates oligomerization (Johnson et al., 2007). TOXCAT has also been shown to correlate to changes in the free energy of association of GpA and point mutations (Duong et al., 2007). Anderson et al. used TOXCAT to study the association of GpA and similar TMHs, suggesting that these proteins associate via a combination of hydrogen bonding and van der Waals interactions (Anderson et al., 2017). TOXCAT is a well-studied system for probing TMH association, determining the impact of individual AAs and their respective forces on the thermodynamics of association.

Recently, TOXCAT has been adapted into the high-throughput assay TOXGREEN. The reporter gene CAT has been replaced with green fluorescent protein (GFP), allowing fluorescent readings to be used to assess the association levels of the TMs of interest and their corresponding mutants (Armstrong & Senes, 2016). The development of this assay allows it to be utilized in high-throughput applications such as fluorescence activated cell sorting (FACS), where a library of TMs can be expressed, sorted, and sequenced through next generation sequencing (NGS). The sequencing data can then be quantified to determine the relative association propensities for each protein present in the library (cite sort-seq).

1.4.3 *In silico* tools

**Computational tools have been developed to help assess our understanding of the forces that drive MP association. These tools allow researchers to predict and design MPs *in silico* that can then be tested using the previously mentioned *in vitro* and *in vivo* experimental techniques. Molecular dynamics (MD) allows researchers to use established statistical and energetic potentials to simulate MP folding over time (Karplus & Petsko, 1990; MacKerell et al., 1998). Structure prediction uses known information from previously solved structures to estimate the structure of MP folded states (Elofsson & von Heijne, 2007). Protein design strategies build on structure prediction, essentially building unknown structures to build simple model systems to assess the current understanding of MP folding (Ghirlanda, 2009). MP design to study TMH systems has been previously successful: peptides were engineered to associate with the TM helix of integrins and a cytokine receptor (Mravic et al., 2024; Shandler et al., 2011; Yin et al., 2007), an integral MP successfully transferred electrons across the lipid bilayer (Korendovych et al., 2010), a 4-helix bundle was designed to transport Zn2+ across the bilayer (Joh et al., 2014), and phospholamban was redesigned using packing interactions and shown to successfully fold (Mravic et al., 2019). By extracting backbone conformations from the PDB, sampling different AA conformations on the interface of a standardized backbone sequence, and assessing different conformations using known energetic functions, I designed thousands of TMHs to study in high throughput. I will further detail the computational tools used to study MP folding and detail my design algorithm in Chapter 3.**

****1.5 Understanding van der Waals as a driving force****

As individual TMHs are threaded into the membrane, an interplay of biophysical forces produces helix-helix association. This process is regulated by an intricate distribution of hydrogen bonding, electrostatic interactions, and van der Waals forces that govern the stabilities of the unfolded and folded states. Each of these interactions are driven by the types of amino acids (AAs) present within the TM. Removing hydrogen bonds within TMs decreases stability (Duong et al., 2007; Gratkowski et al., 2001; He & Hristova, 2008; Li et al., 2006; Stanley & Fleming, 2007). Hydrogen bonding not only regulates the secondary structure of TMs, but has been shown to drive TM helix association when polar amino acids (Ser, Thr) form interhelical hydrogen bonds between opposing helices (Johnson et al., 2007; Zhou et al., 2001). Additionally, electrostatic interactions between positively charged residues (Lys) and electronegative ring amino acids (Tyr, Trp, Phe) promotes association between helices (Johnson et al., 2007). Other charged and aromatic interactions have been shown to contribute similar stability as in water soluble proteins (Bañó-Polo et al., 2013; Burley & Petsko, 1985; Hong et al., 2009; Hong et al., 2007; Ulmschneider et al., 2017). However, hydrogen bonding and electrostatic interactions only highlight a subset of AAs typically present in MPs. The three AAs most frequently found in MPs (Leu, Ile, and Ala) are uncharged and lack the ability to hydrogen bond (Liu et al., 2002), thus TMs constituted of these AAs can only be stabilized by van der Waals forces.

Van der Waals occurs between atoms within close contact, including the interactions between MPs and the hydrophobic tails within the membrane. MP association motifs have been structurally characterized and studied, determining that tight van der Waals packing plays an important role in TMH association (Gurezka et al., 1999; Kim et al., 2004; MacKenzie et al., 1997; North et al., 2006; Russ & Engelman, 1999; Wu et al., 2005). Mutational studies on well packed residues in the core of MPs suggest that changes in packing can destabilize protein structure (Ash et al., 2004; Faham et al., 2004; Joh et al., 2009; Mravic et al., 2019; Yano et al., 2002). However, accounting for van der Waals between MPs and phospholipids is complex, and not many studies have successfully investigated the influence of van der Waals forces on MP stability.

A screenshot of a computer

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The physical properties of atoms are the foundation of intermolecular interactions. Atoms are composed of a nucleus of protons surrounded by an outer electron shell. The electron shell expands a finite distance away from the nucleus, constructing the space occupied by the atom, or the van der Waals radius. This is the closest distance another atom can approach without forming a chemical bond. When atoms are found at a distance smaller than their combined van der Waals radii, the opposing electron shells repulse, resulting in a strongly unfavorable interaction that pushes atoms away. However, as atoms approach this minimum distance, there is a weak attraction between protons in the nucleus of one atom and the electrons of another. This attraction is the core principle behind the van der Waals force: It is a favorable intermolecular interaction occurring between atoms in proximity.

A black and white square with a number and a symbol

Description automatically generated with medium confidenceThe van der Waals force between two atoms can be calculated using the Lennard-Jones (LJ) Potential:

A graph of a function

Description automatically generatedThe LJ potential calculates the intermolecular potential (*V*) between two atoms at a specified distance (*r*), using the strength of attraction between the atoms (*ϵ*) and the distance where the potential is 0 (*σ*). This function expresses the repulsive force as (*σ*/r)12 while the attractive force is represented as (*σ*/*r*)6. As the atoms approach the minimum distance, there is a distance *r* that corresponds to the tightest attraction between the atoms *ϵ*.

Soluble proteins are driven to fold by the hydrophobic effect, where nonpolar amino acids are forced to the core of the folded state. These nonbonded atoms at the core of these proteins are found in tight contact with one another, compounding into a multitude of weak van der Waals interactions known as van der Waals packing. Although van der Waals packing is not a driving force for soluble protein folding, it is a necessary force that is always present in the folded state. For MPs situated in the core of the hydrophobic membrane, the hydrophobic effect does not drive MP folding. This means that MPs must rely on other forces to reach the folded state. Although hydrogen bonding and polar interactions have been found to drive MP folding, the extent at which packing contributes to the folded state is unclear. Like soluble protein folding, MP folding eventuates van der Waals packing. But because MPs are engulfed within the crowded lipid bilayer, it is difficult to tease out the influence that van der Waals packing has on MP folding.

The contribution of van der Waals packing to MP folding can be broken down into three distinct interactions: lipid-lipid packing, lipid-protein packing, and protein-protein packing. Lipid-lipid packing involves individual lipid molecules nudged tightly against each other to keep the bilayer assembled. Lipid-protein packing occurs between these lipid molecules and the lipid exposed protein shell. Protein-protein (or sidechain) packing focuses on the stability gained between fragments of proteins in close contact. Each of these interactions plays a role stabilizing an MP in the bilayer. When an individual protein subunit is inserted into the membrane, it must destabilize the lipid-lipid packing with more favorable lipid-protein packing interactions. For protein-protein packing to occur, these newly formed lipid-protein interactions must be destabilized for a more favorable combination of protein-protein packing and lipid-lipid packing. This assortment of packing interactions takes place to keep the lipid bilayer intact while the MP reaches its folded state. But simultaneously accounting for all these interactions within the thermodynamics of MP folding is impractical using current technologies. Protein-protein (or sidechain) packing is a technically feasible starting point because of the ability to manipulate protein sequence and structure within a controlled environment.

Previous research has demonstrated that disruption of sidechain packing within the core of bacteriorhodopsin destabilizes protein structure (Faham et al., 2004; Joh et al., 2009). In addition, a recent study using MP design has shown that optimized sidechain packing can stabilize the folded state of the 5-helix bundle protein phospholamban (Mravic et al., 2019). Although these studies suggest that sidechain packing plays a role in stabilizing MP structure, there has not been much investigation of the thermodynamic contribution of packing outside of individual MP systems. My research aims to characterize and quantify the extent to which sidechain packing is a driving force for MP folding for the general population of MP structures.

* 1. ****Thesis overview****

My graduate research focused on using computational protein design in combination with high throughput assays to determine the extent at which van der Waals packing contributes to membrane protein association and folding. Prior research on the impact of packing to the folded state of membrane proteins honed-in on singular systems, and I aimed to expand this knowledge to a larger variety of membrane protein structures.

**In Chapter 2**, I present the majority of my graduate schoolwork to be published in the near future. In this paper, I determined that van der Waals packing is a weak driving force that leads to association of MP homodimers. I data mined the PDB for all solved MP structures to determine the best structures for computational design, developed a protein design algorithm and designed 1000s of proteins, and assessed the ability of proteins designed with solely van der Waals packing for their ability to associate using a high-throughput assay. I found that packing drives my designed proteins to associate mildly when compared to GASright proteins associating according to both hydrogen bonding and van der Waals packing.

**In Chapter 3**, I discuss the computational methods that have been used to study MPs in detail. My research aimed to utilize these growing technologies to discover the potential impact of biophysical forces on protein association. With improving experimental technologies, many other studies at the forefront of research utilize a combination of high-throughput experiments and computational analysis. I review similar studies and rationalize their impact on the field. Finally, I detail the computational inspirations for my project that involves data mining of the PDB and discuss the development of my protein design algorithm.

**In Chapter 4**, I describe a variety of future directions for studying van der Waals packing and other uses for my protein design algorithm. I discuss how to expand my protein design algorithm, detailing how to design heterodimers. Finally, I explore how my algorithm can be improved using tools available today.

**In Chapter 5**, I share a collaboration with the SciFun program at UW-Madison, detailing my PhD journey through a chapter written for the public. I describe the premise of my research in leyman’s terms while simultaneously reflecting on moments during graduate school, giving transparent thoughts on how my research affected my physical, emotional, and mental well-being. I showcase a bit of creativity in sharing these thoughts on how science and research has helped me grow during my time in graduate school.

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