

INVESTIGATING THE RECOGNITION
AND INTERACTIONS OF NON-POLAR
 α HELICES IN BIOLOGY

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Contents

Abstract	5
Abstract	6
Lay Abstract	7
Declaration	8
Copyright Statement	9
Acknowledgements	10
1 Introduction	11
1.1 α Helices; Structure And Function	11
1.1.1 Trans-membrane Helix Sequence Composition	11
1.1.2 Hydrophobicity of Trans-membrane Segments	14
1.1.3 Sequence Complexity	16
1.2 α Helices In Membranes	17
1.2.1 The Transmembrane Protein Problem	17
1.2.2 The Importance Of Transmembrane Proteins	19
1.3 Biological Membrane Composition	19
1.3.1 Lipids of the Membrane	19
1.3.2 Membrane Potential	20
1.4 Biogenesis of Trans-membrane Proteins	22
1.4.1 Translocation	22
1.4.2 Translocon Independent Membrane Insertion	22
1.5 Aims of This Thesis.	23

2	Tail-Anchored Protein Datasets	24
2.1	Abstract	24
2.2	Introduction	24
2.3	Methods	24
2.3.1	Filtering the Uniprot database	24
2.3.2	Calculating Hydrophobicity	25
2.3.3	Calculating Sequence Complexity	25
2.4	Results	25
2.4.1	An Up To Date Tail-Anchor Dataset	25
2.4.2	Potential Tail-Anchored SNARE Protein Discovery	25
2.4.3	Biology of Spontaneously Inserting Tail Anchored Proteins	25
3	Protein Classification Based on Intra-membrane Complexity Arrangement	26
3.1	Abstract	26
3.2	Introduction	26
3.3	Methods	26
3.4	Results	26
4	Conclusions	27
4.1	Outlook	27
4.1.1	The hydrophobicity–sequence complexity continuum	27

Word count 22,000

List of Tables

List of Figures

1.1	A cartoon showing the general components of the membrane and a typical Trans-membrane Helix (TMH).	12
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Acronyms

ER Endoplasmic Reticulum. 13, 16, 20, 21

MD Molecular Dynamics. 20

PDB Protein Data Bank. 12

POPC Palmitoyllecithin. 16

SNARE Soluble N-Ethylmaleimide-Sensitive Factor Attachment Receptor. 22, 24

SP Signal Peptide. 18

TM Trans-membrane. 11, 22

TMH Trans-membrane Helix. 11–14, 16–20, 22

TMP Trans-membrane Protein. 11, 13, 17–19

TMS Trans-membrane Segment. 11, 13, 17, 18

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Doctor of Philosophy

Investigating the Recognition and Interactions of Non-Polar α Helices in
Biology

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Abstract

Non-polar helices figure prominently in structural biology, from the first protein structure (myoglobin) through trans-membrane segments, to current work on recognition of protein trafficking and quality control. Trans-membrane α helix containing proteins make up around a quarter of all proteins, as well as two-thirds of drug targets, and contain some of the most critical proteins required for life as we know it. Yet they are fundamentally difficult to study experimentally. This is in part due to the very features that make them so biologically influential: their non-polar trans-membrane helix regions. What is missing in the current literature is a nuanced understanding of the complexities of the helix composition beyond a hydrophobic region of around 20 residues. Currently, it is known that the properties of trans-membrane protein α helices underpin membrane protein insertion mechanisms.

By leveraging large datasets of trans-membrane proteins, this thesis is focused on characterising features of α helices en masse, particularly regarding their topology, membrane-protein interactions, and intramembrane protein interactions.

In this thesis, I make the argument that there are different classifications of trans-membrane α helices. These have markedly different evolutionary pressures, these different classes interact differently with the membrane, and each class serve the protein differently.

Lay Abstract

The survival of each of our cells relies on a cellular barrier to separate themselves from the surrounding environment. This cellular skin can be thought of as the bag that contains all the important machinery required for normal cell function. The barrier works by being chemically very different to both the outside environment, and to the inside of the cell, which in both cases are mostly water. The membrane is fatty, and because of that repels water.

Proteins are the molecular machinery that form much of the cell structure and shape as well as carrying out many of the cell's routine tasks. Around a third of our genome codes for proteins that are permanently embedded in the membrane, but because these proteins are adapted for a life in the water repelling cell wall, they are very hard to study in laboratories which need to look at proteins in water.

In this thesis, we focus particularly on the parts of the protein that are embedded in the water repelling cellular skin. Traditionally, these regions are hard to study, because we must first remove them from the cellular wall, which causes problems since the embedded regions also repel water and this often causes them to stick to one another, making them hard to work with in a laboratory setting.

We analyse thousands of proteins to further our understanding of electrical charges in the embedded regions and find that negative charge on the outside of the cell has been evolutionarily selected across bacteria, animals, and plants. This is especially true for regions that specifically anchor the protein into the cellular wall. Where the embedded regions have additional function, for example ferrying something in or out of the cell, the negative charge "bias" can no longer be seen.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Chapter 1

Introduction

Trans-membrane (TM) biology is a huge and varied field that is ultimately the study of the interface between compartments of the cell; one of the fundamental pillars of life as we know it [1]. Trans-membrane Protein (TMP)s include some of the most critical to life proteins as well as a large number of drug targets. However, the experimental inaccessibility of the TMH has hampered the progress of study compared to their globular structural analogues. Despite progress over the last decade, the understanding of the relationship between the sequence and function of a TMH is incomplete.

In this chapter we will place the TMH problem in context, then describe the important biological aspects of the TMH (the traversing Trans-membrane Segment (TMS) as well as the membrane itself), and discuss tools and methods that allow us to analyse and describe the nuanced differences between these TMH sequences.

1.1 α Helices; Structure And Function

1.1.1 Trans-membrane Helix Sequence Composition

Measurements of the TMH regions have found that they are roughly 20 residues in length; 17.3 ± 3.1 from 160 TMHs [2], 27.1 ± 5.4 residues based on 129 TMHs [3], 26.4 residues based on 45 TMHs [4], 25.3 ± 6.0 residues based on 702 TMHs [5], 24.6 ± 5.6 from 837 TMHs [6], and $28.6 \pm 1.6 \text{\AA}$ to $33.5 \pm 3.1 \text{\AA}$ from 191 proteins depending on membrane types [7]. There are a couple of reasons for this variation. Primarily is that the boundaries of TMHs are extremely hard to precisely identify since it is unclear

exactly how far the TMH rises into the water interface region [8]. Secondly is that it is emerging that different membranes have different thicknesses [9], and that this is directly reflected in the hydrophobic lengths of the TMH [7, 10].

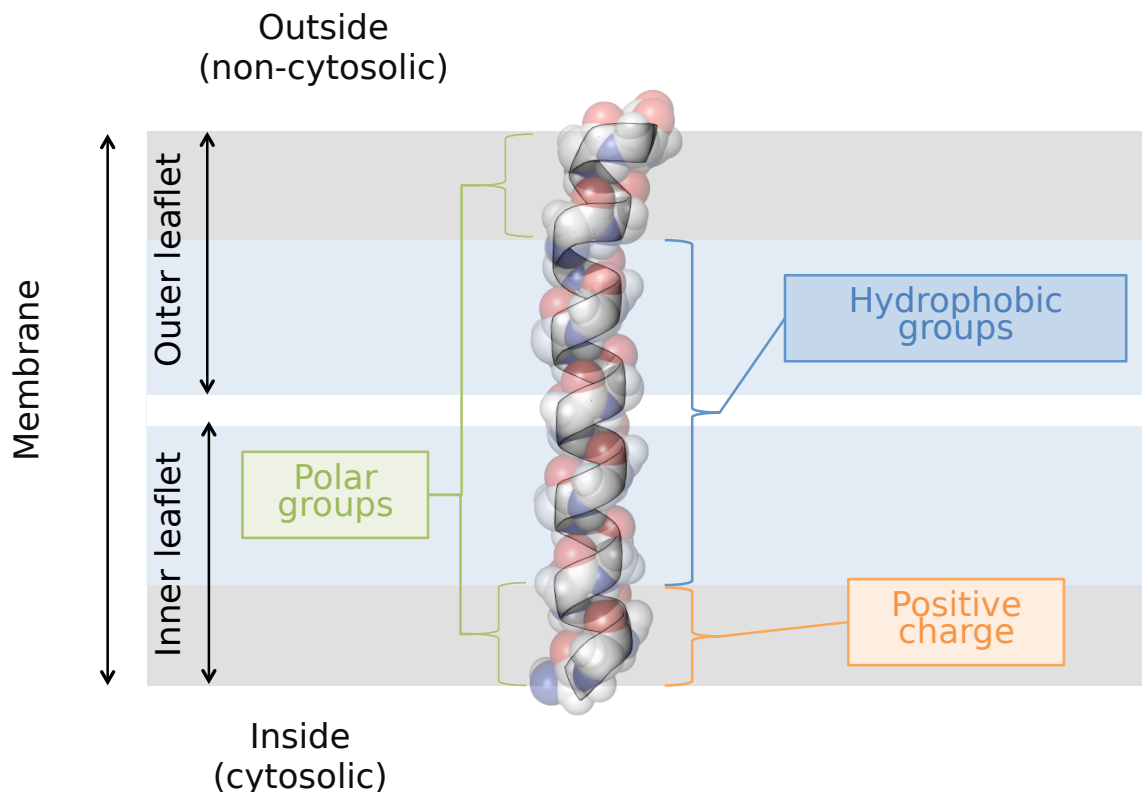


Figure 1.1: A cartoon showing the general components of the membrane and a typical TMH.

The example used here for illustrative purposes is the trans-membrane region of therein (Protein Data Bank (PDB) 2LK9) [11]. Dark grey areas denote the area of lipid head groups. The residues found in these areas are often described as flanking regions and are often in contact with the aqueous interface of the membrane. The helix core is mostly composed of hydrophobic residues. Although the regions labelled here generally hold true in terms of the statistical distribution of polar, non-polar, and charged groups, it is by no means absolute laws and many proteins break these “rules” [6, 7, 10].

The language used to describe TMHs varies somewhat across the literature, primarily due to a changing understanding of TMH general structure and relevance to function over the last 15 years or so. There is a general composition of a TMH despite specific protein and membrane constraints [10].

A study by Baeza-Delgado *et al.* from 2013 [6] looked at TMHs in 170 integral membrane proteins from a manually maintained database of experimentally confirmed TMPs; MPTopo [12]. The group examined the distribution of residues along the

TMHs. As expected, half of the natural amino acids are equally distributed along transmembrane (TM) helices whereas aromatic, polar, and charged amino acids along with proline are biasedly near the flanks of the TM helices [6]. It has been noted that transitions between the polar and non-polar groups at the ends of the hydrophobic core occur in a more defined edge on the cytoplasmic side than at the extracytoplasmic face when counting from the middle of the helix outwards [6]. This is probably reflecting the different lipid composition of both leaflets of biological membranes [6].

A previous study by Sharpe *et al.* from 2010 used 1192 human and 1119 yeast predicted TMHs that were not structurally validated to further explore the difference in TMH and leaflet structure by exploiting the evolutionarily conserved sequence differences between the TMH in the inner and outer leaflets [10]. TMHs from vertebrates and invertebrates were found to be reasonably similar compositionally. The differences in consensus TMH structure implies that there are general differences between the membranes of the Golgi and Endoplasmic Reticulum (ER). The abundance of serines in the region following the luminal end of Golgi TMSs probably reflects the fact that this part of many Golgi enzymes forms a flexible linker that tethers the catalytic domain to the membrane [10].

The “Positive-Inside” Rule

Two publications by von Heijne coined the “Positive-Inside” rule demonstrated the practical value of positively charged residue sequence clustering in topology prediction of TMHs in bacteria [13, 14]. It was clearly defined and shown that positively charged residues more commonly were found on the “inside” of the cytoplasm rather than the periplasm of *E. coli*. More recently still large-scale sequence analysis of TMHs from different organelle membrane surfaces in eukaryotic proteomes, show the clustering of positive charge being cytosolic [6, 7, 10].

The Aromatic Belt

Tyrosine and tryptophan residues commonly are found at the interface boundaries of the TMH and this feature is called the “aromatic belt” [6, 10, 15–17]. Not all aromatic residues are not found in the aromatic belt; phenylalanine has no particular preference for this region [16, 18]. However, it still remains unclear if this is to do with anchorage

or translocon recognition [6].

Snorkeling

Broadly speaking, TMHs are non-polar. However, some contain polar and charged residues in the helix itself. Whilst this might seem thermodynamically unstable at first glance, a molecular dynamic feature called the “snorkel” effect explains in part how this is possible [19, 20]. Simply put, the snorkelling effect involves the long flexible side chain of leucine reaching the water interface region to interact with the polar headgroups of the bilayer even when the α helix backbone is pulled into the hydrophobic layer [21]. This has also been suggested to allow helices to adapt to varying thicknesses of the membrane [22]. More recently it was found that although in simulations the energetic cost of arginine at the centre of the TMH is large, in vivo experimentation with the Sec61 translocon reveals a much smaller penalty [23]. That same study also found that in simulations, snorkeling, bilayer deformation, and peptide tilting combined to be sufficient to lower the thermodynamic stability penalty of arginine insertion so that hydrophobic TMHs with a central arginine residue will readily insert into the membrane.

1.1.2 Hydrophobicity of Trans-membrane Segments

Perhaps the most prevalent and important feature of the trans-membrane regions is the membrane spanning region which is composed mostly of non-polar residues. More recently the hydrophobic group region has been associated with cell localisation and a broad range of biochemical functions [24, 25].

Over the last 50 years or so, there have been many attempts to use hydrophobicity scales of residues to predict structural classifications of proteins. Due to the vast amounts of scales, major efforts have been made to compare them to identify which ones are better for which tasks of identifying structural elements [26, 27]. Simm *et al.* 2016 [26] compared 98 scales and found that the accuracy of a scale for secondary structure prediction depends on the spacing of the hydrophobicity values of certain amino acids but generally that the methods behind the scales don’t affect the separation capacity between β sheets or α helices.

Throughout this thesis, several scales are used to evaluate and estimate hydrophobic values of peptide chains. All the scales aim for quantifying the hydrophobic values of each residue. There are several key differences in their methodology, assumptions, and aims. Ultimately, all the scales are attempting to allow estimation of ΔG_{whf} ; the free energy of a folded helix (f) from the water (w) into the membrane core (h). This free energy measurement is regarded as being currently experimentally inaccessible [28].

Although as a trend most of the scales agree, because of the methodological differences, there are indeed variations of values even after normalisation. Due to these discrepancies, it is preferable and typical amongst the literature to use several scales to verify the observable trends resulting from interpretation from an individual scale. Notably, one of the classic scales, Kyte & Doolittle Hydropathy Scale shows a striking similarity to the modern Hessa's ΔG_{app}^{aa} scale, and that generally the "better" scales count proline as hydrophilic, and focus on helix recognition rather than amino acid analogues [27]. In α helices from soluble proteins, proline is almost always a helix breaker, and α helix prediction scales don't even attempt to quantify a proline scoring penalty. Several of the scales used throughout this thesis are outlined below.

Kyte & Doolittle Hydropathy Scale

A scale based on the water–vapour transfer free energy and the interior-exterior distribution of individual amino acids [29].

Hessa's Biological Hydrophobicity Scale

This is arguably the most biologically relevant scale [27], and is often called the ΔG_{app}^{aa} scale. The scale is based on an experimental method where the free energy exchange during recognition of designed polypeptide TMH by the ER Sec61 translocon occurred [15]. These measurements were then used to calculate a biological hydrophobicity scale. The original study reported positional variance in some residues and is strictly valid only for residues in the core of the TMH. A more refined study quantified the positional dependencies of each amino acid type [30].

White and Wimley Octanol – Interface Whole Residue Scale

This scale is calculated from two other scales; the octanol scale, and the interface scale [31]. This scale is fundamentally based on the partitioning of host-guest pentapeptides (acetyl-WL-X-LL-OH) and another set of peptides (AcWLM) between water and octanol, as well as water to Palmitoyloleoylphosphatidylcholine (POPC).

The Eisenberg Hydrophobic Moment Consensus Scale

The Eisenberg scale is a consensus scale based on the earlier scales from Tanford [32], Wolfenden [33], Chothia [34], Janin [35], Wolfenden [36], and the von Heijne scale [37]. The scales are normalised according to serine [38]. The automatic TRANSMEM annotation currently used in Uniprot is according to TMHMM [39], Memsat [40], Phobius [41] and the hydrophobic moment plot method of Eisenberg and coworkers [38].

1.1.3 Sequence Complexity

Sequence properties that can be analysed by bioinformatics, the sequence complexity and hydrophobicity, of the TMH have been used to predict the role of the TMH as either functional or structural, and as a discrete cluster from other SCOP annotated helices [25]. Those findings demonstrated that the sequence of the TMH holds valuable information regarding biological roles, and forms the basis of our interest in the link between the polarity of a helix and functional activity beyond structural anchorage.

Sequence complexity otherwise referred to as sequence entropy, is essentially an estimate of the linguistic entropy of a string. In the context of biology can be thought of as an estimation of the non-randomness of a sequence. Sequence complexity can be used to analyse DNA sequences [42–44], however here we will focus on the analysis of the complexity of a sequence in protein sequences.

The compositional complexity is measured over sequence windows. If we have an amino acid composition $\{n_i\}_i = \min i, \dots, \max i$ with a window length of $L = \sum n_i$, the total number of sequences can be calculated by dividing a factorial of the length by the product of the compositions, i.e $N = L! / \prod n_i$ possible sequences. The SEG algorithm [45, 46] identifies subsegments of the raw region which have the lowest probability. The algorithm searches for and concatenates sub-threshold segments for

the Shannon entropy-like term in equation 1.1

$$K_2 = -\sum \frac{n_i}{L} \log \frac{n_i}{L} \quad (1.1)$$

The lowest probability subsegment can be defined as $K_1 = \log N/L$. By altering the window lengths, and the thresholds SEG can be optimised to search for subtle compositional deviations, such as coil-coiled regions.

1.2 α Helices In Membranes

1.2.1 The Transmembrane Protein Problem

Because of the experimental hindrance, TMP biology has been relatively slow to emerge. Throughout the 1990s the concept of a TMH was simple and fairly assured: they were greasy peptides of around 30Å in length, often bundled together and oriented perpendicularly to the membrane. By 2006, crystallography had elucidated more than 60 high-resolution structures. Although the classic TMH structures were broadly prevalent, these structures contained a plethora of unusual TMHs. TMSs are capable of partial spanning of the membrane, spanning using oblique angles, and even lying flat on the membrane surface [8, 47]; the classical model was incomplete. Even recently, there is a contingency in the membrane biology field that despite progress over the last decade there is still a lack of information regarding the relationship between TMH sequences and function, TMH structure, intra-membrane TMP assembly, and the behavior of TMHs in the lipid bilayer; the native biological environment of TMHs [1].

Furthermore, the insertion and formation of the unusually orientated TMHs and of the more traditional TMHs have been shown to be underpinned by complex thermodynamic equilibriums and electrostatic interactions [28, 48, 49]. As well as being a biophysically convoluted system, TMHs are biologically functional beyond anchorage in many cases. TMSs have been identified as regulators of protein quality control and trafficking mechanisms, shifting the idea away from TMHs broadly exclusively functioning as anchors [50], and crucially this function beyond anchorage can be revealed by sensitive, careful analysis of the sequence information alone [25].

When predicting the function of any protein, one follows the dicta that function is facilitated by form, and form is determined by the sequence; the more similar the sequences, the more likely that the function is similar. For globular soluble proteins having the same folds induces strict biochemical restrictions on the packing of a hydrophobic protein core which requires similarity of non-polar residue patterns. Sequence analysis of non-globular TMPs has not been studied to nearly the same extent yet homology paradigms are silently extended and applied to them. In the case of Signal Peptide (SP)s or TMSs the physical constraints are similar for all TMPs, and so matching is indeed merely a reflection of the physical environment of the bilayer, not the common ancestry. Worryingly, because of this oversight, it appears that between 2.1% and 13.6% of Pfam hits for SPs or TMSs are indeed false positive results [51].

Over the last decade, Nanodiscs have been routinely used to much more easily obtain crystal structures. Nanodiscs overcome some of the major challenges caused by the hydrophobic helices and a more faithful representation of the biological membranes than alternative model membranes like liposomes [52].

However, critical questions remain: How is the TMH oriented in the membrane, how is the TMH interacting with the membrane, how is the TMH interacting with another TMH in the membrane, does the TMH have functions beyond anchorage and if so what are they?

1.2.2 The Importance Of Transmembrane Proteins

Membrane bound proteins underpin almost every biological process directly, or indirectly, from photosynthesis to respiration. Integral TMP are encoded by around 30% of the genes in the human genome which reflects their biological importance [53]. These proteins allow biochemical pathways that traverse the various biological membranes used in life.

The relationship between the membrane and TMPs is underpinned by complex thermodynamic and electrostatic equilibria. Once inserted the protein doesn't leave the membrane as a result of the TMH being very hydrophobic. This hydrophobicity and the hydrophobicity of the lipid tails means that they self-associate and this association is entropically driven by water. Another way of describing it is that they fiercely dissociate from the water. The overall ΔG for a TMH in the membrane is

-12kcalmol^{-1} [28]; the association of the helix in the membrane is typically spontaneous.

1.3 Biological Membrane Composition

1.3.1 Lipids of the Membrane

The compartmentalization of cellular biochemistry is arguably one of the most significant events to have occurred in evolution and is certainly one of the fundamental prerequisites for life [54]. The proteins that allow life to use this biochemical barrier are perhaps equally important. Together, the lipid bilayer and proteins therein allow complex biochemical systems that facilitate life as we know it.

It is critical to understand that the lipid bilayer and the trans-membrane α helices are inextricably linked, and often what we observe from the α helices reflect the properties of the much harder to study membranes. The lipid membranes influence the local structure, dynamics, and activity of proteins in the membrane in non-trivial ways [55–62], as well as protein folding [63].

There is a rich variety of lipid molecules that make up the biological membranes. The majority of lipids in higher organism membranes are phospholipids, sphingolipids, and sterols. These are composed of a glycerol molecule. Bonded to the glycerol molecule are two hydrophobic fatty acid tail groups and a negatively-charged polar phosphate group. The polar phosphate group is modified with an alcohol group. Water entropically drives the self-association of the lipid molecules. In other words, the bilayer forms from these phospholipid molecules due to the fierce dissociation between the polar water and the hydrophobic tails. Furthermore, the bilayer maximises van der Waals interactions between the closely-packed hydrocarbon chains, which contributes to the stability of the bilayer. This can be seen even in relatively early Molecular Dynamics (MD) simulations [64].

Differences in Membrane Compositions

It has been known for some time that the outer membranes of Gram-negative bacteria are asymmetric in terms of lipid composition. The outer membranes contain

lipopolysaccharide, whilst the inner is a mixture of approximately 25 phospholipid types. Adding to the membrane asymmetry composition story, a thorough analysis of residue composition in yeast and human TMH regions revealed intra-membrane leaflet composition asymmetry in the ER, but not the Golgi [10]. Furthermore, protein-lipid interactions have been shown to be determinants of membrane curvature [61], and undertake complex orientations and conformations to allow for hydrophobic mismatch [65].

1.3.2 Membrane Potential

Simply put, membrane potential is the voltage across a membrane. If the membrane is permeable to a certain type of ion, then the ion will experience an electrical pulling force during the diffusion process that pulls toward the “preferred” biological location. This clearly depends on a chemical component involving both the charge and ion concentration gradient. There are various ways of estimating the membrane potential *ab initio*.

The Nernst equation can be derived directly from the simplified thermodynamic principles (i) the Boltzmann distribution, and (ii) a field charge interaction energy [66]. It is defined as:

$$E_m = \frac{RT}{F} \times \ln \frac{c_{out}}{c_{in}} \quad (1.2)$$

Where charge E_m is the membrane potential, z is the ion charge, c is the concentration of an ion in that cell environment.

One problem in a biological membranes is that the compartments always involve multiple ion channels. The Goldman equation aims to solve this problem by accounting for several ions that contribute to c_{out} and c_{in} (such as K^+ , Na^+ , and Cl^-) simultaneously:

$$E_m = \frac{RT}{F} \times \ln \left(\frac{p_{K^+} \cdot [K^+]_{out} + p_{Na^+} \cdot [Na^+]_{out} + p_{Cl^-} \cdot [Cl^-]_{in}}{p_{K^+} \cdot [K^+]_{in} + p_{Na^+} \cdot [Na^+]_{in} + p_{Cl^-} \cdot [Cl^-]_{out}} \right) \quad (1.3)$$

Where charge E_m is the membrane potential, z is the ion charge, $[i]$ is the ion concentration and p_i is the relative membrane permeability for the actual ion.

However, it is rife with caveats caused by the assumptions of the simplified model.

Such assumptions include ions having point charge, that the potential is constant throughout the solution. This is compounded because it assumes the constant potential is the same as the point of measurement which can be heavily influenced by, for example, a specific adsorption of either part of the redox pair or the competitive adsorption of a supporting ion in solution [66]. Therefore one should be cautious to understand the limitations and variability when extrapolating experimentally determined E_0 , particularly when using such an idealised model in a biological context.

Organelle Membrane Potentials

Several studies have attempted to quantify the various voltages across the intracellular membranes. Negativity was found in the ER, with a voltage between between 75mV to 95mV in the ER membrane [67, 68]. Negativity was found in the mitochondrial matrix with a voltage across the mitochondrial membrane at 150mV [69]. No notable membrane potential has been identified in the Golgi [70, 71].

1.4 Biogenesis of Trans-membrane Proteins

1.4.1 Translocation

Tail-Anchored Proteins Post-Translationally Insert

Tail-anchored proteins are a topologically distinct class of intracellular proteins defined by their single carboxy-terminal transmembrane domain with a cytosolic-facing amino-terminus. Tail-anchored proteins are involved in a range of key cellular functions including protein translocation and apoptosis. Additionally, within the tail-anchored class of proteins are a set of vesicle fusion proteins called Soluble N-Ethylmaleimide-Sensitive Factor Attachment Receptor (SNARE) proteins. There is biomedical interest in SNARE drug delivery mechanisms. SNAREs can fuse liposomes containing various drug payloads into the membrane.

Notably, known SNARE TMHs are highly hydrophobic even compared to other tail anchored TMHs.

1.4.2 Translocon Independent Membrane Insertion

Signal anchored proteins, proteins that contain a single hydrophobic segment that serves as both a mitochondrial targeting signal and a membrane anchor, as well as tail-anchored proteins have been shown to be able to spontaneously insert into the membrane independently from the translocon [48, 72, 73].

It is postulated that there are electrostatic factors in the flanking regions that contribute to this spontaneous membrane insertion. Our experimental collaborators in Stephen Highs group are interested in a small group of tail-anchored proteins that have very polar trans-membrane domains and are capable of liposome membrane insertion without insertion machinery, also known as spontaneous insertion. They have found that chimeric synaptobrevin, one of the first identified SNARE proteins, is capable of spontaneous insertion if the tail anchor domain is replaced by the TM domains belonging to a protein of known spontaneously inserting domains. Their studies have moved the focus of spontaneous insertion away from the loop regions and onto the physicochemical factors of the TMH itself. The idea that SNARE proteins are modular and capable of spontaneous insertion has significant implications for both biomedical application in liposome-based drug delivery and can aid future research for testing complex biological molecular networks [74, 75].

1.5 Aims of This Thesis.

Chapter 2

Tail-Anchored Protein Datasets

2.1 Abstract

2.2 Introduction

This study aims to identify SNARE proteins in eukaryotic proteomes by filtering through large datasets using automatically predicted TrEMBL consensus, and manually annotated SWISS-PROT transmembrane regions. The pipeline generates a list of singlepass proteins with a transmembrane domain close to the C terminal, that are not splice isoforms. A previous study predicted 411 tail anchor proteins [76].

2.3 Methods

The original list UniProt protein database was queried for records containing “TRANSMEM” annotation on June 15, 2016, totaling 75826 records from swissprot, and 12322000 records from TrEMBL.

2.3.1 Filtering the Uniprot database

Steps carried out by Kalbfleisch *et al.* published in Traffic 2007 (8: 16871694) [76], were recreated using up to date tools. The nonredundant human dataset of 145,715 proteins from SwissProt and TrEMBL [77]. 2,478 singlepass proteins were programmatically extracted according to the TRANSMEM count from that list. Then TMDs not within

15AA of the C terminal were removed, resulting in 455 proteins. No splice isoforms were detected according to searching for NON-TER annotation. 195 proteins of the 411 predicted proteins from the previous study were successfully mapped using the Uniprot mapping tools [77]. Duplicate IDs from the previously predicted tail anchored protein were removed from the set. The remaining dataset contained XXX proteins.

2.3.2 Calculating Hydrophobicity

2.3.3 Calculating Sequence Complexity

2.4 Results

2.4.1 An Up To Date Tail-Anchor Dataset

2.4.2 Potential Tail-Anchored SNARE Protein Discovery

2.4.3 Biology of Spontaneously Inserting Tail Anchored Proteins

Chapter 3

Protein Classification Based on Intra-membrane Complexity Arrangement

3.1 Abstract

3.2 Introduction

3.3 Methods

3.4 Results

Chapter 4

Conclusions

4.1 Outlook

4.1.1 The hydrophobicity–sequence complexity continuum

We hypothesize that the hydrophobicity–sequence complexity continuum contains nuanced codes for different functions and that such differentiation of sequence and structural properties will allow assignment to these varying functions. Additionally, we suggest probing functional classification of yet uncharacterized membrane proteins by similarities of combinations of complex TM sets to well studied membrane proteins and finding those classes of TM proteins where this principle is most directly applicable.

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