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

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Transmembrane 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 hydrophobic transmembrane helices. 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 transmembrane protein α helices underpin membrane protein insertion mechanisms and furthermore can be used to predict presence of function in the transmembrane helix itself. By leveraging large datasets of transmembrane proteins, this thesis is focussed on characterising features of α helices en masse, particularly regarding their topology, membrane-protein interactions, and intra-membrane protein interactions.

Herein we expand on the core understanding of the biophysicochemical properties of these helices. We find evidence of a universal "negative-not-inside" rule that complements the famous "positive-inside rule" as well as intramembrane leucine propensity for the inner leaflet.

Furthermore we provide an up-to-date dataset of potential Tail-Anchored proteins, a group of post-translationally inserted proteins.

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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Together my supervisory team have instilled in me an excitement of discovery. Furthermore they have taught me a deep value of inductive reasoning and inference over deduction.

Shout out to all the Biopython devs that saved my sanity!

Preamble

In the 1950s and 1960s, the field of biological philosophy was still emerging. David Hull writes on the matter in his 1969 article entitled *"What Philosophy of Biology is not"*:

> "Periodically through the history of biology, biologists have tried to do a little philosophy..." [1]

I think this accurately summarises my attempts at applying philosophy to science for my philosophical doctorate. I can't help but wish my thesis title was "The ins-and-outs of greasy peptides".

So long, and thanks for all the fish!

List of publications

Journal Articles

Posters

Baker, J. and Warwicker, J. A Bioinformatic Method to Identify Potential SNARE Proteins. *40th FEBS Congress* Late Breaker (2015)

Chapter 1

Introduction

1.1 A Brief History of Transmembrane Proteins in Science

1.1.1 The Difficulties of Studying Transmembrane Proteins

Because of the experimental hinderence, the story of transmembrane proteins has been relatively slow to emerge. In the 1990s and early 2000s the story was seemingly uncomplicated. There were membrane-spanning bundles of non-polar -helices of roughly 20 residues length, with a consistent orientation of being perpendicular to the membrane surface. Since the mid-2000s the elucidation of many more intramembrane helix structures implied a far richer variety of transmembrane helices existed than previously thought, with a range of orientations and intra-membrane biophysical variations. Although the simple helices are broadly prevelant, hundreds of high quality membrane structures have elucidated that Transmembrane Helix (TMH)s can adopt a plethora of lengths and orientations within the membrane. TMHs are capable of partial spanning of the membrane, spanning using oblique angles, and even lying flat on the membrane surface [2, 3] (Figure 1.1).

The insertion and formation of the unusually orientated TMHs and of the more traditional TMHs have been shown to be underpinned by complex thermodynamic equilibria [4]. TMHs have been identified as regulators of protein quality control and trafficking mechanisms, shifting the idea away from TMHs broadly simply functioning as anchors [5]. The story is not as simple as originally thought. There is a contingency

in the field of biological membranes that despite progress over the last decade, there is a still lack of information regarding their structure, assembly, and the behaviour of TMHs in the lipid bilayer; the native biological environment of TMHs [4, 6].

1.1.2 The Importance of Membranes for Life

The compartmentalisation 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 [7]. The proteins that allow life to use this biochemical barrier are perhaps equally important.

1.2 Biological Membrane Composition

It is critical to understand that the lipid bilayer and the transmembrane 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 [8–14]. It has been known for some time that the outer membranes of Gram negative bacteria are asymentric in terms of lipid composition. The outer membranes contain lipopolysccharide, 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 asymetry in the Endoplasmic Reticulum (ER), but not the Golgi [15]. Furthermore proteinlipid interactions have been shown to be determinants of membrane curvature [14], and undertake complex orientations and conformations to allow for hydrophobic mismatch [16].

There is a rich variety of lipid molecules that make up the biological membranes. The majority of lipids in the membrane are phospholipids. 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 modeified with an alcohol group. Water entropicly 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. This

can be seen even in relatively early Molecular Dynamics (MD) simulations [17].

1.3 α Helices in Membranes

1.3.1 Transmembrane Helix Sequence Composition

Membrane bound proteins underpin almost every biological process directly, or indirectly, from photosynthesis to respiration. Integral Transmembrane Protein (TMP) are encoded by around 30% of the genes in the human genome which reflects their biological importance [18]. 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 thermondynamic and electrostatic equilibria. Once inserted the protein doesn't leave the membrane as a result of the transmembrane helix being very hydrophobic. This hydrophobicity, and the hydrophobicity of the lipid tails means that they self associate. A better way of describing it is that they fiercely dissociate from the water. The overall ΔG for a transmembrane helix in the membrane is -12kcal mol1 [4]: the association of the helix in the membrane is typically spontaneous.

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 [21]. 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.

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 [15].

A study by Baeza-Delgado *et al.* from 2013 [22] looked at TMHs in 170 integral membrane proteins from a manually maintained database of experimentally confirmed TMPs; MPTopo [24]. 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

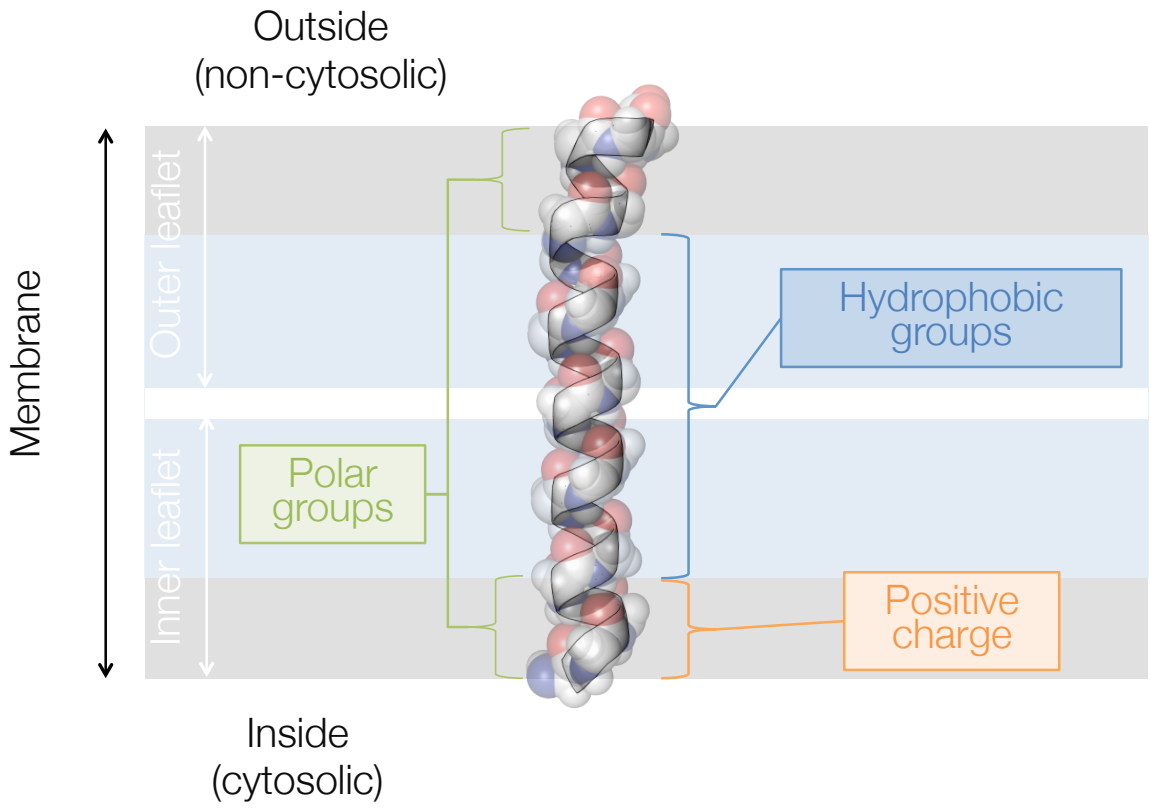

Figure 1.1: A cartoon showing the general components of the membrane and a typical TMH. The example used here for illustrative purposes is transmembrane region of tetherin (PDB 2LK9) [19]. Dark grey areas denote the area of lipid head groups. The residues found in these areas are often described as flanking regions, and are often in contact with the aqueous interface of the membrane. The helix core is mostly composed of hydrophobic residues. More recently the hydrophobic group region has been associated with cell localisation and a broad range of biochemical functions [20, 21]. 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" [15, 22, 23]. Note that the definition of a TM α-helix is not entirely clear; how far the helix rises into the water-interface region to qualify as a TMH for example [2].

with proline are biasedly near the flanks of the TM helices [22]. Transitions between the different types of amino acid at the ends of the hydrophobic core occur in a more defined region on the cytosolic side than at the extra cytosolic face. This is probably reflecting the different lipid composition of both leaflets of biological membranes [22]. A larger study using 1192 human and 1119 yeast predicted TMHs that were not structurally validated further explored the difference in TMH and leaflet structure by exploiting the evolutionarily conserved sequence differences between the TMH in the inner and outer leaflets [15]. 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 ER. The abundance of serines in the region following the lumenal end of golgi TMDs probably reflects the fact that this part of many golgi enzymes forms a flexible linker that tethers the catalytic domain to the membrane [15].

1.3.2 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 [25, 26]. 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 [15, 22, 23].

1.3.3 The Aromatic Belt

1.3.4 Snorkeling

1.4 Biogenesis of Transmembrane Proteins

1.4.1 Translocation

The "inside" was an imprecise term used to indirectly refer to the cytoplasmic space. To understand why the cytoplasm is the key part, one must recall how the membranes are thought to be synthesised.

1.4.2 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 transmembrane helices are highly hydrophobic even compared to other tail anchored transmembrane helices.

1.4.3 Spontaneous 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 indepently from the translocon [27–29].

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 transmembrane 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 its tail anchor domain is replaced by the transmembrane 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 biophysicochemical 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 [30, 31].

1.5 Measures of Hydrophobicity

1.6 Aims of This Thesis.

1. Negative not inside rule

2. Glycosylphosphatidylinositol (GPI) project

3. SNARE and Tail Anchor (TA) project

4. Good and bad helices

Chapter 2

The "negative-not-inside" rule

2.1 Abstract

2.2 Introduction

As the idea of positive residues inside the cytoplasm emerged during the late 1980s, so did the idea of negative residues working in concert with TMH orientation. It was shown that removing a single lysine residue reversed the topology of a model Escherichia coli protein, whereas much higher numbers of negatively charged residues are needed to reverse topology [32]. One would also expect to see a skew in negatively charged distribution if a cooperation between oppositely charged residues orientated a TMH, however there is no conclusive evidence in the literature for an opposing negatively charged skew [15, 22, 23, 33, 34]. However, in *E. coli* negative residues do experience electrical pulling forces when travelling through the SecYEG translocon indicating that negative charges are biologically relevant [35].

2.3 Methods

2.3.1 Normalisation

$$c_r = \frac{(a_{K,r}+a_{R,r})-(a_{D,r}+a_{E,r})}{N}$$

$$p_{i,r} = \frac{a_{i,r}}{\max_r(a_r)}$$

$$q_{i,r} = \frac{100a_{i,r}}{a_i}$$

2.4 Results

2.4.1 Biophysicochemical differences in multi-pass and single-pass helices

Chapter 3

Tail-anchored protein discovery

3.1 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 [36].

3.2 Methods

3.2.1 Filtering the Uniprot database

Steps carried out by Kalbfleisch *et al.* published in Traffic 2007 (8: 16871694) were recreated using up to date tools. The nonredundant human dataset of 145,715 proteins from SwissProt and TrEMBL [36, 37]. 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 [36, 37]. Duplicate IDs from the previously predicted tail anchored protein were removed from the set. The remaining dataset contained XXX proteins.

3.2.2 Calculating hydrophobicity

3.3 Results

3.3.1 An up to date tail-anchor dataset

3.3.2 Potential tail-anchored SNARE protein discovery

3.3.3 Biology of spontaneously inserting tail anchored proteins

Chapter 4

A novel GPI lipid anchor categorised

4.1 Abstract

4.2 Introduction

4.3 Methods

4.4 Results

Chapter 5

The good, the bad, and the ugly helices

5.1 Abstract

5.2 Introduction

5.3 Methods

5.4 Results

Chapter 6

Conclusions

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