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Charge Pair Interactions in Transmembrane Helices and Turn Propensity of the Connecting Sequence Promote Helical Hairpin Insertion

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Abstract

α-Helical hairpins, consisting of a pair of closely spaced transmembrane (TM) helices that are connected by a short interfacial turn, are the simplest structural motifs found in multi-spanning membrane proteins. In naturally occurring hairpins, the presence of polar residues is common and predicted to complicate membrane insertion. We postulate that the pre-packing process offsets any energetic cost of allocating polar and charged residues within the hydrophobic environment of biological membranes. Consistent with this idea, we provide here experimental evidence demonstrating that helical hairpin insertion into biological membranes can be driven by electrostatic interactions between closely separated, poorly hydrophobic sequences. Additionally, we observe that the integral hairpin can be stabilized by a short loop heavily populated by turn-promoting residues. We conclude that the combined effect of TM–TM electrostatic interactions and tight turns plays an important role in generating the functional architecture of membrane proteins and propose that helical hairpin motifs can be acquired within the context of the Sec61 translocon at the early stages of membrane protein biosynthesis. Taken together, these data further underline the potential complexities involved in accurately predicting TM domains from primary structures.

Introduction

Multi-spanning membrane proteins (those including two or more membrane-spanning segments) are important for many biological functions. The basic structural unit of such membrane proteins is a hydrophobic α-helix. In folded proteins, these individual helix-forming sequences are engaged in a rich network of interactions with other helices. Whereas individual helices are formed in response to main-chain hydrogen bonding and the hydrophobic effect, other interactions must be responsible for side-to-side assembly. Such interactions might include hydrophobic packing, electrostatic effects, turns between helices, and binding to components placed in the aqueous environments that surround the membrane.

α-Helical hairpins, consisting of a pair of closely spaced transmembrane (TM) helices that are connected by a short extramembrane or interfacial turn, are the simplest structural motifs found in multi-spanning membrane proteins.[1] This motif is thought to occur relatively frequently in integral membrane proteins and may serve as an important structural and/or functional element.[2]

The insertion of most helical plasma membrane proteins occurs co-translationally, whereby protein

synthesis and integration into the membrane are coupled. For the integration of individual TM sequences into the membrane, it is expected that TM segments will preadopt a helical state,[3,4] due to the significant free-energy penalty of embedding an exposed polypeptide backbone into the hydrophobic membrane core.[5] Similarly, the formation of interhelical hydrogen bonds facilitates the integration of polar residues present in adjacent TM regions. Then, there is no doubt that hydrogen bond interactions can play key roles in helical hairpin stabilization.

While many studies addressing the formation of helical hairpins in membranes have been carried out on model hydrophobic TM segments,[6–9] naturally occurring helical hairpins are not always highly hydrophobic,[10] and the role of helix–helix interactions and turn propensities of the residues interconnecting the two helices in their folding and stability is poorly understood. We previously showed that poliovirus (PV) 2B, which is a small protein involved in PV virulence, is a double-spanning integral membrane protein, in which the two TM segments are interconnected by a short turn forming a putative helical hairpin.[11] As a first step towards understanding its biogenesis, we demonstrated that *in vitro* PV 2B integrates into the endoplasmic reticulum (ER) membrane through the translocon.[11]

Here, we present a detailed investigation on structural determinants underlying helical hairpin formation in the viral membrane protein 2B. Using an *in vivo*-like translation–glycosylation system of the naturally occurring helical hairpin from PV 2B, we have determined the importance of helix–helix interactions for hairpin formation. In addition, we show that the hairpin structure is stabilized by the turn propensity of the amino acid residues in the short loop between the two TM helices. Our results suggest that integral helical hairpins may form in biological membranes driven by electrostatic interactions between marginally hydrophobic sequences and be additionally stabilized by short, tight connecting turns.

Results

Insertion of the viroporin 2B hairpin region into biological membranes

We have recently shown that *in vitro* PV 2B product inserts into the ER membrane as a double-spanning integral membrane protein with an N-/C-terminal cytoplasmic orientation.[11] Such topology is attained upon insertion of a helical hairpin whose constituent TM helices are marginally hydrophobic (Fig. 1a). *In vitro* synthesis of several truncated protein versions indeed put forward that the two hydrophobic regions cooperate to insert into the ER-derived microsomal membranes. Here, we explore the structural grounds for such effect.

As in our previous study of membrane insertion of the PV 2B,[11] we used a well-characterized *in vitro* experimental system based on glycosylation[12] that accurately reports the integration of TM regions into microsomal membranes. Upon insertion, the oligosaccharyl transferase (OST) enzyme modifies the protein of interest. OST adds sugar molecules to an NX(S/T) consensus sequence,[14] with X being any amino acid except proline,[15] after the protein emerges from the translocon channel. Glycosylation of a protein region synthesized *in vitro* in the presence of microsomal membranes therefore indicates the exposure of this region to the OST active site on the luminal side of the ER membrane. When assayed independently, the two hydrophobic regions of the PV 2B did not span the ER-derived membranes,[11] as expected according to the predicted apparent free energy of insertion (Fig. 1a). It has been shown previously that, in some cases, a neighboring TM helix can promote membrane insertion of a marginally hydrophobic TM region[16–19] and that there is a correlation between the polarity of a TM helix and its interaction area with the rest of the protein.[20] Therefore, we investigated the insertion of the full α-helical hairpin region (residues 35–81) in this *in vitro* translation system.

In our first experimental setup, the helical hairpin region was introduced into the "host" protein leader peptidase (Lep) (Fig. 1b), which contains two TM helices (H1 and H2) and a large lumenally exposed C-terminal domain (P2). In this first Lep construct, the 2B hairpin sequence (residues 35–81, Fig. 1a) was placed near the middle of the P2 domain (Fig. 1b) and was flanked by two engineered NXT acceptor sites for *N*-linked glycosylation (G1 and G2), with the G2 site located immediately downstream the hairpin region (see Materials and Methods). It has previously been demonstrated that efficient glycosylation occurs when the acceptor Asn is ~12–14 residues away from the membrane.[21,22] If the hairpin is translocated across the membrane, both G1 and G2 sites will be modified by the lumenally oriented OST; if the helical hairpin is inserted into the membrane, only G1 will receive a glycan (Fig. 1b). If one of the two hydrophobic regions is inserted, only G1 will be modified, but in that case, the large P2 domain will be non-translocated across the microsomal membrane. In this way, a single glycosylation suggests either correct hairpin integration (Fig. 1b, right) or the integration of only one hydrophobic region, whereas double glycosylation reports the non-integration capability of the hairpin region (Fig. 1b, left). Single glycosylation of the molecule results in an increase in molecular mass of about 2.5 kDa relative to the observed molecular mass of Lep