doi:10.1016/j.jmb.2010.01.042 J. Mol. Biol. (2010) 397, 190–201

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Repositioning of Transmembrane α-Helices during Membrane Protein Folding

Anni Kauko†, Linnea E. Hedin†, Estelle Thebaud, Susana Cristobal, Arne Elofsson* and Gunnar von Heijne*

Center for Biomembrane Research and Stockholm Bioinformatics Center, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden

Received 23 November 2009; received in revised form 19 January 2010; accepted 19 January 2010
Available online 25 January 2010

Edited by J. Bowie

We have determined the optimal placement of individual transmembrane helices in the *Pyrococcus horikoshii* Glt_{Ph} glutamate transporter homolog in the membrane. The results are in close agreement with theoretical predictions based on hydrophobicity, but do not, in general, match the known three-dimensional structure, suggesting that transmembrane helices can be repositioned relative to the membrane during folding and oligomerization. Theoretical analysis of a database of membrane protein structures provides additional support for this idea. These observations raise new challenges for the structure prediction of membrane proteins and suggest that the classical two-stage model often used to describe membrane protein folding needs to be modified.

Keywords: membrane protein; Glt_{Ph}; protein folding; membrane insertion

Introduction

How do integral helix-bundle membrane proteins fold? Classically, the rather simple structural principles gleaned from the first available high-resolution three-dimensional structures—canonical hydrophobic transmembrane (TM) α-helices packed against each other to shield polar residues—have been interpreted in terms of a two-stage folding model,[1] where the insertion of individual TM helices into the lipid bilayer in their energetically most favored position is followed by a folding process where preformed helices find their optimal packing interactions.

While the two-stage folding model is still a useful first-order approximation to the folding process, both structural and biochemical studies have begun to unravel a more complex reality.[2] TM helices are no longer seen as rigid rods, but often contain kinks and other kinds of nonhelical irregularities.[3] Reentrant loops dip into the plane of the membrane, but do not span its entire width.[4] During cotranslational membrane insertion, TM helices do not necessarily exit the translocon one by one and can remain close to the translocon channel until the ribosome terminates translation,[5] may change their orientation in the membrane after chain termination,[6,7] or may insert into the membrane only at a late state during folding.[5,8]

Even the basic assumption that TM helices in the folded protein are individually in their equilibrium positions relative to the lipid bilayer—an assumption that often underlies attempts to predict the three-dimensional structure of membrane proteins from their TM topology—may not always hold.[9] We were alerted to this possibility by an analysis of TM helices in the Glt_{Ph} glutamate transporter homolog from the bacterium *Pyrococcus horikoshii* and now show that the TM2, TM4, and TM7 helices in Glt_{Ph} in fact become dramatically repositioned relative to the membrane during the folding and oligomerization process. These findings highlight a hitherto neglected aspect of membrane protein structure prediction, namely, that the positions in the membrane of TM helices in the folded structure do not always correspond to the thermodynamically favored positions in the membrane of the isolated helices. Instead, long-range tertiary interactions might make it more energetically favorable for TM helices to alter their position relative to the membrane during folding, thereby providing a way to introduce polar and charged residues into the membrane domain.

**Corresponding authors.* E-mail addresses: arne@bioinfo.se; gunnar@dbb.su.se.

† A.K. and L.E.H. contributed equally to this work.

Abbreviations used: TM, transmembrane; RM, rough microsome; MGD, minimal glycosylation distance; PDB, Protein Data Bank.

Results

The hydrophobicity of TM helices does not match membrane location in Glt_{Ph}

Glt_{Ph} forms a trimer with a large central cavity that extends partway across the membrane[10] (Fig. 1a). Each monomer is composed of eight TM helices and two reentrant loops, and both the N-terminus and the C-terminus face the cytoplasm. Recent structural studies show that TM1, TM2, TM4, and TM5 in each monomer together form a 'trimerization domain' that does not move relative to the membrane during the transport cycle,[12] confirming an earlier study on the Glt_{Ph} homolog GltT where disulphide cross-linking was used to show that the trimerization domain remains rigid during the transport cycle.[13] TM4 and TM7 both contain short coil segments that break the helical structure near the middle of the membrane. However, as seen by comparing the left and right panels in Fig. 1b, the membrane-buried segments of TM2, TM4, TM7, and TM8 do not coincide with the most hydrophobic segments identified using the experimentally based 'biological' hydrophobicity scale, as embodied in the 'ΔG predictor' program.[14] In addition, for the membrane-buried segments of TM7 and TM8, the predicted apparent free energy of insertion (ΔG_{app}^{pred}) is remarkably high and not typical of a TM helix. As the 'ΔG predictor' has been shown to quite accurately predict the membrane-insertion efficiency of isolated TM helices,[14–16] this suggests that there may be some important rearrangements in the membrane-embedded part of Glt_{Ph} during folding and trimerization.

To better understand the possible rearrangements of the TM segments in Glt_{Ph}, we experimentally determined the apparent free energy of insertion into the endoplasmic reticulum membrane of the membrane-embedded part of each TM segment (ΔG_{app}^{S}), as defined in the OPM membrane protein structure database,[17] and the apparent free energy of insertion of the most hydrophobic segment corresponding to each TM segment (ΔG_{app}^{P}), as identified by the 'ΔG predictor.'

Repositioning of TM helices in Glt_{Ph}

We used a previously described assay[15,18] for measuring ΔG_{app}^{S} and ΔG_{app}^{P}. Briefly, the relevant Glt_{Ph} segment (H-segment) is introduced into the 'host' protein Lep, as shown in Fig. 1c; note that two different Lep constructs are used, depending on the orientation of the TM helix in Glt_{Ph} (N_{in}–C_{out} or N_{out}–C_{in}). Lep has two N-terminal TM helices (TM1 and TM2) and a large C-terminal domain (P2). When expressed in a rabbit reticulocyte *in vitro* transcription/translation system in the presence of dog pancreas rough microsomes (RMs), Lep inserts into the microsomal membrane with both the short N-terminal tail and the large P2 domain located in the lumen of the microsome.[19] In Lep^{I} construct (used for even-numbered Glt_{Ph} TM helices), the H-segment is placed near the middle of the P2 domain and is flanked by two engineered Asn-X-Thr acceptor sites for N-linked glycosylation (G1 and G2). If the H-segment inserts efficiently into the membrane, only the G1 site will be modified by the lumenal oligosaccharyl transferase; if, on the other hand, the H-segment is translocated across the membrane, both the G1 site and the G2 site will receive a glycan. Quantification of the fractions of singly glycosylated (f_{1x}) and doubly glycosylated (f_{2x}) molecules makes it possible to calculate an apparent equilibrium constant, K_{app}, for the membrane insertion of a given H-segment, $K_{app} = \frac{f_{1x}}{f_{2x}}$. The K_{app} value can be converted into an apparent free-energy difference between the noninserted state and the inserted state in the usual way: $\Delta G_{app} = -RT\ln K_{app}$, where R is the gas constant and T is the absolute temperature ($T = 303$ K).

In Lep^{II} construct (used for odd-numbered TM helices), the G2 site will be modified only if the H-segment inserts across the membrane, while the G1 site is always glycosylated; hence, $K_{app} = \frac{f_{2x}}{f_{1x}}$. Since we have found previously that ΔG_{app} values determined using the Lep^{II} construct are ~1 kcal/mol lower than those determined using the Lep^{I} construct and depend on the sequence of the H1 TM helix,[15] all Lep^{II} values reported below have been increased by 1 kcal/mol to make the data obtained with the two Lep constructs comparable and also to make them comparable with the results from the 'ΔG predictor.'

We used the Lep-based glycosylation assay to measure ΔG_{app}^{S} for all the structurally defined membrane-embedded TM helices and ΔG_{app}^{P} for the most hydrophobic regions overlapping TM2, TM4, TM7, and TM8 (Fig. 1d) (see Table 1 and Fig. S1 for sequences). The experimentally measured ΔG_{app}^{S} and ΔG_{app}^{P} values and the corresponding predicted $\Delta G_{app}^{pred,S}$ and $\Delta G_{app}^{pred,P}$ values agree well in most cases. For TM2, TM4, and TM7, $\Delta G_{app}^{P} < \Delta G_{app}^{S}$, as expected; for TM8, ΔG_{app}^{S} is surprisingly low (given $\Delta G_{app}^{pred,S}$ for TM8) and even slightly lower than ΔG_{app}^{P}. One possible explanation for the anomalous behavior of TM8 could be charge-pairing between residues D394 and R397, but further studies will be needed to clarify this issue.

As seen in Fig. 1d, the segments with the lowest ΔG_{app}^{P} values are significantly displaced relative to the structurally defined membrane-embedded TM helices for TM2, TM4, and TM7. This suggests that, for these TM helices, the segment that initially inserts into the membrane is different from the membrane-embedded segment that forms the corresponding TM helix in the folded trimer.

To further substantiate this conclusion, we used a 'glycosylation mapping' approach to map the ends of the membrane-embedded TM4 segment, both when inserted into the Lep constructs and when present in its normal context within Glt_{Ph}. This approach is based on the observation that the oligosaccharyl transferase active site sits at a well-