Membrane Topology and Insertion of the Citrate Transport Protein CitS

van Geest, Marleen

Chapter 6

SUMMARY

INTRODUCTION

Membrane protein topology

Membrane proteins of the plasma membrane of bacterial and eukaryotic cells share a similar architecture; they fold as a bundle of α-helices that is oriented perpendicular to the membrane. The α-helical segments span the hydrophobic core of the phospholipid bilayer and, for energetic reasons, are hydrophobic themselves. The hydrophobic transmembrane segments are connected by loops that contact the water phase and are more hydrophilic. The loops vary considerably in length from a few residues to loops long enough to fold in domain like structures in the periphery of the membrane. A fundamental aspect of the structure is the membrane topology, i.e. the number of transmembrane segments and their orientation in the membrane. The topology is formed during insertion of the protein into the membrane, which is a critical step in the biogenesis of membrane proteins.

Insertion

The process of membrane protein insertion into the bacterial inner membrane is less well understood then membrane protein insertion into the ER membrane of eukaryotic systems. Soluble proteins that have to cross the ER membrane or membrane proteins that have to be inserted into the ER membrane are targeted to the translocon by the signal recognition particle, SRP. The process of targeting starts in the cytosol when the first hydrophobic segment in the nascent chain emerges from the ribosome. The segment is recognized by SRP to which it binds and as a result translation of the nascent chain is arrested. The complex is targeted to the membrane by binding to the membrane bound SRP receptor. Subsequently, SRP is released, translation is resumed and the ribosome binds at the site of the translocon where the nascent chain is inserted into the membrane in a co-translational way. Although soluble proteins are translocated posttranslationally across the bacterial membrane, recent evidence suggests that the pathway for inner membrane protein insertion in bacteria resembles the pathway in the ER. Bacterial SRP and SRP receptor proteins have been identified and were shown to be essential for inner membrane proteins insertion and it was demonstrated *in vitro* that bacterial SRP is able to bind to the first hydrophobic segment of inner membrane proteins. In addition the bacterial translocon is very similar to the ER translocon (chapter 1).

Topogenic sequences

The destiny of each domain in the nascent chain is determined through interactions between the nascent chain and components of the insertion machinery, such as the translocon and the ribosome. A domain in the nascent chain can either be translocated across, inserted into or retained at the cytoplasmic site of the mem-

brane. These options determine the transmembrane topology of the membrane protein. In the most simple view membrane integration of polytopic membrane proteins is thought to be directed by a series of hydrophobic segments in the nascent chain. The segments are alternating signal anchor (SA) or stop transfer (ST) sequences which are inserted into the membrane in a sequential way. Studies with truncated membrane proteins, isolated segments of membrane proteins and model proteins have demonstrated that the insertion of a hydrophobic segment can be overruled by other topogenic signals, indicating that the insertion machinery integrates more than one topogenic signal. Charge distribution around a hydrophobic segment is a well studied topogenic determinant, but also combinations of hydrophobic segments have been shown to determine membrane topology. Interactions between different topogenic signals can result in the exclusion of a hydrophobic segment from the membrane as well as in the insertion of less hydrophobic sequences into the membrane.

Topology prediction

Secondary structure prediction algorithms for membrane proteins are largely based on the physicochemical properties of the membrane and the polypeptide and use the hydropathy properties of the amino acids in the polypeptide. The repeated helix-loop-helix motif in the structure of membrane proteins is a result of alternating stretches of hydrophobic (helix) and hydrophilic (loop) amino acids in the polypeptide. The hydrophobic stretches are reflected in typical peaks in the hydropathy profile of the amino acid sequence. Biochemical approaches to validate predicted topology models are based on techniques involving largely modified or truncated membrane proteins. Both the prediction methods and the experimental methods ignore the steps involved in the biogenesis of membrane proteins that lead to the insertion into the membrane. Therefore, current prediction algorithms are still far from perfect and the use of different biochemical techniques may result in contradictory models. Knowledge of the fundamentals of membrane protein insertion into the membrane is necessary to optimize current prediction and biochemical methods. Only when we are able to recognize all the topogenic sequences present in the nascent chain and if we understand how these sequences interact with each other and with components of the insertion machinery it should become possible to predict the structure of a membrane protein by simply decoding all the topogenic signals present in the polypeptide. Membrane proteins with unexpected topologies are very useful to discover new topogenic sequences and to obtain knowledge in how the insertion machinery integrates different topogenic signals.

MEMBRANE TOPOLOGY AND INSERTION OF CITS

Membrane topology

The sodium ion dependent citrate transport protein of *Klebsiella pneumoniae* (CitS) is a polytopic membrane protein with an unexpected topology. Hydropathy analysis of the amino acid sequence reveals the presence of 12 hydrophobic segments that are long and hydrophobic enough to span the membrane in an α-helical conformation, suggesting a membrane topology with 12 putative transmembrane segments (chapter 2). The membrane topology was determined using different biochemical approaches (chapter 5). Different tags were inserted at

positions that tolerated the insertion after which the sidedness of the tag was determined by proteolytic experiments. In addition, reactivity of cysteine substitution mutants with permeable and impermeable sulfhydryl reagents was tested (chapter 3). The folding of CitS was studied also in the ER membrane, using in vitro translation/insertion of C-terminally truncated CitS molecules engineered in place of the second transmembrane segment of the *E.coli* membrane protein leader peptidase (chapter 4). The membrane topology was found to be the same in the two types of membranes, but different from the 12 transmembrane helix structure common to most secondary transporters. The study revealed that 11 of the 12 putative membrane spanning segments indeed span the membrane while one hydrophobic segment, segment Vb, has a periplasmic location. The protein is further characterized by a cytoplasmic N-terminus and a periplasmic C-terminus (figure 1). One periplasmic and 3 cytoplasmic loops in the CitS molecule are considerably longer than ussually observed in bacteria. The role of these loops in the function and structure of the transporter was investigated (chapter 5).

Insertion of CitS in the ER membrane

In order to find the topogenic signals responsible for the exclusion of hydrophobic segment Vb and to provide insight information in the mode of membrane insertion of CitS, the insertion of CitS into the endoplasmatic reticulum (ER) membrane was investigated (chapter 4). By using *in vitro* translation of model proteins in the presence of dog pancreas microsomes, each of the putative transmembrane segments of CitS was assayed for its potency to insert into the ER membrane, both as isolated segments as well as in the context of COOH-terminal truncation mutants. All 12 segments were able to insert into the membrane as a signal anchor irrespective of their orientation in the wildtype CitS protein. Also segment Vb which is excluded from the *E. coli* and ER membrane in the context of the entire CitS protein inserts into the ER membrane as a signal anchor. In a series of COOH-terminal truncation mutants, the segments inserted into the membrane one after the other, thus in a sequential way, except for segment Vb, which was translocated to the lumen. Also when segment VII and IX were extended with downstream hydrophobic domains, a similar sequential insertion was observed, illustrating that NH_2-terminal fragments of CitS are not required for the insertion of COOH-terminal fragments and that in these constructs the overall orientation of the segments is simply determined by the most NH_2-terminal segment.Using different model proteins, the stop transfer activity of segment Vb was measured in the context of different sequences. Although fully active as a signal anchor, segment Vb does not function efficiently as a stop transfer segment in any of the tested constructs. Apparently, the destiny of segment Vb is influenced by its orientation towards the membrane, suggesting that the insertion machinery is able to discriminate between signal anchor and stop transfer segments. The average hydrophobicity of segment Vb compared to the other hydrophobic segments of CitS is rather low which correlates with its low stop transfer activity, however another CitS segment with an even lower average hydrophobicity (segment IX) exhibits high stop transfer activity, indicating that hydrophobicity is not the only topogenic signal which is responsible for the membrane exclusion of segment Vb. Possibly,

the specific amino acid sequence of a segment plays a role in stop transfer activity. In addition, it was found that the poor stop transfer activity of segment Vb is influenced by the preceding segment. The presence of preceding CitS segment V mediates a 100 % exclusion of segment Vb from the membrane, while the presence of other hydrophobic segments result in significant stop transfer activity of segment Vb. This demonstrates that during insertion topogenic signals in the preceding sequence interact with topogenic signals within segment Vb.

Insertion of CitS in the *E. coli* membrane

Insertion studies in *E.coli* using a serie of COOH-terminal truncation mutants of CitS fused with the C-terminus to the mature part of PhoA demonstrated that the mode of insertion of CitS into the *E. coli* membrane showed remarkable similarities as well as remarkable differences with the mode of insertion into the ER membrane (chapter 2, 3, 4). The set of CitS-PhoA fusion proteins with fusion sites in the hydrophilic area's between the first 8 hydrophobic segments of CitS folded in a similar way in the *E.coli* membrane as in the ER membrane, illustrating that the segments up to segment VIII insert in a sequential way into the *E.coli* membrane, except for segment Vb which is translocated to the periplasm. Apparently, the topogenic signals involved in the exclusion of segment Vb are interpreted in the same way by both insertion machinery's. CitS-PhoA fusion proteins with the fusion sites in between segment VIII and IX however, demonstrated that in the absence of the C-terminal CitS sequences segment VIII is translocated to the periplasm, indicating that insertion of segment VIII into the *E.coli* membrane requires the presence of C-terminal sequences (chapter 2). Cysteine accessibility studies in truncated CitS molecules fused to PhoA demonstrated that insertion of segment VIII into the *E. coli* membrane occurs when the next hydrophobic segment (segment IX) is included in the fusion protein (chapter 4). Thus, downstream segment IX is both essential and sufficient for the insertion of segment VIII in the *E. coli* membrane which illustrates the effect of interactions between different topogenic signals. The different folding of the truncated protein consisting of the first 8 TMSs of CitS in the *E.coli* and ER membrane suggests differences in the bacterial and ER insertion mechanism or is related to different conditions during insertion, f.i. in vivo vs. in vitro, or differences in the reporter system, i.e. alkaline phophatase vs. the P2 domain of leader peptidase.

CONCLUSIONS

The insertion studies done with CitS strongly suggest that the insertion of membrane proteins in the bacterial membrane or in the ER membrane do not follow a simple sequential insertion mechanism in which the hydrophobic segments insert one after the other into the membrane as they emerge from the ribosome. It appears that insertion is a controlled and more complex mechanism in which other factors than hydrophobicity play a role, such as the orientation of a segment towards the membrane and the presence of up- and downstream sequences, and in which different topogenic signals interact. Insertion of transmembrane segments that depend on the presence of neighboring segments has been reported in membrane topology studies of polytopic membrane proteins in the ER membrane, but never before in case of a functional protein in the bacterial membrane.

Summary

Unknown topogenic signals and interactions between them interfere with present structure prediction methods and biochemical methods to assess membrane protein topology. In order to succesfully predict the membrane topology of a membrane protein, a fundemental knowledge of the insertion proces is necessary.