Hydrophobic forces drive spontaneous membrane insertion of the bacteriophage Pf3 coat protein without topological control

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Bacterial integral inner membrane proteins are either translocated across the lipid bilayer using an energy-driven enzyme, such as the Sec translocase, or they might interact directly with the membrane due to hydrophobic forces. We report that the single-spanning Pf3 coat protein is spontaneously inserted into the membrane of *Escherichia coli* and requires the electrical component of the membrane potential ($\Delta\Psi$) to translocate its N-terminal region. This results in a final $N_{out}C_{in}$ orientation of the protein in the cytoplasmic membrane, due the potential-driven translocation of the aspartyl residue at position 18 in the hydrophilic N-terminal tail. Uncharged protein tails are only translocated when the hydrophobic transmembrane region of the protein has been extended. An extended transmembrane anchor allows membrane insertion in the absence of an electrochemical membrane potential, but also causes the loss of a strict determination of the topology.

Keywords: bacteriophage Pf3/electrophoretic force/membrane potential/membrane translocation/Sec translocase

Introduction

Prior to the assembly of viral progeny, the *Pseudomonas aeruginosa* phage Pf3 coat protein is integrated into the inner membrane of the bacterial host cell. The membrane insertion process has been studied in *Escherichia coli* and is one example of non-enzymic (Sec-independent) protein insertion (Dalbey *et al.*, 1995; Kuhn, 1995). Membrane integration of the Pf3 coat protein occurs in a strictly N_{out} orientation and is dependent on the membrane potential (Rohrer and Kuhn, 1990; Kiefer *et al.*, 1997). Using site-directed mutagenesis, the charged amino acid residues flanking the 18-amino acid hydrophobic region were converted into neutral or oppositely charged residues. As a result, the orientation of the protein was completely reversed. This suggests that these charged residues control the orientation of the protein (Kiefer *et al.*, 1997). Surprisingly, the contribution of the negatively charged residues was essential for membrane insertion and the positively charged residues had only a minor passive effect on the orientation, although it is thought that positively charged residues control the topology of membrane proteins in general (Dalbey, 1990).

The distribution bias of charged residues in integral

proteins of *E.coli* has led to the 'positive-inside' rule (von Heijne, 1989), suggesting that charged residues flanking a hydrophobic anchor region determines its orientation in the membrane. Two major factors that interact with the charge of a protein have been discussed. The electrochemical membrane potential that renders the periplasm positively charged should favour the translocation of negatively charged residues and disfavour positively charged residues. Kinetic effects on the translocation of charge mutants of the double-spanning leader peptidase (Andersson and von Heijne, 1994) and the M13 procoat protein (Cao *et al.*, 1995; Schuenemann *et al.*, 1999) suggested that an electrophoretic mechanism is a topogenic factor, by preferably pulling negatively charged regions of the protein across the membrane. Additional experiments with mutants of the M13 procoat protein (Kusters *et al.*, 1994) and the leader peptidase (van Klompenburg *et al.*, 1997) showed a decent influence of the negatively charged phospholipids on the translocation event. The positively charged residues prevented translocation most likely by a strong electrostatic interaction between the negatively charged lipid surface and the positive charges of the protein. To distinguish clearly between the electrophoretic versus the electrostatic mechanisms, we studied the membrane insertion of an uncharged mutant of the Pf3 coat protein showing a non-oriented topology. Re-addition of charged residues demonstrated that the positively charged residues have a strong (potential-independent) electrostatic effect, whereas the negatively charged residues show an electrophoretic response only when the hydrophobicity of the membrane anchor region is limited. Hence, both mechanisms to control the orientation of the Pf3 coat protein are effective, but their individual contribution to the overall driving force of the translocation depends very much on the length or hydrophobicity of the transmembrane region.

Results

Membrane insertion of the Pf3 coat protein is independent of the Sec translocase

To analyse whether the translocation of Pf3 coat protein requires a proteinaceous factor in the membrane, we investigated the insertion of the protein into isolated inverted inner membrane vesicles (INV) from *E.coli*. The vesicles were either left untreated (INV) or were pretreated with either trypsin (T-INV) or urea (U-INV) prior to the *in vitro* translocation experiment. [³⁵S]methionine-labelled Pf3 coat protein was synthesized in an *E.coli* translation system and cotranslational insertion into the INV was analysed after 30 min at 37°C by digestion with 0.5 mg/ml proteinase K for 30 min at 25°C (Figure 1, lower panel). Approximately 30% of the synthesized material was shifted to a proteolytic fragment of a slightly lower

Fig. 1. Spontaneous membrane insertion of Pf3 coat protein into *E. coli* inner membrane vesicles (INV). The Pf3 coat protein and pre-LamB were transcribed from pT7-7 and pET3 derived plasmids, respectively. *In vitro* translation with [³⁵S]methionine was performed in an S-135 supernatant at 37°C for 30 min. The reactions were programmed with preLamB (upper panel) and Pf3 coat protein (lower panel) mRNA and analysed before (odd numbered lanes) or after (even numbered lanes) incubation with 0.5 mg/ml proteinase K for 30 min at 25°C. *Escherichia coli* inverted membrane vesicles were added cotranslationally (lanes 3–8). Pretreatment of the vesicles was either with 250 µg/ml trypsin (lanes 5 and 6) or with 5 M urea for 30 min prior to the translocation reaction (lanes 7 and 8).

molecular weight which includes the N-terminal region of the protein carrying the labelled methionine residue at position 1 (lane 4). Membrane insertion of the Pf3 coat protein into trypsin-treated vesicles (lane 6) or urea-washed vesicles (lane 8) was also observed. As a control, the Sec-dependent pre-LamB was analysed in parallel (Figure 1, upper panel). Upon addition of the untreated vesicles pre-LamB was partially cleaved to the mature form (LamB) by the vesicle-bound leader peptidase and became protease resistant (lane 4). In contrast, upon addition of the T-INV no cleavage occurred and the pre-LamB protein was completely sensitive to the externally added proteinase K (lane 6). Since both proteins require the electrochemical membrane potential, we analysed the trypsin-treated vesicles with oxonol and found that the trypsin treatment did not grossly affect the potential of the vesicles. In fact, we found that the efficiency of translocation of the T-INV was slightly enhanced for the Pf3 coat protein, presumably because the vesicle lipid surface was more accessible (compare lanes 4 and 6). Pretreatment of the vesicles with urea to remove peripheral membrane components such as SecA, blocked translocation of pre-LamB but not of the Pf3 coat protein (compare lanes 8 of both panels).

Membrane insertion of the Pf3 coat protein requires the electrical potential $\Delta\psi$ across the cytoplasmic membrane

Dissipation of the potential by addition of the ionophor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) results in the accumulation of the non-translocated form of the Pf3 protein (Kiefer *et al.*, 1997). If the vesicles were treated with the K⁺/H⁺ exchanger nigericin, the electrical gradient ($\Delta\psi$) was not affected but the proton gradient (ΔpH) was destroyed, as verified by oxonol fluorescence measurements (data not shown). Under these conditions, the Pf3 coat protein was efficiently translocated. This was proven by its resistance to externally added protease (Figure 2, compare lanes 4 and 6). Additional treatment of the vesicles with valinomycin to destroy the

Fig. 2. Membrane insertion of the Pf3 coat protein into *E. coli* inner membrane vesicles is independent of the proton gradient (ΔpH) but strongly depends on the electrochemical membrane potential ($\Delta\psi$). *In vitro* translation-translocation of the [³⁵S]methionine-labelled Pf3 coat protein was performed in the absence (lanes 1–4) or presence of 0.5 µg/ml nigericin only (lanes 5 and 6) or 1 µg/ml valinomycin and 0.5 µg/ml nigericin (lanes 7 and 8). Translocation of the Pf3 coat protein was monitored by proteinase K digestion (lanes 2, 4, 6 and 8).

electrical gradient resulted in the complete inhibition of membrane translocation (Figure 2, lane 8), suggesting that the membrane insertion of Pf3 predominantly requires $\Delta\psi$.

A single negatively charged residue orients the Pf3 coat protein in the membrane

The role of charged amino acids in membrane insertion and orientation of the Pf3 coat protein was investigated with the mutant 4N. In this mutant all four originally charged residues (the two aspartyl residues at positions 7 and 18 in the N-terminal region as well as the arginyl residue at position 37 and the lysyl residue at position 40 in the C-terminal region) were converted to asparagine residues (Figure 3A). The resulting 4N mutant was completely inhibited for translocation into inverted inner membrane vesicles (Figure 3B). This was also observed *in vivo* if proteinase K was added externally to the spheroplasts (data not shown). Since the membrane potential of exponentially growing *E. coli* cells is approximately –200 mV inside, it has been suggested that this drives the translocation of negatively charged residues within the Pf3 coat protein region (Kiefer *et al.*, 1997). We therefore tested whether re-introducing a single charged amino acid residue into the 4N mutant is sufficient to restore membrane insertion and orientation. A unique aspartic acid residue was introduced at positions 7, 18, 37 and 40, respectively, and the resulting Pf3 mutant proteins 7D, 18D, 37D and 40D were analysed for translocation. Translocation of the N-terminal region was monitored by protease accessibility of the [³⁵S]methionine-labelled protein and translocation of the C-terminal region by accessibility of the [³H]phenylalanine-labelled protein (Figure 3B). A single negatively charged amino acid residue stimulated translocation and orientation of the Pf3 coat protein, whereas a positively charged residue had no significant effect. Positioning the charged residue close to the hydrophobic region at position 18 or 37 had a stronger effect than the more distant positioning in the mutant 7D.

The membrane potential is required to retain the Pf3 coat protein in a transmembrane state

The next question we asked in terms of the energetics of the translocation process was whether the electrochemical membrane potential is required only for the initial translocation event or whether there is a thermodynamic

Fig. 3. The orientation of the Pf3 coat protein is controlled by a single negatively charged residue. Mutants were generated at amino acid positions 7, 18, 37 and 40. (A) The primary amino acid sequence of the Pf3 coat protein is shown. The sequence in italics represents the hydrophobic transmembrane region, the underlined portion of the protein is an epitope tag that has been introduced into the original Pf3 sequence for optimal performance of the C-terminal protease protection assays and is also present in all other mutants. The nomenclature of the mutants is exemplified. (B) *In vitro* translocation experiments were performed using [³⁵S]methionine for monitoring N-terminal translocation (black columns) and [³H]phenylalanine for C-terminal translocation (white columns). The percentage of protease-protected material was calculated with the AIDA software (Raytest) on a Fuji BioImager BAS1500.

requirement of the potential to retain the protein in the translocated form. *Escherichia coli* BL21 cells bearing a plasmid encoding the 18D mutant were pulse-labelled with [³⁵S]methionine for 3 min and chased with non-radioactive methionine. The culture was then divided into three portions, one was kept on ice, the second was treated with CCCP and incubated for an additional 3 min at 37°C, whereas the third was incubated for 3 min at 37°C without the addition of CCCP. The cells were then converted to spheroplasts (Cao *et al.*, 1995) and the topology of the protein was analysed by protease mapping and immunoprecipitation (Figure 4). After the 3 min pulse-labelling period >90% of the Pf3 protein was accessible from the periplasmic side by the protease and therefore in the N_{out} transmembrane orientation (Figure 4, lane 2). Treatment with CCCP for an additional 3 min resulted in the proteinase-resistant form (Figure 4, lane 5), which was not observed for the cells incubated without CCCP (Figure 4, lane 8). This suggests that the membrane potential is indeed required to keep the protein in the transmembrane configuration, possibly by stretching the transmembrane α -helix.

Extension of the hydrophobic region of the Pf3 coat protein allows membrane insertion without any charged residue

To investigate whether an extension of the transmembrane helix changes the energetics of the membrane insertion of

Fig. 4. The translocated Pf3 coat protein requires the electrochemical membrane potential to remain stably in the transmembrane state. *Escherichia coli* BL-21 cells bearing the pT7-plasmid coding for the 18D mutant of the Pf3 coat protein were labelled with [³⁵S]methionine for 3 min at 37°C and chased with 100 μ g/ml cold L-methionine for 5 min. A portion of the culture was chilled on ice and immediately analysed by protease mapping to ensure that the majority of the 18D protein had been translocated (lanes 1–3) as shown by its protease accessibility. The remaining culture was divided and incubated at 37°C for an additional 5 min, one portion in the presence of 40 μ M CCCP (lanes 4–6), the other portion untreated (lanes 7–9). All reactions were then put on ice. The chilled samples were converted to spheroplasts by osmotic shock (Kiefer *et al.*, 1997) and treated with 1 mg/ml proteinase K for 1 h on ice (lanes 2, 5 and 8) or treated with proteinase K in the presence of 4% Triton X-100 (lanes 3, 6 and 9). All samples were precipitated with 20% TCA, immunoprecipitated with an antibody to Pf3 and analysed by SDS-PAGE.

the Pf3 coat protein, additional leucyl residues were placed into the centre of the membrane anchor of the 4N mutant protein. Either one, two or three leucyl residues were placed between Ile26 and Ile27 (Figure 5A). Whereas one and two added leucyl residues had little effect upon membrane insertion (Figure 5B, lanes 3–6), the addition of three leucyl residues substantially restored membrane insertion of the Pf3 coat protein (Figure 5B, lanes 7 and 8). In contrast to the wild-type Pf3 coat protein, the translocation of the 3L-4N mutant also occurred in membrane vesicles that were treated with CCCP to dissipate the membrane potential (lanes 9 and 10).

Strikingly, even the potential-dependent wild-type protein (Figure 6A, lanes 1–4) became potential independent when three leucine residues (mutant 3L-wild type) were introduced (Figure 6A, lanes 5–8) into the hydrophobic segment. The increased hydrophobicity of the 3L-wild type protein obviously facilitates its translocation, thus rendering it independent of the driving force, the membrane potential. In addition, the original N_{out}C_{in} membrane orientation was strongly maintained. The positively charged C-terminal region of the membrane-inserted 3L-wild type protein was completely accessible to the protease whereas the negatively charged N-terminal region was protected (compare lane 6 in Figure 6A with lane 2 in Figure 6B). Likewise, the topology of the 3L-wild type protein was not altered after the loss of the membrane potential by treatment with CCCP (Figure 6A, lanes 7 and 8, and 6B, lanes 3 and 4). Taken together, these results

Fig. 5. Membrane translocation of the uncharged 4N-Pf3 coat protein mutants with extended hydrophobic regions. **(A)** Site-directed mutagenesis was used to introduce up to three additional leucyl residues between the two isoleucyl residues at positions 26 and 27 in the centre of the hydrophobic region. **(B)** N-terminal translocation of the hydrophobic mutant Pf3 proteins into *E. coli* inner membrane vesicles was analysed by *in vitro* expression and [³⁵S]methionine labelling of the 4N (lanes 1 and 2), 1L-4N (lanes 3 and 4), 2L-4N (lanes 5 and 6) and 3L-4N (lanes 7–10) mutants. Inverted membrane vesicles were added cotranslationally and the samples were analysed by protease digestion. The membrane insertion of the 3L-4N mutant was also analysed for its dependency on the electrochemical potential by addition of 100 μ M CCCP (lanes 9 and 10). The arrow indicates the protease-protected Pf3 coat protein fragment.

show that the extended hydrophobic region in the Pf3 coat protein render its translocation independent of the membrane potential, but it still shows a defined orientation. This is in contrast to the mutant 3L-4N that was also inserted independently of the membrane potential (Figure 6B, lanes 5–8; for N-terminal translocation of this mutant refer to Figure 5B, lanes 7–10). The neutral hydrophobic mutant (3L-4N) did not show a distinct membrane orientation, hence, the extended hydrophobic region led to an equal distribution of both possible orientations (Figure 7A, 3L-4N). Since the hydrophobic Pf3 coat protein was correctly oriented, with only the N-terminal region being translocated to the *trans* side of the membrane when all the originally charged residues were changed back (Figure 7A, 3L-wild type), we investigated whether the negatively or the positively charged residues are topologically important. Intriguingly, in contrast to the charged mutants with unaltered hydrophobic regions (Figure 3B), the positively charged residues of the C-terminal region in the hydrophobic mutants were found to be sufficient in completely orientating the protein, as shown by the mutant 3L-37R40K. This mutant has a positively charged C-terminal region corresponding to the wild-type sequence and an uncharged N-terminal segment. The negatively charged N-terminal residues, however, had no topological role in the hydrophobic mutant (mutant 3L-7D18D) resulting in an equal distribution of the two possible orientations (Figure 7). The N-terminal region of the mutant 3L-7D18D corresponds to the wild-type situation whereas the C-terminal region is uncharged. The topological orientation of the 3L-7D18D mutant corresponded to the neutral mutant 3L-4N. How does the

Fig. 6. Increased hydrophobicity renders membrane insertion of the Pf3 coat protein independent of the electrochemical membrane potential. **(A)** Membrane insertion of the Pf3 wild type protein (lanes 1–4) and the wild type protein with an extended hydrophobic region (3L-wild type, lanes 5–8) into *E. coli* inverted membrane vesicles was analysed *in vitro* by labelling the protein with [³⁵S]methionine and a subsequent protease digestion assay as described (see Figure 1) in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of 100 μ M CCCP. **(B)** C-terminal translocation of the 3L-wild type (lanes 1–4) and the 3L-4N Pf3 coat protein mutant was assayed by *in vitro* translation in the presence of [³H]phenylalanine, cotranslational addition of *E. coli* INV and protease digestion. Translocation was examined in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of 100 μ M CCCP.

electrochemical potential influence the orientation of the hydrophobically extended Pf3 proteins? Dissipation of the membrane potential with CCCP showed that the 3L-wild type protein predominantly translocated the N-terminal region (Figure 7B). Thus, the orientation of the 3L-wild type protein was not altered after dissipation of the membrane potential. This also holds for the other hydrophobic mutants of Pf3, as summarized in Figure 7B. Conclusively, the orientation of the hydrophobically extended proteins in INV was not significantly changed after dissipating the electrochemical membrane potential. We assume that the positively charged residues interact electrostatically with the phospholipid headgroups and thereby hinder translocation across the membrane.

Discussion

Hydrophobic membrane insertion of the Pf3 coat protein

On the basis of this work, we suggest that membrane insertion of the Pf3 coat protein can use two distinct driving forces to translocate protein regions across the lipid bilayer (Figure 8). The first mechanism is independent of the electrochemical membrane potential and simply uses the hydrophobic force resulting in the transmembrane configuration of the protein. For the mutant without any

Fig. 7. Orientation of the Pf3 coat protein with an extended hydrophobic region is determined by the positively charged residues. (A) N-terminal translocation of [^{35}S]methionine-labelled Pf3 coat mutant proteins (black columns) and C-terminal translocation of [^3H]phenylalanine-labelled proteins (white columns) into inverted *E. coli* inner membrane vesicles was analysed. (B) The membrane insertion and orientation of the hydrophobic mutant 3L-Pf3 coat proteins was analysed in the absence of an electrochemical membrane potential after the addition of 100 μM CCCP to the *in vitro* translocation samples.

charged residues (3L-4N) there was no principal preference which of the tails that flank the hydrophobic region was translocated to the *trans* side of the membrane and we found that approximately equal portions had $\text{N}_{\text{out}}\text{C}_{\text{in}}$ and $\text{C}_{\text{out}}\text{N}_{\text{in}}$ orientations. This shows that the helix dipole alone is not sufficient to determine the orientation of the translocating protein chain in the membrane.

In trying to understand the membrane insertion thermodynamically, we were interested to investigate the free energy that allows the membrane translocation of the Pf3 coat protein. Membrane insertion of a protein can be understood as a three-step process, where, in the first step the protein binds laterally to the membrane interface, in a second 'transition' step the hydrophobic anchor region and one hydrophilic tail insert into the hydrocarbon core of the lipid bilayer, and in the third step the hydrophilic tail is released into the aqueous *trans* side. White and Wimley (1998) have calculated the free energy change for peptides to enter the interface of a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayer membrane and estimated the ΔG° values for each of the 20 amino acyl residues. With these values, the hydrophobic anchor region of the Pf3 coat protein shows a $\Delta G^\circ = -6.7$ kJ/mol (see Materials and methods). We therefore think that the hydrophobic region of the Pf3 coat protein binds readily to the membrane interface. Little is known about the free energy to transfer a peptide from the interface to the lipid

hydrocarbon core. One basis for an estimation are the water/oil distributions of amino acids by Engelman *et al.* (1986). However, these values (termed the GES scale) do not consider the energetic contribution of the peptide backbone. The free energy to transfer a peptide bond of an α -helix into the bilayer has not been determined precisely, but +5 kJ/mol (1.2 kcal/mol) is a good assumption based on computational and experimental studies (Ben-Tal *et al.*, 1996; White and Wimley, 1999). For estimating the change in free energy of the hydrophobic region of the Pf3 coat protein we therefore added to the values of Engelman and colleagues +5 kJ/mol for each amino acid (see Materials and methods). Since the conformation of the Pf3 coat protein is 40% α -helical in an aqueous and 40–75% α -helical in a hydrophobic environment (Thiaudiere *et al.*, 1993) we did not consider the energetic contribution of a conformational change for the translocation process that might occur. To integrate the 18 residue hydrophobic region of the Pf3 coat protein into the membrane gives a free energy gain of $\Delta G^\circ = -74$ kJ/mol and in the case of the extended hydrophobic region by three additional leucines the gain is $\Delta G^\circ = -94$ kJ/mol. For a successful membrane translocation of the protein the hydrophobic effect has to serve as an energy source to translocate one of the hydrophilic tails.

Translocation of the hydrophilic tail regions

To translocate a hydrophilic region across a lipid bilayer it has to overcome a high energy barrier. In the transition state, the presence of either the N-terminal or C-terminal region of the wild-type Pf3 coat protein in the hydrophobic core of the lipid bilayer would cost $\Delta G^\circ = \sim 128$ kJ/mol or ~ 130 kJ/mol, respectively. In this calculation we also considered the integration of the charged amino and carboxyl groups at the terminus of either tail, which contribute 21 and 18 kJ/mol, respectively (see Materials and methods). The high energy cost of translocating the N-terminal tail explains why the hydrophobic anchor region does not provide enough energy for translocation. Extending the hydrophobic region by three leucines, however, lowers the required free energy of activation to $\Delta G^\ddagger = 34$ kJ/mol, which is low enough for the translocation of the N-terminal region in a time range of seconds. The positively charged C-terminal region is blocked for translocation due to the electrostatic forces at the interface of the *cis* side. The translocation of the N-terminal and C-terminal tails with uncharged residues would cost 91 and 82 kJ/mol, respectively. Indeed, with the mutant 3L-4N we found that the two tails were translocated with about the same probability. Since the extended hydrophobic region provides -94 kJ/mol, no activation energy would be required for the membrane insertion of the neutral tails.

Surprisingly, the hydrophobic anchor region of the wild type, which provides a free energy of $\Delta G^\circ = -74$ kJ/mol, was not capable of translocating the uncharged tails of the 4N mutant across the membrane (which would require an activation energy of < 20 kJ/mol). We suggest that the actual length of the hydrophobic anchor region is limiting for successful insertion of the 4N mutant and that parts of the hydrophilic tail would be located in the hydrocarbon core region of the membrane as drawn in Figure 8A. Although the hydrophobicity of the anchor region is

Fig. 8. Two different mechanisms for membrane insertion and orientation. (A) The electrophoretic mechanism involves $\Delta\Psi$ and a negatively charged residue to translocate the hydrophilic and negatively charged tail. For a defined orientation the hydrophobicity of the transmembrane region has to be limiting. (B) With the hydrophobic mechanism, orientation of the spontaneously inserting protein is achieved by positively charged residues in one of the tail regions. These residues interact electrostatically with the negatively charged phospholipid head groups and hinder the translocation, independent of a membrane potential.

sufficient, the short length of this region prevents a stable transmembrane conformation.

In the final step of translocation the hydrophilic tails leave the hydrocarbon core to enter the membrane interface or the aqueous *trans* compartment. This is an energetically favoured process and the estimated free energy gain from the bilayer to water is between $\Delta G^\circ = -82$ and -128 kJ/mol for the uncharged or charged tails, respectively. We have previously explored the free energy change experimentally with purified M13 procoat and coat protein into lipid vesicles (Soekarjo *et al.*, 1996). For the double-spanning M13 procoat protein the partition coefficient into lipid vesicles corresponded to a ΔG° value of -43 kJ/mol. Other attempts to determine the free energy of membrane insertion experimentally were performed with polyalanine fused to bovine pancreatic trypsin inhibitor (Moll and Thompson, 1994) and of a 36 residue fragment of bacteriorhodopsin (Hunt *et al.*, 1997). They estimated a change in the free energy to $\Delta G^\circ = -21$ and -25 kJ/mol, respectively. Our assumptions on the energy costs of the inserting peptide bonds and charged groups are still too hypothetical to allow a detailed quantitative analysis of the entire translocation process.

Electrophoretic contribution to protein insertion

The second mechanism of Pf3 coat protein insertion uses the electrochemical membrane potential as an additional energy source for translocation. The presence of a single negatively charged amino acid residue close to the hydrophobic region was sufficient for efficient membrane insertion. The negatively charged residue positioned at residue 7 or 18 in the N-terminal portion resulted in the translocation of the N-terminal region, whereas negatively charged residues at 37 or 40 led to the translocation of the C-terminal tail. The electrophoretic movement can be explained by the force on a charge in an electrical field. One negatively charged aspartyl residue is moved by the Coulomb energy to the positively charged periplasmic side. With the membrane potential across the *E. coli* membrane being $\Delta\Psi = -0.2$ V, the energy of one charged residue can be calculated as $\Delta\Psi F \approx 20$ kJ/mol, where F is the Faraday constant. Together with the hydrophobic partitioning this contributes to the transfer of the charged tail across the bilayer and to overcoming the energy

barrier, which includes a positive intramembrane potential (Flewelling and Hubbell, 1986; Clarke, 1997). For the wild-type Pf3 protein this Coulomb energy can provide the additional energy required for the translocation of the N-terminal region.

The membrane potential retains the Pf3 coat protein in a transmembrane state

The electrochemical potential is not only used kinetically, i.e. as an electrophoretic driving force, but also thermodynamically, to keep the Pf3 coat protein in a transmembrane state. We observed that the N-terminal hydrophilic region of the transmembrane Pf3 coat protein 18D partially falls back into the cytoplasm if the membrane potential is destroyed by CCCP (Figure 4). This suggests that the membrane potential holds the protein permanently in the transmembrane conformation and that the hydrophobicity alone is not sufficient to keep the protein transmembrane. This feature might be quite specific for the Pf3 coat protein since in the biological cycle of the phage-infected cell the protein has to leave the membrane to become assembled into phage particles. A tight anchoring of the coat protein in the membrane might therefore hinder the assembly of the phage. Backsliding of a translocated N-terminal tail has been observed with a leader peptidase mutant (Delgado-Partin and Dalbey, 1998) and might also occur in other proteins.

Orientation of the Pf3 coat protein can be achieved by two mechanisms

Interestingly, the helix dipole of the translocating protein responds to the membrane potential to a very minor degree. If the orientation of the uncharged 3L-4N mutant is analysed, the proportion of the N-terminal versus C-terminal translocation is about equal. Even in the presence of a membrane potential the two orientations were found about equally. This implies that the helix dipole cannot define the orientation of a membrane protein, but that it requires flanking charged residues to assure the correct orientation. In essence, orientation can be achieved in two distinct ways (Figure 8), either by the active movement of one negatively charged tail powered by the electrical gradient across the membrane or, passively, by preventing the movement of a positively charged tail due

to the electrostatic interactions with the phospholipid head groups at the *cis* side of the membrane (Gallusser and Kuhn, 1990; van Klompenburg *et al.*, 1997; van de Vossenberg *et al.*, 1998). The results obtained with the 3L-4N mutant prove that hydrophobicity is the major driving force for spontaneous membrane insertion and no charged residue is required for translocation. As expected, the hydrophobicity does not determine the orientation of the protein and which of the tails is translocated. We observed that in 3L-4N about equal portions had translocated the N-terminus or C-terminus into the INV regardless of an electrochemical membrane potential. Even in case of a negatively charged tail (3L-7D18D) no orientational preference was observed. We would expect the same for a mutant with negatively charged C-terminal tail. These findings may explain why exceptions to the 'positive inside rule' exist. For example, a mutant of the M13 procoat protein with four positively charged residues in the periplasmic loop region efficiently translocated across the membrane in the correct orientation (Kuhn *et al.*, 1990). The strong hydrophobicity of the two transmembrane anchors of the procoat protein can overcome the repulsive force of the membrane potential (positive outside) acting on the positively charged residues. Detailed studies with the M13 procoat protein verified that the membrane potential has only a small inhibitory effect on the translocation of positively charged residues and only becomes evident if the hydrophobicity and length of the anchor regions have been reduced (Schuenemann *et al.*, 1999).

It is conceivable that other membrane proteins use the membrane potential and a negatively charged residue for orientation. The analysis of the charge bias of bacterial membrane proteins by Wallin and von Heijne (1998) showed that there is a low preference for negatively charged residues in periplasmic regions which could have a topological function. For the mitochondrial Oxa-1 protein it was shown that negatively charged residues are essential for membrane insertion (Rojo *et al.*, 1999). In addition, a reversible orientation of a membrane protein in response to the membrane potential might provide a useful biological switch. A reversible membrane topology has recently been described for the SecE protein and it was shown that the topology of SecE was affected when the glutamic acid residue was mutated to an arginine (Nishiyama *et al.*, 1996).

Materials and methods

Strains and plasmids

Escherichia coli strain BL21(DE3)*lysS* (Studier *et al.*, 1990) was used for the *in vivo* experiments, *E. coli* strain MRE600 (DSM3901) was used for the preparation of the *in vitro* S-135 high speed extracts and the inner membrane vesicles. The Pf3 mutants were cloned into the pT7-7 vector as described (Kiefer *et al.*, 1997) and the pET3-lamB expression vector was a kind gift from J. Rosenbusch.

Mutagenesis

Site-directed mutagenesis was performed on the plasmid DNA with two complementary mutagenic 30mers by 16 polymerization cycles with 2.5 U Pwo DNA polymerase. The parental DNA was digested with 7 U *DpnI* for 2 h at 37°C and the sample was subsequently transformed into highly competent cells. The mutants were identified by DNA sequencing. All Pf3 coat mutants used in this study contained a C-terminal epitope tag of seven neutral amino acid residues (LLHPVQL; see Figure 3),

which replaced the penultimate phenylalanyl residue of the natural Pf3 coat protein sequence. Due to the fact that the original C-terminal Pf3 tail is protease resistant this short extension was necessary to perform the C-terminal protease digestion assays (Kiefer *et al.*, 1997).

In vitro translation and translocation

The inner membrane vesicles (INVs) and high speed cell extracts were prepared according to Müller and Blobel (1984), except that HEPES buffer pH 8.0 was used throughout the whole procedure instead of triethanolamine acetate buffer. Where indicated, INVs were treated with either trypsin or urea basically according to Swidersky *et al.* (1990). Treatment of the INVs with 250 µg/ml trypsin was performed for 60 min on ice, the protease was inhibited with 0.5 mM PMSF and the INVs were collected by ultracentrifugation at 130 000 g for 10 min on a 500 mM sucrose cushion in the Beckman airfuge. The pelleted INVs were resuspended in the same volume of a buffer containing 250 mM sucrose, 50 mM HEPES pH 7.5 and 1 mM dithiothreitol. Urea treatment of the vesicles was performed accordingly with 5 M urea for 60 min on ice. *In vitro* translation was performed as described (Kiefer *et al.*, 1997) by programming a high speed *E. coli* supernatant with *in vitro* transcribed mRNA (Promega Riboprobe T7 transcription kit) for the respective protein. Translation was incubated at 37°C for 30 min. For translocation experiments INVs (~20–25 µg total protein) were added cotranslationally. Where indicated, 100 µM CCCP (final concentration) was added to the cell extract 3 min before the addition of INVs. Translation reactions were started by adding the radioactive amino acid and stopped by the addition of 5% trichloroacetic acid (TCA) on ice. Protease protection essays were performed by the addition of 0.5 mg/ml proteinase K (Sigma) to the *in vitro* samples and incubation at 25°C for 30 min. Proteolysis was stopped by the addition of 2 mM PMSF and 5% TCA on ice.

In vivo protease mapping and topology analysis

Escherichia coli BL21(DE3)*lysS* cells were transformed with the respective pT7 plasmid coding for the various Pf3 mutant proteins. Cells (500 µl) were grown at 37°C in minimal medium without methionine to the mid-log phase, induced with 1 mM isopropyl-β-D-thiogalactopyranoside for Pf3 coat protein synthesis for 10 min and labelled with 50 µCi [³⁵S]methionine for 3 min. Cells were chased with 50 µg cold L-methionine and 150 µl were withdrawn, chilled on ice, converted to spheroplasts and analysed by protease mapping with 1 mg/ml proteinase K as described (Kiefer *et al.*, 1997). The remaining culture was divided into two and further incubated for 5 min at 37°C. One half received 40 µM CCCP [100 mM stock solution in dimethylsulfoxide (DMSO)], the other half was the control receiving only DMSO. Samples were then chilled on ice and subjected to protease mapping. All samples were immunoprecipitated by a Pf3 antibody and analysed by urea-SDS-PAGE.

Membrane potential measurements

Oxonol VI (Molecular Probes, Eugene, OR) was used as a fluorescent probe to monitor the membrane potential in the *E. coli* inverted membrane vesicles (INV). Fluorescence measurements were performed in a JASCO FP-750 instrument with an excitation wavelength of 599 nm and an emission wavelength of 634 nm. Samples were in 10 mM HEPES pH 8.0, 200 mM KCl, 10 mM MgCl₂ and contained 80 µg/ml (protein content) INV, 2 µM oxonol VI (1 mM stock solution in DMSO), 2.5 mM ATP, 1 µM nigericin (1 mM stock solution in methanol) and valinomycin (1 mM stock solution in ethanol), respectively. Measurements were carried out at 25°C.

Determination of standard free energy of translocation

The standard free energy to transfer a protein region into the membrane bilayer, $\Delta G_{\text{TL}}^{\circ}$ values, were calculated using a modified GES scale (Engelman *et al.*, 1986) which takes the free energy cost of the insertion of the peptide bonds into account. The free energy to translocate a peptide bond was taken as 5 kJ/mol (Ben-Tal *et al.*, 1996; White and Wimley, 1999). The free energy to translocate a peptide-bonded residue is $\Delta G_{\text{TL}}^{\circ} = \Delta G_{\text{GES}}^{\circ} + 5$. The values in kJ/mol are: A: -1.7; D: 43.5; F: -10.5; G: 0.8; H: 17.6; I: -8; K: 41.8; L: -6.7; M: -9.2; N: 25.1; P: 5.8; Q: 22.2; R: 56.5; S: 2.5; T: 0; V: -5.9; W: -3. The free energy to translocate a terminal amino group and carboxyl group was estimated as 21 and 18 kJ/mol, respectively (Engelman *et al.*, 1986).

Quantification of translocation efficiency

The proteins were separated on 22% SDS-acrylamide gels containing 5 M urea, the gels were dried, exposed on a standard phosphorimager plate usually overnight and quantified on a Fuji BAS1500 BioImager

using the AIDA software (Raytest). For ^3H -labelled proteins the gels were blotted onto a nitrocellulose membrane and the blot was exposed for 3–5 days on a tritium imaging plate and quantified. The translocation rate was calculated from the ratio of the protease-protected material to the total translation product. Experiments were repeated two or three times.

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References

- Andersson, H. and von Heijne, G. (1994) Positively charged residues influence the degree of SecA dependence in protein translocation across the *E. coli* inner membrane. *FEBS Lett.*, **347**, 169–172.
- Ben-Tal, N., Ben-Shaul, A., Nicholls, A. and Honig, B. (1996) Free-energy determinants of α helix insertion into lipid bilayers. *Biophys. J.*, **70**, 1803–1812.
- Cao, G., Kuhn, A. and Dalbey, R.E. (1995) The translocation of negatively charged residues across the membrane is driven by the electrochemical potential—Evidence for an electrophoresis-like membrane transfer mechanism. *EMBO J.*, **14**, 866–875.
- Clarke, R.J. (1997) Effect of lipid structure on the dipole potential of phosphatidylcholine bilayers. *Biochim. Biophys. Acta*, **1327**, 269–278.
- Dalbey, R. (1990) Positively charged residues are important determinants of membrane protein topology. *Trends Biochem. Sci.*, **15**, 253–257.
- Dalbey, R.E., Kuhn, A. and von Heijne, G. (1995) Directionality in protein translocation across membranes: the N-tail phenomenon. *Trends Cell Biol.*, **5**, 380–383.
- Delgado-Partin, V.M. and Dalbey, R.E. (1998) The proton motive force, acting on acidic residues, promotes translocation of amino-terminal domains of membrane proteins when the hydrophobicity of the translocation signal is low. *J. Biol. Chem.*, **273**, 9927–9934.
- Engelman, D.M., Steitz, T.A. and Goldmann, A. (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.*, **15**, 321–353.
- Flewell, R.F. and Hubbell, W.L. (1986) The membrane dipole potential in a total membrane potential mode. Applications to hydrophobic ion interactions with membranes. *Biophys. J.*, **49**, 541–552.
- Gallusser, A. and Kuhn, A. (1990) Initial steps in protein membrane insertion. Bacteriophage M13 procoat protein binds to the membrane surface by electrostatic interaction. *EMBO J.*, **9**, 2723–2729.
- Hunt, J.F., Rath, P., Rothschild, K.J. and Engelman, D.M. (1997) Spontaneous, pH-dependent membrane insertion of a transbilayer α -helix. *Biochemistry*, **36**, 15156–15176.
- Kiefer, D., Hu, X., Dalbey, R. and Kuhn, A. (1997) Negatively charged amino acid residues play an active role in orienting the Sec-independent Pf3 coat protein in the *E. coli* membrane. *EMBO J.*, **16**, 2197–2204.
- Kuhn, A. (1995) Bacteriophage Pf3 and M13 as model system for Sec-independent protein transport. *FEMS Microbiol. Rev.*, **17**, 185–190.
- Kuhn, A., Zhu, H.-Y. and Dalbey, R.E. (1990) Efficient translocation of positively charged residues of M13 procoat protein across the membrane excludes electrophoresis as the primary force for membrane insertion. *EMBO J.*, **9**, 2385–2388.
- Kusters, R., Breukink, E., Gallusser, A., Kuhn, A. and de Kruijff, B. (1994) A dual role for phosphatidylglycerol in protein translocation across the *Escherichia coli* inner membrane. *J. Biol. Chem.*, **269**, 1560–1563.
- Moll, T.S. and Thompson, T.E. (1994) Semisynthetic proteins: model systems for the study of the insertion of hydrophobic peptides into preformed lipid bilayers. *Biochemistry*, **33**, 15469–15482.
- Müller, M. and Blobel, G. (1984) *In vitro* translocation of bacterial proteins across the plasma membrane of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **81**, 7421–7425.
- Nishiyama, K., Suzuki, T. and Tokuda, H. (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. *Cell*, **85**, 71–81.
- Rohrer, J. and Kuhn, A. (1990) The function of a leader peptide in translocating charged amino acyl residues across a membrane. *Science*, **250**, 1418–1421.
- Rojo, E.E., Guiard, B., Neupert, W. and Stuart, R.A. (1999) N-terminal tail export from the mitochondrial matrix. Adherence to the prokaryotic 'positive-inside' rule of membrane protein topology. *J. Biol. Chem.*, **274**, 19617–19622.
- Schuenemann, T.A., Delgado-Nixon, V.M. and Dalbey, R.E. (1999) Direct evidence that the proton motive force inhibits membrane translocation of positively charged residues within membrane proteins. *J. Biol. Chem.*, **274**, 6855–6864.
- Soekarjo, M., Eisenhawer, M., Kuhn, A. and Vogel, H. (1996) Thermodynamics of the membrane insertion process of the M13 procoat protein, a lipid bilayer traversing protein containing a leader sequence. *Biochemistry*, **35**, 1232–1241.
- Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.*, **185**, 60–89.
- Swidersky, U.E., Hoffschulte, H.K. and Müller, M. (1990) Determinants of membrane-targeting and transmembrane translocation during bacterial protein export. *EMBO J.*, **9**, 1777–1785.
- Thiaudiere, E., Soekarjo, M., Kuchinka, E., Kuhn, A. and Vogel, H. (1993) Structural characterization of membrane insertion of M13 procoat, M13 coat and Pf3 coat proteins. *Biochemistry*, **32**, 12186–12196.
- van de Vossenberg, J.L.C.M., Albers, S.-V., van der Does, C., Driessen, A.J.M. and van Klompenburg, W. (1998) The positive inside rule is not determined by the polarity of the $\Delta\psi$. *Mol. Microbiol.*, **29**, 1125–1127.
- van Klompenburg, W., Nilsson, I., von Heijne, G. and de Kruijff, B. (1997) Anionic phospholipids are determinants of membrane protein topology. *EMBO J.*, **16**, 4261–4266.
- von Heijne, G. (1989) Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature*, **341**, 456–458.
- Wallin, E. and von Heijne, G. (1998) Genome-wide analysis of integral membrane proteins from eubacterial, archaean and eukaryotic organisms. *Protein Sci.*, **7**, 1029–1038.
- White, S.H. and Wimley, W.C. (1998) Hydrophobic interaction of peptides with membrane interfaces. *Biochim. Biophys. Acta*, **1376**, 339–352.
- White, S.H. and Wimley, W.C. (1999) Membrane protein folding and stability: physical principles. *Annu. Rev. Biophys. Biomol. Struct.*, **28**, 319–365.

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