The Active Protein-conducting Channel of *Escherichia coli* Contains an Apolar Patch

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Protein translocation across the cytoplasmic membrane of *Escherichia coli* is mediated by translocase, a complex of a protein-conducting channel, SecYEG, and a peripheral motor domain, SecA. SecYEG has been proposed to constitute an aqueous path for proteins to pass the membrane in an unfolded state. To probe the solvation state of the active channel, the polarity sensitive fluorophore N-(2-(iodoacetoxy)ethyl)-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole was introduced at specific positions in the C-terminal region of the secretory protein proOmpA. Fluorescence measurements with defined proOmpA-DHFR translocation intermediates indicate mostly a water-exposed environment with a hydrophobic region in the center of the channel.

Translocase is a protein complex that mediates the secretion of preproteins across the bacterial cytoplasmic membrane. It consists of the membrane-integral heterotrimeric SecYEG complex (1), which forms a transmembrane protein-conducting channel (PCC), and the peripherally associated cytosolic ATPase SecA (2, 3), which functions as a motor protein. The PCC is evolutionarily conserved and is found in all domains of life. It mediates both the transport of secretory proteins across the membrane, as well as the insertion of membrane proteins into the membrane (4). Secretory proteins are synthesized as precursors (preproteins) with a characteristic N-terminal signal sequence that is recognized by translocase (5). Preproteins are kept translocation-competent during synthesis (6) and are targeted to the PCC-bound SecA (7–9) by the cytosolic chaperone SecB (10). Successive rounds of binding and hydrolysis of ATP by SecA result in the stepwise translocation of the unfolded preprotein through the PCC (11–13). In addition, the proton motive force facilitates translocation by an unknown mechanism (11, 14).

A critical requirement for the PCC is to maintain the membrane barrier for ions and other small solutes both under idle and active conditions. This is particularly important in bacteria and Archaea as the cytoplasmic membrane plays a key role in proton and sodium ion-based energy transduction. Only recently, an understanding is emerging how the membrane integrity is preserved during protein translocation. Cryo-electron microscopy study indicates that the active PCC bound to the ribosome consists of a dimer of SecYEG (15, 16), whereas both structural and biochemical studies indicate an oligomeric, possibly dimeric, assembly of the SecA-bound PCC (17–19). X-ray structural analysis indicates that the SecY protein of a single SecYEG complex has a clamshell-like shape that encompasses a central channel. The channel in this monomeric complex is constricted and closed at the center by a ring of hydrophobic amino acid residues, termed the pore. In addition, a periplasmic re-entrance loop (TM2a) closes the pore at the extracellular side like a plug. The pore and the plug domains have been proposed to seal the channel under idle conditions (20).

The plug is a flexible structure that can be cross-linked to SecE at the periplasmic membrane interface peripheral to the translocation pore (21). It has been proposed that the channel is opened upon the insertion of the signal sequence forming a wedge at the membrane lateral opening of the clamshell. The recently proposed peristalsis model suggests that channel opening is induced by SecA (or the ribosome) rather than the signal sequence (22). In this model, SecA controls the juxtapositioning of two SecYEG protomers of a dimeric complex with their lateral gates facing each other (15, 16). In either model, the enlargement of the pore will result in a destabilization of the plug, which is subsequently displaced, enabling the vectorial membrane passage of unfolded preproteins. Biochemical studies indeed show that the plug domain is more readily cross-linked to SecE in the presence of a proOmpA preprotein (23). However, the role of the plug as a sealing entity seems questionable, as it is not essential for viability and preprotein translocation (24, 25). Recent evidence suggests that in the absence of a plug, other periplasmic loops of SecY substitute for the plug domain to seal the periplasmic exit site (26).

Cross-linking experiments have shown that a ribosome-bound proOmpA translocation intermediate preferentially localizes in the neighborhood of pore residues at the center of the PCC, while minimizing contact with the remainder of the structure (27). A close association of the hydrophobic pore with the preprotein may provide a way to prevent ions and solutes from leaking through the channel (1, 20, 27). Indeed, in a recent
been attributed to the chaperone BiP (32), whereas at the cytoplasmic reticulum membranes argue that the channel is hydrophilic throughout (29, 30). Moreover, fluorophores positioned along the PCC spanning part of the nascent polypeptide chain (28). However, the presence of a functional hydrophobic constriction in the translocation channel in its active state remains to be demonstrated experimentally, because previous constrictions throughout the channel. Our results demonstrate that a hydrophobic locality exists in the active PCC that corresponds to the central region of the translocation channel.

E. coli DH5α
E. coli SF100
pET610
pET2311
pET2520
pET401
pEK27
pET75
pET77
pET78
pET79
pET80
pET81
pET82
pEK501
pEK502
pEK503
pEK504
pEK505
pEK506
pEK507
pEK508
pEK509
pEK510
pEK511
pEK522

| Strain/plasmid       | Characteristic                              | Source                        |
|----------------------|---------------------------------------------|-------------------------------|
| E. coli DH5α         | supE44, ΔlacI169 (Δ80lacZam15) hisD17, recA1, endA1, gyrA96 thi-1, relA1 | 54                            |
| E. coli SF100         | F′, ΔlacX74, gale, gaiK, thi, rpsL, strA ΔproA(pruvU), ampR T             | 55                            |
| pET610               | SecYiEG                                      | 56                            |
| pET2311              | SecYiN(G67C)EG; derived from pET610          | J. de Keyzer, unpublished data|
| pET2520              | SecYiN(S282C)EG; derived from pET607         | E. O. van der Sluis, unpublished data|
| pET401               | DHFR(C7S)                                    | E. O. van der Sluis, unpublished data|
| pEK27                | proOmpA(S66C,C290S,C302S)-DHFR              | This study                    |
| pET75                | proOmpA(S245C,C290S,C302S)-DHFR              | This study                    |
| pET77                | proOmpA(S266C,C290S,C302S)-DHFR              | This study                    |
| pET78                | proOmpA(S275C,C290S,C302S)-DHFR              | This study                    |
| pET79                | proOmpA(C302S)-DHFR                          | This study                    |
| pET80                | proOmpA(C290S)-DHFR                          | This study                    |
| pET81                | proOmpA(C302S)-DHFR                          | This study                    |
| pET82                | proOmpA(A315C,C290S,C302S)-DHFR              | This study                    |
| pEK501               | proOmpA(S245C,C290S,C302S)-DHFR(C334S); derived from pET75 | This study                    |
| pEK502               | proOmpA(S266C,C290S,C302S)-DHFR(C334S); derived from pET76 | This study                    |
| pEK503               | proOmpA(S275C,C290S,C302S)-DHFR(C334S); derived from pET77 | This study                    |
| pEK504               | proOmpA(S282C,C290S,C302S)-DHFR(C334S); derived from pET78 | This study                    |
| pEK505               | proOmpA(C302S)-DHFR(C334S); derived from pET80 | This study                    |
| pEK506               | proOmpA(C290S)-DHFR(C334S); derived from pET81 | This study                    |
| pEK507               | proOmpA(G315C,C290S,C302S)-DHFR(C334S); derived from pET79 | This study                    |
| pEK508               | proOmpA(C290S,C302S)-DHFR(C334S); derived from pET82 | This study                    |
| pEK509               | proOmpA(C290S,C302S,C305S)-DHFR(C334S); derived from pET83 | This study                    |
| pEK510               | proOmpA(C290S,C302S,C305S)-DHFR(C334S); derived from pET84 | This study                    |
| pEK511               | proOmpA(C290S,C302S,C305S)-DHFR(C334S); derived from pET85 | This study                    |
| pEK522               | proOmpA(C290S,C302S,C305S)-DHFR(C334S); derived from pET86 | This study                    |

Materials—SecA (39), SecB (40), and proOmpA-DHFR (37, 38, 41) were overexpressed, isolated, and purified as described. Overexpression of SecYEG and subsequent isolation of inverted inner membrane vesicles (IMVs) were done essentially as described (42). Chemicals used were reagent grade or better. Premixed acrylamide/bisacrylamide 37.5:1 was from Bio-Rad. N-(2-(Iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole, Oregon Green 488 maleimide, fluorescein-5-maleimide, and tris-(2-carboxyethyl)phosphine hydrochloride were from Invitrogen. Absolute ethanol and protease K were from Merck. 1,4-Dithiothreitol (DTT) was from Roche Applied Science. 4-Amino-N20-methylpyrrolo-[1-glutamic acid (methotrexate), β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (β-NADPH), nucleotides, and sodium tetrathionate dihydrate (Na2S4O6) were from Sigma.

SecYEG Channel Polarity

In Escherichia coli, most secretory proteins translocate in a post-translational manner. It is unknown if any of the PCC ligands, such as SecA, contribute to sealing of the translocation channel. Previous studies have demonstrated that disulfide-bridged loops of up to 13 amino acids introduced into the preprotein proOmpA do not block translocation provided that a proton motive force is present (35, 36). The translocation of such bulky structures requires a high flexibility of the channel, and the pore needs to widen considerably while maintaining a close seal with the preprotein.

To examine the molecular environment of the PCC of E. coli, while active in SecA-mediated post-translational translocation, we have inserted the environmentally sensitive probe 1-thio-N-(2-(iodoacetoxo)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (NBD) at defined positions inside the channel, while conjugated to a proOmpA-DHFR translocation intermediate (37, 38). NBD was conjugated to the protein by means of cysteine-scanning mutagenesis and thiol chemistry. By varying the position of NBD along the chain of the translocation interme-

ExPERIMENTAL PROCEDURES

Materials—SecA (39), SecB (40), and proOmpA-DHFR (37, 38, 41) were overexpressed, isolated, and purified as described. Overexpression of SecYEG and subsequent isolation of inverted inner membrane vesicles (IMVs) were done essentially as described (42). Chemicals used were reagent grade or better. Premixed acrylamide/bisacrylamide 37.5:1 was from Bio-Rad. N-(2-(Iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole, Oregon Green 488 maleimide, fluorescein-5-maleimide, and tris-(2-carboxyethyl)phosphine hydrochloride were from Invitrogen. Absolute ethanol and protease K were from Merck. 1,4-Dithiothreitol (DTT) was from Roche Applied Science. 4-Amino-N20-methylpyrrolo-[1-glutamic acid (methotrexate), β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (β-NADPH), nucleotides, and sodium tetrathionate dihydrate (Na2S4O6) were from Sigma.

Strains and Plasmids—The strains and plasmids used in this study are listed in Table 1. E. coli strain SF100 was used for overexpression of wild-type and mutant SecYEG, which contained a hexahistidine tag at the N terminus of SecY. E. coli strain DH5α was used for overexpression of proOmpA-DHFR. All plasmids contained the gene of interest behind the isopropyl β-D-galactopyranoside-inducible T7 promoter, as well as an ampicillin resistance marker. The positions of proOmpA correspond to the mature protein minus the signal sequence for consistency (38).

ProOmpA-DHFR is a fusion construct of E. coli proOmpA and murine DHFR (43). The latter contained an endogenous cysteine (Cys3) at position Cys334 in the proOmpA-DHFR proteins. This aminoacyl residue was substituted for a serine using
standard cloning techniques. The Sall/HindIII restriction fragment of pET401, containing cysteine-less DHFR, was cloned into vectors pET75-pET82, creating vectors pEK501-pEK508. Mutations I300C, D301C, and L303C were introduced into cysteine-less pEK508, creating pEK509, pEK510, and pEK511, respectively (Table 1).

Fluorescent Labeling of Proteins—ProOmpA-DHFR was denatured in buffer A: 50 mM Tris-HCl, pH 7.4, 8 M urea at a final concentration of 0.1 mg/ml. Thiols were reduced with 25 μM tris-(2-carboxyethyl)phosphine hydrochloride for 30 min at room temperature. Labeling was performed with 250 μM of fluorophore for 30 min (fluorescein, Oregon Green) or 2 h (NBD) at room temperature under constant stirring. Reactions were quenched with 2.5 mM DTT for 10 min at room temperature, and free probe was removed by precipitation of the protein with 10% (w/v) trichloroacetic acid. The protein was resuspended in 1:10 of the labeling volume of buffer A, and aliquots were flash-frozen with liquid nitrogen and stored at −80 °C. Labeling was analyzed by 12% SDS-PAGE and imaged using a Lumi-imager F1 (Roche Applied Science), with a bandpass filter at 520 nm (±20 nm).

**In Vitro Translocation Assay**—The activity of IMVs was analyzed by means of a standard translocation and protease protection assay. ProOmpA-DHFR (37, 38) was used as a substrate, which optionally contained a fluorescent label conjugated to a unique cysteine in the proOmpA moiety. Standard translocation reactions were performed in a total volume of 50 μL. Folding of the DHFR domain was induced by diluting the proOmpA-DHFR (0.5 mg/ml), denatured in buffer A, 25-fold into a buffer containing 50 mM Heps-KOH, pH 7.6, 30 mM KCl, 2 mM DTT, 18 μg/ml SecB, 1 mM NADPH, and 50 μM methotrexate, with a final volume of 25 μL. The mixture was incubated for 5 min on ice, another 5 min at 37 °C, and stored at 4 °C. The folding mixture was then added to 25 μL of the translocation buffer and kept at 4 °C. The translocation buffer contained 50 mM Heps-KOH, pH 7.6, 30 mM KCl, 5 mM MgCl2, 10 mM DTT, 0.5 mg/ml bovine serum albumin, 200 μg/ml IMVs, 20 μg/ml SecA, 18 μg/ml SecB, 1 mM NADPH and 50 μM methotrexate and optionally 20 mM creatine phosphate and 20 μg/ml creatine kinase. Typically, 0.5 μg of proOmpA-DHFR was used in the translocation assay. Reactions were started by the addition of 2 mM of nucleotide in 100 mM Tris-HCl, pH 8.0, and the mixture was incubated for 30 min at 37 °C. Thereafter, when indicated, samples were digested for 15 min with 0.1 mg/ml protease K on ice, and proteolysis was terminated by the addition of 10% trichloroacetic acid and further incubation for 30 min at 4 °C. Samples were centrifuged in a table-top centrifuge for 30 min at 4 °C, washed with ice-cold acetone, and resuspended in SDS sample buffer. After 12% SDS-PAGE, labeled protein was visualized in gel with a Lumi-imager F1 (Roche Applied Science), using a bandpass filter at 520 nm.

**Fluorescence Assay**—Spectrophotometric assays were performed in a total volume of 150 μL, by scaling up the standard translocation reaction three times but leaving out the energy-regenerating system of creatine kinase/creatine phosphate. The reaction mixture was incubated for 5 min, and started by the addition of 2 mM nucleotide in 100 mM Tris-HCl, pH 8.0. The reaction was followed in time for 10 min on an Aminco-Bowman series II spectrophotometer. Measurements were performed at 37 °C, using a 4 nm slit width. F/Fo was calculated by dividing the average emission intensity after addition of nucleotide by the average intensity before addition of nucleotide at 530 nm (±2 nm), both after subtraction of the cysteine-less sample.

**Disulfide Cross-link Assay**—The standard translocation reaction was scaled up five times and performed in a total volume of 250 μL, in the absence of DTT. After translocation for 30 min at 37 °C, the reactions were cross-linked by the addition of 0.5 mM tetraethionate or received 10 mM DTT as a control. Incubation was for 30 min at 37 °C. To remove nontranslocated preprotein, the reaction mixture was centrifuged through 200 μL of buffer B: 50 mM Tris-HCl, pH 8.0, 0.2 M sucrose, 50 mM KCl, 1 mM NADPH and 50 μM methotrexate in a Beckman Airfuge at 210,000 × g for 30 min at room temperature. The pellet was resuspended into nonreducing SDS-PAGE sample buffer, separated on 7.5% SDS-PAGE, and blotted according to standard laboratory techniques, using a Bio-Rad semi-dry blotting system. Blots were developed with polyclonal antibodies against proOmpA or SecY and an alkaline phosphatase assay and imaged on a Lumi-imager F1 (Roche Applied Science).

**Miscellaneous**—Protein determinations were performed by means of the Lowry method, using the Protein DC assay (Bio-Rad) and bovine serum albumin as a standard. The enzyme activity of DHFR was measured using a spectrophotometric NADPH oxidation assay (37).

**RESULTS**

Overexpression of ProOmpA-DHFR and Labeling of Cysteines—To generate in vitro defined translocation intermediates trapped inside the PCC, a fusion protein of proOmpA with DHFR was used as a model secretory protein. Upon the folding of the DHFR moiety, a stable translocation intermediate can be obtained after translocation of the proOmpA-DHFR into inner membrane vesicles (IMVs) (37). In the folded DHFR, the endogenous cysteine (Cys7) is not accessible to fluorescent maleimides but becomes readily labeled upon urea denaturation of the protein. Because labeling of this position interferes with folding of DHFR, a proOmpA–DHFR mutant was used in which Cys7 of DHFR was replaced by a serine. The C7S (C334S in the proOmpA–DHFR protein) substitution did not affect the folding properties of the enzyme when refolded from a urea-denatured state, as verified by NADPH oxidation (data not shown) and protease protection (see below). In a previous study, the C7S/S42C/N49C triple mutant of DHFR was reported to be highly sensitive to protease and not able to bind the substrate analog methotrexate (43). Therefore, the mutations at positions 42 and 49 seem to infer the labile phenotype. Next, we introduced unique cysteines in the C-terminal tail of the cysteine-less proOmpA(C290S, C302S) fused to DHFR(C334S), further referred to as cysteine-less proOmpA–DHFR (Fig. 1). This region of proOmpA was previously found to cross-link to SecY when trapped in the PCC as a translocation intermediate (38). Therefore, this region must be in proximity to SecY.

All the proOmpA–DHFR mutants were overexpressed at high levels and purified to homogeneity. To verify the accessi-
bility of the newly introduced cysteines, urea-denatured proteins were labeled with the Oregon Green 488 maleimide (OG) fluorophore. This reagent is very reactive toward thiols and efficiently labeled all the single cysteine proOmpA-DHFR proteins with essentially no signal with the cysteine-less proOmpA-DHFR protein (supplemental Fig. 1, A and B). A second incubation with the spectrally distinct fluorophore Texas Red C_{2}-maleimide indicated a labeling efficiency (>80%) for all the cysteine proteins. Similar results were obtained with the fluorescent dye NBD (supplemental Fig. 1, C and D). These results show that each of the proOmpA-DHFR proteins is labeled efficiently with a fluorophore at specific cysteine positions. In particular with NBD and to a lesser extent with OG, position-specific differences in fluorescence intensity were observed between the proOmpA-DHFR proteins in the gel, whereas the same amount of protein was loaded (supplemental Fig. 1, A–D), and the same extent of labeling was obtained as verified by absorbance measurements. This indicates that in particular NBD is responsive to the local environment of the polypeptide chain in the gel.

Generation of Fluorescent Translocation Intermediates—The fluorescently labeled proOmpA-DHFR proteins were subjected to translocation experiments, using a protease protection assay. In this assay, preproteins translocated into the IMVs become protected against digestion from externally added protease. Because of the presence of a fluorophore, translocated proteins can be visualized directly in gel after SDS-PAGE. When proOmpA-DHFR was first unfolded in urea and subsequently diluted into translocation buffer, the protein was completely translocated in the presence of ATP, yielding protease-protected proOmpA-DHFR at the expected position in the gel, i.e. 58 kDa for the cysteine proteins (Fig. 2A). However, when the DHFR moiety first was allowed to fold by pre-dilution and incubation in a buffer containing SecB and the folding ligands NADPH and methotrexate, it becomes too large to pass the channel and will not translocate to its full length. Under those conditions, the folded DHFR has the additional property that it becomes protease-resistant, and only the junction between proOmpA and folded DHFR is partially protease-accessible (37, 38). Therefore, a proOmpA-DHFR translocation intermediate yields two protein bands on a gel after protease digestion, i.e. at the position of the full-length proOmpA-DHFR preprotein (58 kDa) and as protected proOmpA (37 kDa, I_{27}) (Fig. 2C) (37). With all fluorescent proOmpA-DHFR proteins, the level of translocation intermediate formation was similar, showing that the presence of the label at the different positions does not dramatically alter the translocation efficiency (Fig. 2, C and D).

Next, the conditions resulting in the formation of the fluorescent translocation intermediates were analyzed in more detail. The translocation intermediate was observed only after energizing the system with ATP and not when ADP or AMP-PNP, a nonhydrolyzable ATP analog, were added (Fig. 2E, lanes 2 and 3). The amount of translocation intermediate depended on the level of SecYEG expression in the membranes, with significantly higher levels observed with membranes containing overproduced SecYEG as compared with wild-type IMVs (Fig. 2E, compare lanes 1 and 4). To show that the full-length proOmpA-DHFR protease-protected band (Fig. 2, C and E, lane 1) represents a true translocation intermediate, the preprotein was forced to shift to other intermediate positions. Here, the DHFR domain was first unfolded by removing the folding ligands by means of a centrifugation step through a sucrose cushion (37). Next, the sample was subjected to thermal incubation at 37 °C and mechanically sheared by bath sonication, whereupon the IMVs were protease-treated and analyzed by SDS-PAGE. This treatment resulted in reduced amounts of full-length proOmpA-DHFR and increased levels of proOmpA and OmpA (Fig. 3, lane 5). In addition, distinctive protease-resistant bands appeared that were smaller and larger than proOmpA (Fig. 3) and that were previously indicated as the intermediates I_{26} (13) and I_{46} (37) These data suggest that in the absence of ATP and upon unfolding of the DHFR moiety, the proOmpA-DHFR intermediate can move in position within

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**Figure 1.** Outline of the proOmpA-DHFR fusion constructs. The signal sequence (SS), OmpA, and DHFR moieties are indicated, as well as the positions of the unique cysteines. The β-barrel and C-terminal periplasmic domains of OmpA are indicated separately. Numbering of the residues is without the signal sequence for consistency. The positions that tested positive (+) and negative (−) in a cross-link analysis with SecY and the I_{27} translocation intermediate are indicated (37).

**Figure 2.** Translocation and protease protection of fluorescently labeled proOmpA-DHFR. Translocation of urea-denatured (A) and prefolded OG-labeled proOmpA-DHFR (C). Lanes 1–8, 80% of the protease K-treated sample. B and D, 10% of nonprotease-digested sample as a standard of the reactions shown in A and C. E, nucleotide and SecYEG dependence of the formation of the proOmpA(Cys^{629})-DHFR-OG fluorescent translocation intermediate. Lanes 1–6, 80% of the protease-treated sample, and lane 7, 10% of nondenatured sample as a standard.
the channel. Taken together, these results demonstrate the efficient generation of fluorescently modified translocation intermediates of proOmpA-DHFR in IMVs of E. coli.

**Solvation of Translocation Intermediates**—The polarity-sensitive fluorophore NBD has appreciable fluorescence only in a hydrophobic environment and is almost completely quenched in aqueous solution (45, 46). With all NBD-labeled proOmpA-DHFR proteins, the fluorophore was quenched to background levels when the protein was diluted from urea into an aqueous buffer, both in the presence and absence of SecB (Fig. 4 showing proOmpA(Cys301)-DHFR). Apparently, with none of the positions, the binding of the proOmpA moiety to SecB resulted in a shielding of NBD from the solvent, consistent with the notion that SecB binds only to the β-barrel domain of proOmpA and not to the C terminus (47). The NBD fluorescence increased 7-fold when the protein was diluted into the apolar solvent (99%) ethanol (Fig. 4). These results show that NBD is a sensitive reporter of the polarity of the environment when conjugated to the proOmpA-DHFR preprotein, and that the change of fluorescence intensity can be used as a measure to assess the polarity of the environment.

To analyze the solvation state of the various positions of the PCC-entrapped proOmpA-DHFR translocation intermediate, NBD-labeled proteins were subjected to a translocation experiment. For this purpose, first the translocation of NBD-labeled proOmpA(Cys301) was followed in time in the absence or presence of the folding ligands methotrexate and NADPH. When translocation was initiated with ATP, no change in NBD fluorescence was observed when the unfolded proOmpA-DHFR was used as a substrate (Fig. 5D, line 4). On the other hand, with the methotrexate/NADPH-stabilized proOmpA-DHFR, a substantial NBD fluorescence increase was observed (Fig. 5D, line 1), whereas no change was detectable when ATP was replaced for ADP (line 2) or the nonhydrolyzable ATP analog AMP-PNP (line 3). These data demonstrate that the accumulation of a stable translocation intermediate can be detected fluorescently with NBD and that trapping of the protein results in a altered environment of the NBD probe at position 302. In contrast, when conditions are used that lead to complete translocation, no fluorescence change is observed showing that the fluorescence changes are not because of pre- or post-translocation folding or unfolding.

Next, the NBD fluorescence was recorded before and after the formation of a translocation intermediate labeled at different positions with NBD, and nonsaturating preprotein amounts were used so that the intermediate levels increased linearly with the amount of preprotein added (data not shown). Therefore, the emission intensity change can be normalized relative to the fluorescence intensity of the proOmpA-DHFR preprotein before translocation. The majority of the NBD conjugates tested remained quenched and thus solvent-accessible in the intermediate state (Fig. 5A). Again position 302 showed a
changes in fluorescence (Fig. 5B). Under these conditions, only low levels of intermediate were formed (Fig. 2E). Taken together, these data demonstrate that the positions 300–303 of proOmpA-DHFR experience a change in solvation upon formation of the translocation intermediates. This change corresponds to an increased hydrophobicity of the environment.

Localization of Translocation Intermediates—To determine the position of the aminoacyl residues of the proOmpA-DHFR translocation intermediates within the PCC, an oxidative cross-link approach was employed. Previously, a ribosome-bound proOmpA translocation intermediate could be cross-linked to both the pore and the plug domains (27). Therefore, a SecY mutant was used with a cysteine at position 67 (F67C) in the plug and one with a cysteine at position 282 (S282C) just below the pore. These mutants supported the formation of proOmpA-DHFR translocation intermediates with similar efficiency as with wild-type SecY (data not shown). An efficient cross-link between proOmpA-DHFR and the F67C mutant was observed for positions 300–303 (Fig. 6 showing proOmpA(Cys302)-DHFR). The cross-linked product, which migrated at a position in SDS-PAGE corresponding to a molecular mass of about 125 kDa, was identified with both anti-SecY (Fig. 6, A, lane 6, and B, lanes 2 and 4) and anti-proOmpA antibodies (Fig. 6C, lane 5). Cross-linking was not observed under reducing conditions (Fig. 6, B, lane 1, and C, lane 1) or when no translocation intermediate was formed, i.e. when ATP was omitted (Fig. 6, B, lane 5, and C, lane 4). Because the F67C mutant still contained the endogenous cysteines at positions 329 and 385, a control reaction was performed with IMVs containing overproduced levels of wild-type SecY. With those membranes, no cross-linked products were formed (Fig. 6B, lane 3). A lower efficiency of cross-linking occurred between the proOmpA-DHFR proteins with a unique cysteine at positions 300–303 and Cys282 of SecY (see Fig. 6C, lanes 1 and 2). In the gels, an anti-proOmpA IgG-reactive protein band was observed that was not present when proOmpA-DHFR was reduced. This band corresponds to the oxidized dimeric form of proOmpA-DHFR. Taken together, these data suggest that positions 300–303 of the proOmpA-DHFR translocation intermediate localize near the plug of SecY.
In this study, we have employed proOmpA-DHFR translocation intermediates to probe the polarity of the interior of the PCC. For this purpose, a cysteine-less proOmpA-DHFR derivative was used as a template to introduce unique cysteines into the C-terminal region of proOmpA. By means of a cross-linking approach, this region was shown previously to be in proximity to SecY and SecA when present as a translocation intermediate (38). By the use of thiol chemistry, we have introduced the polarity-sensitive fluorophore NBD at defined positions into the proOmpA-DHFR protein. Because the fluorescence characteristics of this probe are highly sensitive to the polarity of the environment, NBD has been applied successfully to identify water-accessible regions of proteins (46). ProOmpA is a widely used substrate for protein translocation studies, and the protein can be covalently modified with bulky fluorophores (44) or cross-linkers (27) without obstructing translocation. Indeed, the introduction of the NBD fluorophore into proOmpA-DHFR did not affect the translocation ability of the preprotein.

The fluorescence intensity of the NBD fluorophore is responsive to solvation changes of the fluorophore (45). When the proOmpA-DHFR proteins were trapped in the PCC as translocation intermediates, a significant increase in fluorescence occurred when NBD was conjugated to unique cysteine positions 300–303 in the C-terminal domain of proOmpA but not with up- and downstream positions. The NBD fluorophore was almost quenched when the proOmpA-DHFR protein was present in solution, either bound or unbound to SecB, as the signal was barely further reduced by the presence of 40 mM KI.3 With the formation of the translocation intermediate, the fluorescence increased up to 1.6-fold for NBD conjugated to the Cys301. Concomitantly, a blue shift in the emission maximum was observed. In 99% ethanol, the fluorescence increase of NBD-labeled proOmpA-DHFR was 7-fold relative to aqueous buffer, whereas an up to 12-fold increase has been reported for NBD in the lipid bilayer (46) or 100% ethanol (45). The fluorescence increase, however, is not linear with the hydrophobicity of the environment. The efficiency of the formation of translocation intermediates is about 20% for the total added proOmpA-DHFR. Therefore, the recalculated fluorescence increase for the active fraction is about 4-fold. This implies that NBD bound to proOmpA(Cys303)-DHFR encounters a significant hydrophobic environment inside the PCC, which corresponds to about 80% ethanol (45). The hydrophobic region encountered is at least four consecutive amino acids long but cannot be defined more precisely as the polypeptide chain is likely not completely immobilized as translocation intermediates can move in the pore (11). The secondary structure of the trapped segment is not known, but our data clearly demonstrate that the Cys300–Cys303 segment of the proOmpA-DHFR intermediate is surrounded by a hydrophobic environment of the PCC.

To determine the location of the Cys300–Cys303 segment of the proOmpA-DHFR inside the PCC, we use an oxidative cross-linking method. A relatively strong cross-link could be demonstrated between Cys302 of proOmpA-DHFR and a cysteine at position 67 of SecY. This position is part of TM2a, the re-entrance loop that has been proposed to function as a plug at the periplasmic face of the membrane (20). This mutant is also known as PrlA3, which is one of weaker Prl mutants that suppresses signal sequence defects in preproteins (48). Oxidative cross-link studies have shown that the plug moves toward SecE at the periplasmic membrane interface (21, 23). However, a recent molecular dynamics simulation suggest that the plug may not be nearly as far displaced upon protein translocation and remains near the channel center (28). In this respect, when NBD is directly conjugated to Cys97, it records a highly hydrophobic environment, whereas it becomes partially quenched upon the insertion of a translocation intermediate.3 This indicates that the plug normally localizes to the hydrophobic environment of the pore and becomes more water-accessible in the open state.

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3 R. Bol and A. J. M. Driessen, unpublished results.
In a previous study on the polarity of the PCC, using ribosome-bound nascent preproteins labeled with NBD that were translocated into microsomes, it was concluded that the Sec61 translocon forms an entirely water-filled channel (29, 31), which is sealed at the trans side by BiP and at the cis side by the ribosome (30, 32–34). Our data confirm that most of the positions along the PCC-entrapped proOmpA-DHFR translocation intermediate are indeed accessible to water. However, a polypeptide segment around position 302 of the proOmpA-DHFR intermediate encounters a hydrophobic patch that maps to the plug near the central region of the channel (Fig. 7B). This is consistent with structural information showing that the pore and plug domains are hydrophobic entities of the translocation channel. Yet studies show that the PCC remains active in vitro even when the plug domain is fixed into a permanent open state through cross-linking (23) or even by complete removal (24, 25). However, recent studies suggest that in the absence of the plug domain other loops substitute for the plug (26) suggesting a more pivotal role of the plug region in sealing the channel.

The positions 298–303 of proOmpA include a short hydrophobic segment in the C terminus (Fig. 7A). Even more pronounced segments (Fig. 7A, H1 and H2) tend to stall the prehydrophobic region of the PCC.

In conclusion, our studies demonstrate that the active PCC, containing a preprotein substrate, does not form a continuous water-filled pathway. It contains a hydrophobic patch in the central region that contacts preproteins during membrane transit. These observations lend further support for the hypothesis that the ion seal during translocation is provided by a tight alignment of the translocating polypeptide with the central hydrophobic constriction of the channel.

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