The SecYEG translocon of *Escherichia coli* mediates the translocation of preproteins across the cytoplasmic membrane. Here, we have examined the role of the proposed lateral gate of the translocon in translocation. A dual cysteine cross-linking approach allowed the introduction of cross-linker arms of various lengths between adjoining aminoacyl positions of transmembrane segments 2b and 7 of the lateral gate. Oxidation and short spacer linkers that fix the gate in the closed state abolished preprotein translocation, whereas long spacer linkers support translocation. The cross-linking data further suggests that SecYEG lateral gate opening and activation of the SecA ATPase are coupled processes. It is concluded that lateral gate opening is a critical step during SecA-dependent protein translocation.

Translocation of preproteins across the cytoplasmic membrane in *Escherichia coli* is mediated by the Sec translocon (for a recent review see Ref. 1). Preproteins targeted for secretion contain a signal sequence that is removed upon translocation. Their synthesis and translocation are uncoupled events (2), and directly after synthesis at the ribosomes, preproteins are targeted post-translationally to the Sec translocon by the molecular chaperone SecB (3). SecB transfers the preprotein to the motor protein SecA bound at the SecYEG pore complex (4, 5). SecA utilizes cycles of ATP binding and hydrolysis to bind and release the translocating protein resulting in its stepwise translocation across the membrane (6–8). In addition, the proton motive force facilitates translocation when the preprotein is released by SecA (6, 9). Various models for SecA-mediated translocation have been proposed wherein SecA functions as a power-stroke device (10) or as a directed molecular ratchet (11). Cryoelectron microscopy of the *E. coli* SecYEG complex bound to a translating ribosome (18) suggests that the ribosome-bound SecYEG is organized as a dimer with a front-to-front organization (18). It was proposed that individual pores of the dimer have distinct functions in protein translocation, i.e. vectorial protein translocation and lateral release of TMs into the membrane (19). Freeze-fracture rotational shadowing electron microscopy has provided evidence for oligomeric forms of SecYEG, and suggest that SecA recruits SecYEG monomers to form a dimeric complex (20). Within this dimeric SecYEG complex, only a single pore seems sufficient for translocation of preproteins (10).

The mechanism by which the translocase coordinates protein translocation is only poorly understood. SecA has been proposed to insert the signal sequence into the SecYEG pore where it may latch between TM2b and TM7 of the SecY lateral gate. This would result in a widening of the central pore constriction and a subsequent displacement of the periplasmic plug domain. Next, adjoining polypeptide segments of the preprotein may enter the opened aqueous pore, but it is not clear if under those conditions the lateral gate remains open or is closed. Despite this vast amount of experimental data available...
SecY Lateral Gate Opening

on the function of the SecYEG complex, the exact role of the putative lateral gate remains unknown. Thus far, the only study on its dynamics and role during translocation concerns a molecular dynamics simulation (21) that does not take SecA or ribosome binding into account. Here we have investigated the function and the dynamics of the proposed lateral gate located between TM2b and TM7 in protein translocation. The data demonstrate that the lateral gate needs to open to allow for SecA-mediated preprotein translocation.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals—Purification of SecA, SecB, proOmpA, and proOmpA-dihydrofolate reductase (DHFR) as well as the isolation of cytoplasmic membrane vesicles (IMVs) containing overproduced levels of SecYEG were performed as described (22). ProOmpA (S245C) was labeled with fluorescein (FL) or Texas Red maleimide (InvitrogenTM) as described (23). The cross-linking reagents bis-maleimidoethane (BMOE) and bis-maleimidohexane (BMH) as well as the reducing agent Tris(2-carboxyethyl)phosphine (TCEP) were obtained from Pierce. Sodium tetrathionate (NaTT) was from Sigma and dibromobimane (bBBr) was from Invitrogen. The reducing agent 1,4-dithiothreitol (DTT) was obtained from Roche Applied Science. Enzymes for DNA manipulation were obtained from Promega, Roche, and Fermentas and all other chemicals were from Sigma.

Bacterial Strains and Plasmids—All strains and plasmids used are listed in Table 1. All DNA manipulations were performed using E. coli DH5α to maintain plasmids and constructs. Cysteine mutations were introduced into a Cys-less SecY using the Stratagene QuikChange® site-directed mutagenesis kit using plasmid pEK1 as template. Mutations were confirmed by sequencing. Plasmids expressing the mutated SecYEG complex were created by exchanging the NcoI-ClaI fragment in pEK20 by the cysteine containing pET651 harboring the F279C mutation, was used as template. Mutations were introduced into SecYEG using the Stratagene QuikChange® kit using plasmid pEK1 as template. Mutations were confirmed by sequencing. Plasmids expressing the mutated SecYEG complex were created by exchanging the NcoI-ClaI fragment in pEK20 by the cysteine containing the NcoI-ClaI fragment of the pEK1 derivative. Plasmid pET651 harboring the F279C mutation, was used as template to generate the F279C/M83C and F279C/I86C double cysteine mutants. E. coli strain SF100 or NN100 (SF100, unc−) was used for overproduction of the various SecYEG complexes.

OmpT was expressed from plasmid pND9 in strain SF100 and expressed under its own temperature-sensitive promoter (24). After overnight incubation at 37 °C, outer membranes containing high levels of OmpT were isolated by differential centrifugation. Briefly, cells were harvested at 7,500 rpm for 15 min in a JLA 10.500 (Beckman) rotor, resuspended in a buffer containing 50 mM Tris-HCl, pH 8, and 1 mM EDTA, whereupon the suspension was passed twice through a cell disruptor at 8,000 p.s.i. Cell debris was removed by centrifuging at 4,000 × g for 15 min in a SS-34 (Beckman) rotor. The supernatant was transferred to a Ti45 ultracentrifuge rotor (Beckman) and centrifuged for 1 h at 40,000 × g and 4 °C. The outer membrane pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 8, and 250 mM sucrose and stored in small aliquots at −80 °C.

Chemical Cross-linking and OmpT Assay—IMVs containing overproduced levels of the SecYEG mutants were diluted to 1 mg/ml, whereupon the cross-linkers NaTT (1 mM), bBBr (1 mM), BMOE (300 μM), or BMH (600 μM) were added, respectively. The reducing agents DTT and TCEP were used at a final concentration of 30 and 5 mM, respectively. For optimal cross-linking, reactions were performed in a maximum volume of 30 μl and afterward pooled for analysis. After 30 min at 37 °C, IMVs were sedimented through a sucrose cushion in 50 mM Tris-HCl, pH 8, and 0.8 M sucrose for 20 min at 80,000 rpm in a TLA 120.1 rotor at 4 °C. The pellet was resuspended in 50 mM Tris-HCl, pH 8, 20% glycerol and used for further reactions.

An OmpT protease assay was used to assess the cross-linking between cysteine residues in TM2b and TM7 of SecY. Outer membranes containing overexpressed levels of OmpT were diluted to a final concentration of 1 mg/ml in 50 mM Tris-HCl, pH 7, 0.1% Triton X-100. The OmpT solution (7.5 μl) was mixed with the cross-linked IMVs (12 μl) and incubated for 30 min at 37 °C. Where indicated, the oxidized cysteine residues were reduced by the addition of an excess DTT (30 mM final concentration), and the samples were separated by 12% SDS-PAGE. SecY was visualized by staining with Cooamassie Brilliant Blue R-250 or SYPRO® Ruby (Invitrogen) where indicated. Visualization with SYPRO Ruby staining was done with a Roche Lumi-Imager F1 station using bond pass filters of 520 (FL) and 600 nm (Texas Red). Western blotting was performed using antibodies directed against the N-terminal hexa-histidine tag in SecY (Amersham Biosciences).

Cross-linking in the Presence of the ProOmpA-DHFR Translocation Intermediate—Folding of the DHFR domain and translocation of the proOmpA-DHFR into IMVs was performed as described (25). After 30 min of translocation at 37 °C, the different cross-linkers were added to the reaction mixture and incubation was continued for another 30 min at 37 °C. To analyze cross-linking of the cysteine residues, IMVs were treated with OmpT as described above.

Translocation ATPase Assay—The SecA ATPase activity during translocation was determined by measuring the amount of released free phosphate using the malachite green assay (26). Typically, proOmpA (33 μg/ml) was added to translocation buffer containing SecA (20 μg/ml), SecB (400 μg/ml), 1 mM ATP and IMVs (12.5 μg/ml). P, release was measured after 30 min. Measurements were done in triplicate and corrected for background ATPase activity in the absence of proOmpA.

Other Techniques—In vitro translocation of proOmpA was performed as described (23). Typically, fluorescently labeled proOmpA was diluted into translocation buffer containing SecA (20 μg/ml), SecB (400 μg/ml), ATP (1 mM), and IMVs containing SecYEG derivatives. After 8 min at 37 °C, the translocation reaction was terminated on ice by treatment with proteinase K, and analyzed by SDS-PAGE and in-gel UV fluorescence using a Roche Lumi-Imager F1 station using bond pass filters of 520 (FL) and 600 nm (Texas Red), respectively. Protein concentrations were determined with the Bio-Rad RC DC protein assay kit using bovine serum albumin as a standard.

RESULTS

Introduction of Cysteines in the Putative Lateral Gate of SecYEG—To date, no direct evidence exist for the functioning of the putative lateral gate between TM2b and TM7 of SecY (17). This gate has been suggested to play a role in channel
opening, release of the signal sequence of translocating proteins, as well as in the insertion of transmembrane helices of nascent integral membrane proteins into the lipid bilayer (17). To investigate the functioning of the lateral gate we identified amino acid positions in TM2b and TM7 in E. coli SecY (Fig. 1A) that based on the M. jannaschii SecYEβ crystal structure (17)
SecY Lateral Gate Opening

TABLE 1
Overview of strains and plasmids used in this study

| Strain/plasmid                               | Relevant characteristic | Source       |
|---------------------------------------------|-------------------------|--------------|
| E. coli DH10a                                | supE44, ΔlacI169 (Δ80lacZ, M15), hisD417, relA1, endA1, gyrA96 thi-1, relA1 | Ref. 39      |
| E. coli SE100                                | F−, ΔlacX74, galE, galK, thi, rpsL, strA, ΔprotA (pvull), ΔompT | Ref. 40      |
| E. coli NN100                                | SF100, unc              | Ref. 41      |
| E. coli BL21 (DE3) Rosetta                   | F−, ompT hsdS(r− m−) gal dcm (DE3) pRARE2 (CamR) | Novagen      |
| pND9                                        | OmpT                    | Ref. 24      |
| pET80                                       | ProOmpA(C290S)-DHR      | Ref. 25      |
| pET36                                       | ProOmpA(S245C)          | F. Bonardi†  |
| pEK1                                        | Cysteine-less SecY      | Ref. 42      |
| pEK20                                       | Cysteine-less SecYEG    | Ref. 42      |
| pET61                                       | SecY(F279C)EG           | This study   |
| pFE-SecY1                                   | SecY(M83C)EG            | This study   |
| pFE-SecY4                                   | SecY(I86C)EG            | This study   |
| pFE-SecY5                                   | SecY(S87C)EG            | This study   |
| pFE-SecY7                                   | SecY(I283C)             | This study   |
| pFE-SecY10                                  | SecY(F286C)EG           | This study   |
| pFE-SecY13                                  | SecY(I283C/S87C)EG      | This study   |
| pFE-SecY16                                  | SecY(F286C/S87C)EG      | This study   |
| pFE-SecY16                                  | SecY(F279C/M83C)EG      | This study   |
| pFE-SecY62                                  | SecY(F279C/I86C)EG      | This study   |

† F. Bonardi, unpublished data.
‡ A. Kaufmann, unpublished data.

(Fig. 1B) would be within disulfide bonding distance of each other. As controls, we also selected amino acids that are predicted to be too far apart to form a disulfide bond. The selected amino acids were replaced by cysteine residues via site-directed mutagenesis using a cysteine-less (Cys-less) SecY as template. Subsequently, the various single cysteine mutants in TM2b were combined with single cysteine mutants in TM7 to form double cysteine mutant pairs (Table 1). From the possible combinations, we specifically selected the pair F286C/S87C, as the homologous amino acids (Asn268 and Thr80, respectively) in the M. jannaschii SecYE form hydrogen bonds that need to be broken to open the lateral gate (17) (Table 1). The various SecY mutants were cloned into a secYEG expression vector and expressed in E. coli strain SF100. SDS-PAGE analysis and immunoblotting using an antibody against the N-terminal histidine tag in SecY showed that all mutants were (over)expressed in E. coli strain SF100. SDS-PAGE and SecY showed that all mutants were (over)expressed at similar levels (see Fig. 2 and supplemental Fig. S2). In vitro translocation experiments using fluorescein (FL)-labeled proOmpA as substrate and IMVs from cells overproducing the different single and double cysteine mutants showed that under reducing conditions all mutants translocate proOmpA to similar levels as the Cys-less SecYEG (supplemental Fig. S1).

Chemical Cross-linking of the Putative Lateral Gate Formed by TM2b and TM7—To visualize the cross-linking between cytoines introduced in TM2b and TM7, an assay was developed based on the specific cleavage of SecY by the outer membrane protein OmpT. OmpT cleaves SecY in the fourth cytoplasmic domain (C4) between two arginine residues at positions 255 and 256 (Fig. 1A) (27). Cleavage results in a N-terminal fragment of SecY with a molecular mass of 22 kDa that is readily detected by Coomasie Brilliant Blue-stained SDS-PAGE (Fig. 2A and supplemental Fig. S2). When TM2b is cross-linked to TM7, OmpT will cleave SecY but the two halves will not be separated on non-reducing SDS-PAGE and SecY will migrate as a full-length protein.

To oxidize the cytoine pairs in SecY, the hydrophilic oxidizer NaTT was used to form a disulfide bond. The use of the strong and hydrophobic oxidizer copper phenanthroline was avoided as this agent can cause protein aggregation, whereas there is the risk of a formation of thiol-copper-thiol bridge rather than a disulfide bond. DTT was used as a reducing agent to reverse disulfide bond formation. After NaTT treatment, IMVs were incubated with the protease OmpT and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 2A). Under both oxidizing and reducing conditions, OmpT treatment of the IMVs containing Cys-less SecY resulted in the complete cleavage of SecY as seen as a disappearance of full-length SecY with the concomitant formation of the 22-kDa N-terminal fragment (Fig. 2A, lanes 15 and 17). Moreover, addition of the reducing agent DTT after OmpT digestion (Fig. 2A, lane 18) did not affect the cleavage pattern (Fig. 2A, compare lane 17 versus 18). It should be noted that samples containing DTT were loaded on a separate SDS-PAGE gel to prevent the DTT from diffusing and affecting neighboring cross-linked samples. For this reason there is a slight difference in migration of full-length SecY and the derived N-terminal fragment of the same samples with and without DTT (see Fig. 2). The same results as shown for Cys-less SecY were obtained with all single cysteine mutants (Fig. 2A, lanes 1–12, and supplemental Fig. S2, lanes 1–18). In contrast, after oxidation of IMVs containing the double cysteine mutant SecY F286C/S87C, OmpT treatment resulted in the formation of a fuzzy band that corresponds to full-length SecY without appearance of the 22-kDa N-terminal fragment. This was not due to an incomplete digestion or NaTT-induced protein aggregation, as addition of DTT to the sample after OmpT treatment resulted in the conversion of full-length SecY into the 22-kDa N-terminal fragment (Fig. 2A, lane 24). This indicates that NaTT treatment results in the formation of a disulfide bond between the selected cytoine pair at positions 286 and 87. The fuzzy character of the protein band corresponding to OmpT cleaved but full-length SecY still indicates an altered conformational state of SecY in SDS-PAGE, a phenomenon often seen in cross-linking of membrane proteins. In comparison with the other double cysteine mutants, disulfide bond formation was very efficient with the mutants F286C/S87C and weaker for the F279C/M83C (compare Fig. 2A, lanes 19–24 versus supplemental Fig. S2, lanes 19–30) and I283C/S87C (data not shown) pairs. Cross-linking was absent with the...
negative control, mutant F279C/M83C (supplemental Fig. S2, lanes 19–24), confirming the SecYEG structure that indicated that these residues are not within disulfide bonding distance. For this reason, the SecY F286C/S87C mutant was used in the remainder of this study.

To verify the oxidation and OmpT digestion, formation of the N-terminal 22-kDa SecY fragment was validated by Western blotting using an antibody directed against the His6 tag (Fig. 2B). OmpT treatment of NaTT-oxidized IMVs containing Cys-less SecYEG and single cysteine mutants resulted in the complete conversion of SecY into the 22-kDa N-terminal fragment (Fig. 2B, lanes 1–18). On the other hand, in oxidized IMVs containing the F286C/S87C mutant, substantial levels of intact SecY remained after OmpT treatment (Fig. 2B, lane 21). The full-length SecY was converted into the 22-kDa N-terminal SecY fragment after DTT treatment (Fig. 2B, lane 24). The small amount of N-SecY fragment in Fig. 2B, lane 21, indicates incomplete disulfide bond formation. However, because of the blotting transfer of full-length SecY, the N-terminal fragment and the cross-linked products likely differ, data quantification was based on the full-length SecY visualized by SDS-PAGE. NaTT-mediated oxidation of the F286C/S87C SecY mutant pair results in a cross-linking efficiency of at least 80% (refer to Fig. 4A, lane 4, for cross-linking efficiency).

Oxidative Cross-linking of the Lateral Gate Abolishes ProOmpA Translocation and SecA Translocation ATPase Activity—To determine whether cross-linking of the cysteines in the SecY F286C/S87C mutant has an effect on protein translocation, we analyzed the in vitro translocation of FL-labeled proOmpA into IMVs that had been oxidized with NaTT or reduced with DTT. With IMVs containing Cys-less SecYEG, NaTT treatment had little effect on proOmpA translocation (Fig. 3A, compare lanes 3 and 4), nor did it affect proOmpA translocation into IMVs containing the SecY single Cys-286 (Fig. 3A, lane 10) and Cys-87 (Fig. 3A, lane 8), although the latter showed a reduced activity that can be attributed to the mutation. In contrast, NaTT treatment of IMVs containing the double cysteine mutant F286C/S87C was nearly completely abolished to the very low levels found for the endogenous wild-type levels of SecYEG (Fig. 3A, compare lane 6 versus 2). The activity of the F279C/M83C mutant was reduced to a lesser extent by NaTT oxidation in line with the observed weaker disulfide bonding efficiency (supplemental Fig. S3, lanes 3 and 4). As expected, proOmpA translocation into NaTT-treated IMVs containing the negative control SecY F279C/M83C was not affected (supplemental Fig. S3, lanes 5 and 6). These conditions also do not lead to the formation of a disulfide bond between the two positions.

Because proOmpA translocation is a SecA-dependent process, the effect of the lateral gate cross-linking on the proOmpA-stimulated ATPase activity was determined. IMVs containing the Cys-less, single cysteine 87 and 286, and the double cysteine F286C/S87C SecYEG mutant were treated with NaTT, and analyzed for the proOmpA-stimulated SecA translocation ATPase activity. NaTT treatment of IMVs containing Cys-less SecYEG did not significantly affect the SecA translocation
ATPase activity (Fig. 3A). Similar results were obtained with IMVs containing the single cysteine mutants SecY Cys-87 and Cys-286, although the activity of the SecY Cys-87 mutant was reduced as compared with the Cys-less control. The latter explains the reduced proOmpA-stimulated ATPase activity of the untreated double cysteine mutant SecY F286C/S87C (Fig. 3B), and possibly signifies a functional role of Ser-87 in translocation. Importantly, upon NaTT treatment of IMVs containing the double cysteine mutant SecY F286C/S87C, the proOmpA-stimulated ATPase activity of SecA was completely abolished. These data demonstrate that immobilization of the lateral gate of the translocon by disulfide-bonded cross-linking inhibits SecA-mediated preprotein translocation and the SecA translocation ATPase activity.

**Immobilization of the Lateral Gate of SecY by Chemical Cross-linkers with Varying Length—NaTT forms a disulfide bond between the two cysteine residues with an approximate distance of 2 Å. Oxidation is reversible by the reductant DTT. To analyze the effect of the cross-linking distance of the two cysteines on protein translocation, chemical cross-linkers were employed that have spacer arms with increasing lengths (supplemental Fig. S5). bBBr forms a covalent bond between thiol groups and has a rigid cross-linking distance of 5 Å (28, 29). bBBr has the particular characteristic that it becomes fluorescent when both of its alkyllating groups have reacted with thiols (30). BMOE is a rigid cross-linker that contains a thioether-linked spacer of ~8 Å, whereas BMH is a longer yet more flexible cross-linker with a maximal ~13-Å spacer arm (31). With the SecY F286C/S87C mutant, bBBr showed similar cross-linking efficiency as NaTT at 84 and 81%, respectively (Fig. 4A, compare lane 5 and 4). The cross-linking efficiencies of the longer cross-linking molecules BMOE and BMH were somewhat lower, i.e. 56 and 59%, respectively (lanes 6 and 7), but clearly discernable upon OmpT treatment. None of these cross-linkers interfered with the efficiency of OmpT digestion as validated with the Cys-less SecYEG complex digested by OmpT in the presence of these compounds (Fig. 4A, lanes 9–13). In addition, the cross-link between cysteines 286 and 87 after bBBr treatment could be directly demonstrated by UV exposure of a SDS-PAGE gel showing a bright fluorescent band at the position of full-length SecY only with the F286C/S87C mutant (Fig. 4B, lane 4) and that is absent in the bBBr-treated single cysteine and Cys-less mutants (lanes 1–3). The direct fluorescent visualization provides further evidence for the formation of a cross-link between Cys-286 and Cys-87 in addition to the OmpT assay.

IMVs containing the Cys-less and F286C/S87C mutations were treated with cross-linkers NaTT, bBBr, BMOE, and BMH as well as with the reductant TCEP, respectively. TCEP instead of DTT was used here as it is capable of reducing thiol in the presence of reactive maleimides. Next, in vitro translocation assays were performed using Texas Red-labeled proOmpA as substrate. Texas Red-proOmpA has an emission spectrum that does not overlap with the emission spectrum of bBBr, thus allowing for the simultaneous detection of proOmpA translocation and cross-linking of the lateral gate in SecY F286C/S87C. At the limiting amounts of IMVs used, the activity of SecYEG is rate-determining for translocation (supplemental Fig. S4) and thus the activity can be quantified and compared. None of the chemical cross-linkers affect the translocation of proOmpA into the Cys-less SecYEG IMVs (Fig. 4C, lanes 8–12). As shown before, NaTT treatment of SecY F286C/S87C IMVs reduced translocation to the levels observed with wild-type IMVs containing only endogenous levels of SecYEG (Figs. 3A, lane 6, and 4C, lane 2). A strong reduction in activity was also observed when SecY F286C/S87C IMVs were treated with bBBr (Fig. 4C, lane 3), whereas the longer cross-linkers BMOE and BMH had little effect on proOmpA translocation (Fig. 4C, lanes 4 and 5). Quantification of the data shows that translocation efficiency increases with an increasing length of the spacer arm reaching...
the same level of activity as untreated IMVs with the longest cross-linker BMH (Fig. 4D, black bars).

We also addressed the reversibility of oxidation and cross-linking. Addition of DTT after the cross-linking reaction restored translocation to normal levels only for the NaTT-oxidized F286C/S87C IMVs (Fig. 4D, gray bars). As expected, DTT had no significant effect on the translocation of proOmpA into IMVs treated with the irreversible cross-linkers bBBr, BMOE, and BMH. It should be noted that none of these compounds affected the activity of the single Cys-87 mutant (supplemental Fig. S6). Thus the inhibition of proOmpA translocation is due to a constrained lateral gate and is not caused by alkylation of a single cysteine position.

Next we performed SecA ATPase assays using SecY F286C/S87C IMVs treated with the various cross-linkers. Whereas these compounds did not affect the basal SecA ATPase activity of the IMVs (data not shown), the proOmpA-stimulated ATPase activity increased when the length of the spacer arm of the cross-linker (Fig. 4E) matches their effect on proOmpA translocation. Although the reduced efficiency of BMH cross-linking will contribute to the observed translocation and SecA translocation ATPase activities, it does not explain the entirely undisturbed activities with the BMH cross-linked F286C/S87C SecY (Fig. 4D and E). Therefore, these data lend strong support for the notion that SecYEG lateral gate opening and activation of the SecA ATPase are linked processes.

**Cross-linking of the Lateral Gate of a Translocon Containing a Protein Translocation Intermediate—**A pre-existing disulfide bond between positions 286 and 87 of the lateral gate of SecY prevents preprotein translocation. To access the proximity of these positions during translocation, a translocation intermediate was generated using a proOmpA-DHFR fusion protein (25, 32, 33). Upon addition of ligands NADPH and methotrexate, the DHFR moiety folds and blocks further translocation yielding a translocation intermediate that is stably arrested in the translocase. To ensure that proOmpA-DHFR efficiently blocks the F286C/S87C SecYEG pore, translocation was performed with proOmpA-DHFR in excess of the translocation sites whereupon IMVs were re-isolated and incubated with Texas Red-proOmpA for a second round of translocation.
Membranes were re-isolated by ultracentrifugation, resuspended in translocation buffer with SecA (20 mM ATP (and 10 mM NADPH and 10 mM methotexrate for proOmpA-DHFR), and assayed for a second round of translocation of Texas Red-proOmpA in the presence of 2 mM ATP. Membranes were re-isolated by ultracentrifugation, resuspended in translocation buffer with SecA (20 mM ATP, 2 mM ATP (and 10 mM NADPH and 10 mM methotexrate for proOmpA-DHFR), and assayed for a second round of translocation using Texas Red-proOmpA. Lane 1 shows a 10% Texas Red-proOmpA standard. B, F286C/S87C SecYEG IMVs treated with OmpT, and DTTrreduced F286C/S87C SecYEG IMVs, respectively. The cross-linking fragment of SecY are indicated by arrows. These data demonstrate that Cys-87 in TM7 are in close proximity when the pore is occupied by a translocation intermediate. Likely because of geometrical constraints, these positions can no longer be cross-linked by the chemical reagents with longer spacer arms.

DISCUSSION

In this study, we have addressed the role of the lateral gate in translocation of preproteins across the cytoplasmic membrane. On the basis of homology and sequence alignment with M. jannaschii SecY (17) we have engineered pairs of cysteine residues into the putative TM2b/TM7 lateral gate region of E. coli SecY. This allowed site-specific disulfide-bonded cross-linking of positions that were predicted to be in close vicinity, whereas more remotely introduced cysteines did not yield cross-links. Recently, two additional SecYE translocon structures have been described, notably from bacteria known to contain SecA as opposed to the archaeon M. jannaschii (14, 34). The high resolution structure of SecYE from Thermus thermophilus is in an antibody-stabilized pre-open state (34), whereas the structure of SecYE from T. maritima is with SecA bound in an intermediate state of ATP hydrolysis (35). Strikingly, compared with the M. jannaschii SecYEβ structure, the T. maritima SecA-bound SecYE structure shows a partial opening of the lateral gate region around TM2b and TM7. Our biochemical cross-linking data for E. coli SecYE on the lateral gate region are in line with this structural observation, and importantly, we demonstrate that the opening of this lateral gate is required for protein translocation and activation of the SecA ATPase.

The introduction of double cysteine mutants in TM2b and TM7 allowed an efficient cross-linking of the lateral gate. When fully oxidized, translocation is completely abolished. However, when a cross-linker is introduced with sufficient spacer length (larger than 5 Å), translocation occurred unrestricted. These observations support two main conclusions: (i) translocation of proOmpA occurs via a single translocon in line with a previous report (10); and (ii) the lateral gate region needs to open up to be in close vicinity, whereas more remotely introduced cysteines did not yield cross-links. Recently, two additional SecYE translocon structures have been described, notably from bacteria known to contain SecA as opposed to the archaeon M. jannaschii (14, 34). The high resolution structure of SecYE from Thermus thermophilus is in an antibody-stabilized pre-open state (34), whereas the structure of SecYE from T. maritima is with SecA bound in an intermediate state of ATP hydrolysis (35). Strikingly, compared with the M. jannaschii SecYEβ structure, the T. maritima SecA-bound SecYE structure shows a partial opening of the lateral gate region around TM2b and TM7. Our biochemical cross-linking data for E. coli SecYE on the lateral gate region are in line with this structural observation, and importantly, we demonstrate that the opening of this lateral gate is required for protein translocation and activation of the SecA ATPase.
SecYEG translocons seems unlikely because the presence of, for instance, the BMH cross-link in the lateral gate of one of the pores will prevent diffusion of the polypeptide substrate to the other pore.

Strikingly, there is a strong cooperativity between the ability of SecA to hydrolyze ATP and the opening of the lateral gate. Oxidative cross-linking of the lateral gate resulted in a complete loss of the SecA translocation ATPase activity, whereas chemical modification of the lateral gate with bimolecular cross-linkers with longer spacer arms (8–13 Å) supported SecA translocation ATPase activity up to the level observed with an unconstrained lateral gate. A recent study on the role of the E. coli SecA ATPase two-helix finger suggested that the helical scaffold domain, that harbors the two-helix finger, moves up and down inside the translocon with ATP hydrolysis cycles resulting in the pushing of the preprotein substrate into the translocon (35). Two cysteine positions on proOmpA could be cross-linked to both position 282 of SecY together with a cysteine on the tip of the two-helix finger (35). This is very close to cross-linked positions 286 and 87 of the lateral gate as shown in this study. This further highlights the importance of the region. Tsukazaki et al. (34) suggested that SecYE predominantly assumes the closed conformation, whereas binding of SecA induces a conformational change of the translocon from the closed to the pre-open state where a swinging of TM8 possibly opens an opening of the lateral gate region. Taken together, our data shows that a tight physical interaction between TM2b and TM7 is incompatible with preprotein translocation lending strong support for the hypothesis that the clamshell structure of the SecYEG translocon needs to open for the SecA-dependent initiation of protein translocation.

A recent molecular dynamic simulation study modeled conformational changes in SecYEG upon translocation initiation where a force of 2–3 nN is required to move TM2b and TM7 apart (21). Two independent events are proposed to require this amount of force. The first event is the opening of the gate to around 2 to 5 Å, which involves a latch-like movement of SecE and which requires a large force. The second event is the displacement of the plug domain (TM2a), which allows the lateral gate to open to 6–9 Å. Cross-linking studies suggested that the signal sequence from a translocating protein binds the region between TM2b and TM7 (36) and based on this observation it was proposed that the signal sequence inserts between TM2b and TM7 resulting in a displacement of the plug and the concomitant translocation of the mature preprotein domain. Our biochemical data are consistent with the molecular dynamics simulation as a SecY channel in which the gate is fixed by a cross-linker with a 8-Å or longer spacer arm allows normal translocation of proOmpA, whereas a cross-linker of 5 Å or shorter abolishes protein translocation. Interestingly we also observed that TM2b and TM7 can be efficiently cross-linked utilizing the oxidizer NaTT and the short cross-linker bBBr even when the pore contains a preprotein translocation intermediate. As the major fraction of the preprotein translocation intermediate is processed by leader peptidase (32) (data not shown), the signal sequence is no longer present in the complex. In contrast, the longer cross-linkers BMOE and BMH were no longer able to cross-link the cysteine positions in TM2b and TM7 when the pore was occupied by a translocation intermediate, despite their hydrophobicity that may facilitate access of the cysteine positions via the lipid phase. This suggests that the lateral gate region is in a near to closed conformation with the translocation intermediate present. Most likely when SecA encounters the DHFR domain at the C terminus of the proOmpA-DHFR fusion, SecA remains in an ADP-bound state and is unable to unfold the DHFR domain (37). The latter may correspond to a state of the SecA ATPase in which the two-helix finger is not inserted into SecY (35). Possibly, under these conditions the SecA-SecY interaction corresponds to a more energetically favorable pre-open state (34). In the T. maritima SecA-SecYE complex structure (14), the opening in the lateral gate between TM7 and TM2b is about 5 Å. Likely, longer cross-linkers such as BMOE and BMH will not be able to efficiently cross-link these selected positions when the flexibility of this region is constrained by the SecA transition state and/or the protein translocation intermediate. When the folding ligands of DHFR (NADPH and methotrexate) are removed further translocation of the DHFR domain occurs in an ATP and SecA-independent manner (33). Therefore, with a stalled and processed translocation intermediate, the SecY translocon most likely returns to a pre-open state with bound SecA, which corresponds to a narrowed lateral gate region.

In conclusion, for the first time we have provided experimental evidence for a catalytic requirement for the opening of the lateral gate at the interface of TM2b and TM7 of SecY during SecA-dependent protein translocation. Importantly, SecA activation and lateral gate opening are coordinated events consistent with an allosteric mechanism of channel opening. Eventually, signal sequences may leave the translocon via the TM2b and TM7 gate. Taken together, the SecYEG translocon seems to be a highly flexible structure that undergoes specific conformational changes during protein translocation. Future studies should address the function of the lateral gate in the insertion of membrane proteins.

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FIGURE S1. **The SecYEG translocon is active in proOmpA translocation after the introduction of single and double cysteines in TM2b and TM7.** FL-proOmpA was diluted into translocation buffer containing SecA (20 μg/ml), SecB (400 μg/ml), ATP (1 mM) and *E. coli* SF100 IMVs containing wild-type levels of SecYEG (WT) (lane 2), overexpressed levels the Cys-less SecYEG complex (lane 3), or bearing the indicated single- (lanes 4-9, 14) or double-cysteine mutations (lanes 10-13). After 8 minutes at 37°C, translocation reactions were terminated on ice by treatment with proteinase K. Samples were precipitated with TCA and protease protected material was analyzed by SDS-PAGE and in-gel UV fluorescence. Lanes 1 and 8 show a 20% FL-proOmpA standard.
FIGURE S2. **Disulfide crosslinking of cysteines in the lateral gate formed by TM2b and TM7.** IMVs containing different SecYEG derivatives were treated with the oxidizer NaTT (1 mM) as indicated, and incubated with the protease OmpT. Crosslinking of the cysteine residues in SecY was analyzed by SDS-PAGE and CBB staining. In order to prevent the reduction of the disulfide bond during electrophoresis, samples series which contained DTT were analyzed on a separate gel.
FIGURE S3. ProOmpA translocation by IMVs containing the C286/87 and C286/87 SecYEG complexes. IMVs containing the indicated SecYEG derivatives were oxidized or reduced with NaTT (+) and DTT (-), respectively. Translocation of FL-proOmpA was assayed as described in the legend to Fig. S1. Lane 2 shows the translocation by IMVs containing wild-type levels of SecYEG (WT). Lane 1 shows a 20% FL-proOmpA standard.
FIGURE S4. Limiting amounts of IMVs allow detection of small changes in translocation efficiency. Translocation reaction with TR-proOmpA and the indicated amounts of IMVs containing the SecY 286/87 mutant were performed as described in the legend of Fig. S1. Lanes 1 and 2 show 10 and 20% of TR-proOmpA standards, respectively.

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FIGURE S5. **Chemical structures of crosslinkers used in this study.** Dibromobimane (bBBr) is a rigid molecule that reacts covalently with two thiol groups with a spacer distance of 5 Å. bBBr becomes fluorescent when both alkylating groups have reacted with thiols. BMOE (bis-maleimidoethane) is a rigid homo-bifunctional thiol-crosslinker with an 8 Å spacer arm. BMH (bis-maleimido hexane) is a semi-rigid homo-bifunctional thiol-crosslinking reagent with a spacer arm that has a maximal length of 13 Å.

![Chemical structures of crosslinkers](image)

**Crosslinker distance:**
- dibromobimane: 5 Å
- BMOE: ~8 Å
- BMH: maximal ~13 Å

*du Plessis et al., Figure S5*
FIGURE S6. Crosslinkers with increasing length do not influence the proOmpA translocation activity of the single cysteine SecY mutant C87. IMVs containing single cysteine C87 SecY mutant were reduced with TCEP whereupon samples were treated with different chemical crosslinkers (bBBr, BMOE an BMH) and used in an *in vitro* translocation reaction with TR-proOmpA as substrate. Translocation was assayed as described in the legend to Fig. S1.

du Plessis et al., Figure S6