Cysteine-directed crosslinking demonstrates that helix 3 of SecE is close to helix 2 of SecY and helix 3 of a neighboring SecE

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Summary

Preprotein translocation in *Escherichia coli* is mediated by translocase, a multimeric membrane protein complex with SecA as peripheral ATPase and SecYEG as translocation pore. Unique Cysteines were introduced into transmembrane segment (TMS) 2 of SecY and TMS 3 of SecE to probe possible sites of interaction between the integral membrane subunits. The SecY and SecE single Cys mutants were cloned individually and in pairs into a secYEG expression vector and functionally overexpressed. Oxidation of the single Cys pairs revealed periodic contacts between SecY and SecE that are confined to a specific α-helical face of TMS 2 and 3, respectively. A Cys at the opposite α-helical face of TMS 3 of SecE was found to interact with a neighboring SecE molecule. Formation of this SecE dimer did not affect the high affinity binding of SecA to SecYEG and ATP hydrolysis, but blocked preprotein translocation and thus uncoupled the SecA ATPase activity from translocation. Conditions that prevent membrane deinsertion of SecA markedly stimulated the interhelical contact between the SecE molecules. The latter demonstrates a SecA-mediated modulation of the protein translocation channel that is sensed by SecE.

Introduction

In the well studied general secretory pathway of *Escherichia coli*, preproteins are targeted to the cytoplasmic membrane either as ribosome-bound nascent chains by signal recognition particle and FtsY (56,134) or as completely synthesised polypeptides. Proteins which are secreted through the post-translational pathway can be kept translocation competent by the export-dedicated molecular chaperone SecB (79,591). Both targeting routes converge at a membrane protein complex termed translocase (68). Translocase consists of a peripherally membrane-associated ATPase, SecA (549), and the SecYEG heterotrimeric integral membrane protein complex (226). The dimeric SecA is activated for SecB recognition when bound to the membrane at SecYEG (79,113). SecB donates the preprotein to SecA, and is released into the cytosol upon the exchange of SecA-bound ADP for ATP (104). The latter reaction elicits a conformational change that permits SecA domains to insert into the membrane with
the concomitant insertion of the preprotein (363). The inserted preprotein is released from its association with SecA upon the hydrolysis of ATP (343) and SecA reverses to its membrane surface-bound state. SecA may rebind the partially translocated preprotein, and complete translocation by multiple cycles of ATP binding and hydrolysis (343,347). In the absence of SecA association, translocation may also be driven by the proton motive force (340,343).

The translocase holoenzyme is formed by only SecA, SecY and SecE (227,274). However, SecG co-purifies with the SecYE complex (226,279) and its presence markedly enhances the efficiency of in vitro preprotein translocation (255). SecD, SecF and YajC are integral membrane proteins that assemble into a complex, which interacts with SecYEG (274). They may add to the fidelity of the translocation reaction as overexpression of the SecDFYajC complex stabilises SecA in the membrane-inserted state (285,286). SecD and SecF are not essential for preprotein translocation, but in their absence, cells are no longer able to sustain a proton motive force and are cold sensitive for growth (282,287). In vitro experiments have demonstrated that the SecYEG complex suffices to support efficient SecA-dependent preprotein translocation (226,261,379).

The SecYEG complex shares functional and structural characteristics with the Sec61p protein-conducting channel of the eukaryotic endoplasmic reticulum (401,403). High-resolution electron microscopy images of the mammalian and yeast Sec61p complex show ring-like oligomeric structures, which are formed after interaction with the ribosome (474,494). These structures seem to consist of two to four Sec61p trimers with a central pore. Recently, it has been shown that the SecYE of Gram-positive bacterium Bacillus subtilis shows quasi-pentagonal structures, which resemble the Sec61p complex (380). These structures are thought to consist of an oligomeric assembly of three SecYE subunits.

Both biochemical and genetic data have demonstrated that SecY and SecE interact, but the exact sites of interaction are not known. Synthetic lethality of various combinations of SecY (prlA) and SecE (prlG) signal sequence suppressor mutants suggests an interaction between the periplasmic loop 1 (P1) of SecY and P2 of SecE, and indicates that transmembrane segment (TMS) 7 and TMS 10 of SecY are in close proximity of TMS 3 of SecE (219,102). The cytoplasmic domain 4 (C4) of SecY has been suggested to interact with C2 of SecE (232,249). To obtain detailed insight into the molecular architecture of the SecYEG complex, we have carried out a Cysteine scanning mutagenesis. This is a powerful technique that has been used to reveal helix packing and structure-function relationships in polytopic membrane proteins (592,593). Based on the interaction between P1 of SecY and P2 of SecE and the observation that TMS 1 and 2 of SecE are not essential for its function (241), we have selected TMS 2 of SecY and TMS 3 of SecE to introduce single Cysteine residues. By combining single Cys mutants we were able to directly demonstrate specific contacts between these TMSs. In addition, a specific helical face of TMS 3 of SecE interacts with a neighboring SecE molecule. The latter interaction is stimulated when SecA membrane deinsertion in the presence of a preprotein is blocked, and suggests an oligomeric structure of the SecYEG complex where at least two SecE subunits are in close proximity.

**Results**

*Construction and activity of single Cysteine mutants of SecY and SecE*

To investigate the interaction between SecY TMS 2 and SecE TMS 3, we have employed a
Cysteine scanning mutagenesis approach. The *E. coli* SecY contains two endogenous Cysteines, *i.e.*, Cys329 and Cys385 located in TMS 8 and 9, respectively (Figure 1). SecE and SecG are devoid of Cys residues. A Cys-less SecY was constructed by replacing Cys329 and Cys385 by serine residues using site-directed mutagenesis. The Cys-less SecY was subsequently used to introduce five (F78C, A79C, L80C, G81C and I82C) unique Cys residues into a consecutive stretch of TMS 2 as to cover at least one turn of this putative α-helical segment. Likewise, five (S105C, L106C, I107C, L108C and W109C) unique Cys residues were introduced into TMS 3 of SecE. These mutations are predicted to be located close to the periplasmic face of the membrane (Figure 1, 238). The single Cys SecY and SecE mutants were cloned either individually or as pairs into the *secYEG* expression vector under control of the *trc* promoter with a N-terminal his-tag on SecY (202) (See Table 1). Inner membrane vesicles (IMVs) derived from cells expressing the SecYEG complex were checked by SDS-PAGE, CBB staining and western blotting using pAbs against SecY, SecE and SecG. With each of the constructs, SecY, SecE and SecG were overexpressed to the same level as the wild-type SecYEG complex. This is shown in Figure 2A for the Cys-less SecYEG and the complexes that bear the single Cys SecE mutations, but identical results were obtained for the individual SecY mutants, and the SecE-SecY mutant combinations. Since SecY is only stable when overexpressed together with SecE (234), it appears that with each of the Cys mutants a stable SecY-SecE interaction is achieved. IMVs were analysed for the *in vitro* translocation of 125I-labeled proOmpA (Figure 2B) and for the SecA translocation ATPase activity in the presence of proOmpA (Figure 2C). These assays were performed in the presence of DTT to prevent possible oxidation of the Cys residues. In all cases, the activities of the mutant SecYEG complexes were similar to that of the wild type. IMVs were also tested for the translocation of 125I-Δ8proOmpA, a proOmpA derivative with a defective signal sequence due to the deletion of Ile-8. In contrast to IMVs of the *prlA4* strain
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(216) expressing the PrlA4 SecY at wild-type levels, none of the overexpressed mutants was able to translocate Δ8proOmpA (data not shown). This suggests that the mutagenesis has not yielded any strong prlA or prlG mutants. In summary, the Cys-less SecYEG, the single Cys mutants of SecY and SecE, and the pairs of SecY and SecE mutants are normally overexpressed and are functionally active.

TMS 2 of SecY and TMS 3 of SecE are interacting transmembrane segments

To identify interhelical contacts between SecY and SecE, the membranes containing the SecYEG complex with the pairs of single Cys mutants of SecY and SecE were oxidised with 1 mM Cu²⁺(phenanthroline)₃. The reaction was then quenched with 10 mM neocuproine, and protein profiles were analysed by SDS-PAGE in the absence of reducing agents, western blotting and immunodetection using pAbs directed against SecY and SecE. Out of a total of 25 Cys pairs, only the combinations of SecY(F78C) with SecE(L108C), SecY(A79C) with SecE(L108C), and SecE(I82C) with SecE(S105C) yielded a slowly migrating protein band after oxidation that reacted both with pAbs directed against SecY and SecE (Figure 3). This putative crosslinked product of SecY and SecE showed an apparent molecular mass of 50 kDa on SDS-PAGE, and its formation was reversed by the addition of DTT (data not shown). The crosslinked product was not observed in membranes containing the Cys-less SecYEG (See Figure 4) or single Cys mutants of SecY or SecE. Modeling of TMS 2 of SecY and TMS 3 of SecE reveals that all detected crosslinks are confined to a distinct helical face, and that the interaction reappears after a single turn of both helical segments (Figure 9). The latter periodicity indicates that TMS 2 of SecY and TMS 3 of SecE are indeed α-helical and that both TMSs are in close proximity, i.e., within disulfide bonding distance.

SecE(L106C) contacts a neighboring SecE molecule

In all samples that contained the SecE(L106C) mutant, even in combination with the Cys-less SecYEG, the oxidising conditions also yielded a highly specific protein band with an apparent molecular mass of 28 kDa (Figure 3). This crosslinked product stained only with the pAb directed against SecE, and not with the SecY- or SecG-specific antibodies. The 28 kDa crosslinked product disappeared upon incubation with DTT, and was not found with wild type SecE. Based on its size, it may represent an oxidised dimer of SecE(L106C) molecules. To exclude crosslinking of SecE...
L106C) with another membrane protein of unknown identity, both the SecYE(L106C)G and Cys-less SecYEG complex were purified to homogeneity (379). The SecE(L106C) molecule co-purified with SecG and the his-tagged Cys-less SecY in the same stochiometry as the wildtype SecYEG complex (data not shown), which confirms that the SecE mutant interacts normally with SecY and SecG. Moreover, under oxidising conditions, the reconstituted SecYE(L106C)G complex again yielded the 28 kDa protein band (Figure 4), while the crosslinked band was not observed with the

![Figure 3](image)

Figure 3. Identification of specific cross-links between unique cysteines in TMS 2 of SecY and TMS 3 of SecE. IMVs derived from SF100 cells over-expressing the SecYEG complex containing pairs of the indicated Cys mutants in TMS 2 of amino-terminally His-tagged SecY and the following Cys mutants in TMS 3 of SecE: S105C (lane 1), L106C (lane 2), I107C (lane 3), L108C (lane 4) and W109C (lane 5). IMVs were oxidized for 30 min on ice in the presence of Cu^{II}(phenanthroline)_3, and subsequently quenched with an excess of neocuproine. Samples were analyzed by immunoblotting using pAbs directed against SecY and SecE. Resultant cross-linked products with apparent molecular masses of 50- (SecY-E) and 28-kDa (SecE-E) are indicated. SecY stains as a double band due to the presence of the endogenous SecY, overexpressed His-tagged SecY, and proteolytic loss of the His-tag. The protein band that runs in all samples at 30 kDa and slightly above the (SecE-E) cross-link is outer membrane protein A (OmpA), which is nonspecifically detected by the anti-SecE pAb. Note that this band is not present in the oxidized purified SecYE(L106C)G complexes shown in Figure 4.

![Figure 4](image)

Figure 4. The 28 kDa cross-linked product represents a SecE dimer. Proteoliposomes reconstituted with the purified Cys-less SecYEG and SecYE(L106C)G complex (6.5 g/mL) were oxidized for 30 min on ice in the presence of Cu^{II}(phenanthroline)_3, as indicated, and subsequently quenched with an excess of neocuproine. Samples were separated on SDS-PAGE in the absence or presence of DTT, blotted and immunostained with pAbs directed against SecE. The position of 28 kDa cross-linked SecE product is indicated (SecE-SecE).
Cys-less SecYEG complex. This unequivocally demonstrates that oxidation of the SecYE(L106C)G complex results in dimerisation of SecE. It is important to stress that the apparent ratio between monomeric SecE and the species crosslinked to SecY or SecE on western blots depended on the applied blotting conditions. Longer blotting times resulted in a loss of signal of the polypeptide band representing monomeric SecE, whereas shorter blotting times hardly revealed any crosslinked SecE. Therefore, even though the amount of SecE is equal in all samples, the total of immunostained SecE varies. The blotting conditions applied were optimal to visualise the crosslinked products without complete loss of the monomeric SecE signal. As the unique dimerisation of the SecE(L106C) mutant was observed after purification and reconstitution of SecYE(L106C)G complex, it unlikely results from a loose interaction with SecY or an over-stoichiometric expression level of the mutant SecE molecule. The latter is also apparent from the overexpression level of SecE(L106C), which is the same as for the other SecE Cys mutants.

When each of the single Cys SecE mutants was overexpressed separately, i.e., without SecY and SecG, the oxidation-induced formation of the 28 kDa crosslinked product was no longer unique for the SecE(L106C) but occurred with all constructs (Figure 5). Apparently, uncomplexed SecE is in a conformation in which crosslinking of Cysteines in TMS 3 readily occurs upon oxidation. However, upon association with SecY, SecE is oriented in such a manner that except for SecE(L106C), all Cys mutants are protected from disulfide bond formation. These results therefore suggest that SecE(L106) crosslinks with another SecE molecule within the SecYEG complex (Figure 9) as confirmed by the experiments with the purified SecYEG (Figure 4).
Oxidation of SecYE(L106C)G inhibits translocation

To establish whether the formation of the SecE(L106C) dimer had any influence on the activity of the SecYEG complex, Cys-less SecYEG or SecYE(L106C)G were treated with Cu$^{2+}$(phenanthroline)$_3$ and analysed for SecA-dependent proOmpA translocation. These studies were performed both with IMVs (data not shown) and proteoliposomes reconstituted with the purified SecYEG complexes (Figure 6). The translocation of proOmpA with SecYE(L106C)G was greatly reduced by oxidation, whereas the Cys-less SecYEG allowed translocation up to a translocation intermediate (I$_{31}$) with a molecular weight of 31 kDa. The latter is due to the presence of a disulfide bridge in the carboxyl-terminus of proOmpA that prevents further translocation (343). In IMVs, this intermediate exhibits an apparent molecular mass of 29 kDa due to removal of the signal peptide by leader peptidase. Upon the addition of DTT, proOmpA translocation with Cys-less SecYEG and SecYE(L106C)G occurred equally effective (Figure 6). These results demonstrate that the oxidation-induced dimerisation of SecE(L106C) inactivates translocase in a reversible manner. The effect of oxidation of the SecY-SecE single Cysteine pairs was not further analysed due to the low efficiency of disulfide bond formation.

Dimerised SecE(L106C) uncouples the SecA ATPase activity

The effect of the oxidation of the SecY-bound SecE(L106C) was further examined by
measuring the SecA membrane binding and insertion, and the SecA translocation ATPase activity. SecA binds with high affinity to the SecYEG complex, and with low affinity to the membrane lipids. The binding of 125I-labeled SecA to urea-treated IMVs that harbor overexpressed cys-less SecYEG or SecYE(L106C)G was not affected by the oxidation with Cu2+(phenanthroline)3, but it was effectively released after the addition of an excess of unlabeled SecA (Figure 7A). This demonstrates that the dimerisation of the SecY-bound SecE does not interfere with the number of high affinity SecA binding sites. Next, we determined whether the SecYEG- and precursor-stimulated SecA ATPase activity was influenced by the oxidation of SecE(L106C). Surprisingly, the amount of ATP hydrolysis by SecA in the presence of proOmpA was identical to that observed with the Cys-less SecYEG complex (Figure 7B). Since the oxidation of the SecE(L106C) results in a block of translocation, the SecA translocation ATPase appears uncoupled from translocation. Membrane insertion of SecA was analysed by the formation of a protease-protected 125I-labeled 30 kDa fragment upon interaction with the nonhydrolysable ATP analogue AMP-PNP in the presence of urea-treated IMVs bearing Cys-less SecYEG or SecYE(L016C)G (Figure 7C). Under reducing conditions, both the Cys-less SecYEG and SecYE(L016C)G complex allowed formation of the proteolytic 30 kDa SecA fragment. Oxidising conditions strongly, but not completely, inhibited 30 kDa formation with the SecYE(L106C)G complex. These data demonstrate that SecA retains the ability to bind with high affinity to the oxidised SecYE(L106C)G complex and to hydrolyse ATP. Since membrane insertion is not completely prevented under oxidising conditions, it cannot be excluded whether the remaining membrane insertion activity relates to incomplete oxidation of SecE(L106C) or...
residual activity of the oxidised SecYE(L106C)G channel. We conclude, however, that the oxidised SecYE(L106C)G channel must be in a close-to-functional state to allow SecA binding and an uncoupled translocation ATPase activity.

**SecA modulates the intrahelical contact between neighboring SecE molecules**

To examine whether the formation of the Sec(L106C) dimer was modulated by translocation conditions, conditions were explored that abolished dimer formation. When urea-treated membrane vesicles bearing SecYE(L106C)G were extensively pre-reduced with 5 mM DTT, diluted in buffer without DTT, and subsequently oxidised by the addition of Cu$^{2+}$ (phenanthroline)$_3$, only a low level of SecE(L106C) dimer was formed (Figure 8, lane 2). We used this method of pre-reduction as an assay to find conditions that modulate the efficiency of dimer formation, and in this manner to detect dynamic changes in the subunit interactions of SecYEG during translocation. The yield of dimer formation was not affected by the addition of SecA irrespective of the presence or absence of proOmpA (lanes 4 and 3, respectively) nor by the subsequent addition of ATP (lane 5). However, a dramatic elevation of the SecE dimer formation occurred when the nonhydrolysable ATP-analogue AMP-PNP was added to the solution instead of ATP (lane 6). This phenomenon strictly depended on the presence of preprotein, since in the absence of proOmpA, no stimulation of dimer formation was observed (lane 7). We then tested whether other conditions leading to the stable insertion of SecA had an effect on the SecE(L106C) crosslinking. Indeed, a preprotein-dependent stimulation of dimerisation was observed when ATP-$\gamma$-S was used as a nonhydrolysable ATP analogue (lane 8 and 9). Non-hydrolysable ATP-analogues block translocation in its initial stage, leading to the processing of the signal sequence by leader peptidase (343) and a stabilization of the SecA membrane-inserted state (363). To block SecA in its membrane-inserted state during later stages of the translocation reaction, the ATPase inhibitor azide was added 25 min after the addition of ATP (lane 11). Azide interferes with the SecA ATPase activity (201), and enforces the formation of membrane-inserted SecA by preventing its deinsertion (216). This effect appears strongest during an ongoing translocation reaction (lane 11), as the addition of azide before initiation of translocation with ATP did not result in increased SecE(L106C) dimerisation (lane 10). Also in the case of the azide-induced SecA membrane insertion, the effect on the SecE crosslinking was dependent on the presence of preprotein (lane 12). Under the same set of conditions, we were unable to detect a change in the yield of the SecY-SecE crosslink using the SecY(A79C) and SecE(L108C) mutant pair (data not shown). Furthermore, none of the other SecE single Cys mutants showed dimer formation upon the conditions described above. These results demonstrate that translocase undergoes dynamic changes that influence the proximity of two SecY-bound SecE molecules. This phenomenon is coupled to the membrane insertion of SecA and takes place only with active translocase, i.e., in the presence of preprotein.

**Discussion**

In this manuscript we provide direct evidence for an interaction between TMS 2 of SecY and TMS 3 of SecE of *E. coli*, and demonstrate that the SecYG-bound SecE interacts with a neighboring SecE molecule. SecA influences the latter interaction in a preprotein and nucleotide-dependent manner. For this study, we have used a Cys-less SecYEG complex to
allow Cysteine scanning mutagenesis. The two endogenous Cys residues of SecY were replaced for serine residues, yielding a fully functional SecYEG complex that was subsequently used to introduce unique Cys residues into TMS 2 of SecY (F78 to I82) and TMS 3 of SecE (S105 to W109). For simplicity, the SecY interaction for the second SecE molecule is not shown.

Figure 9. Schematic representation showing the periodic sites of interaction between TMS 2 of SecY and TMS 3 of SecE, and the identified site of interaction between TMS 3 of neighboring SecE molecules. In black are indicated the mutagenized amino acids residues that are part of the amino acid sequences 77-85 and 102-110 of SecY and SecE, respectively. For simplicity, the SecY interaction for the second SecE molecule is not shown.

could be demonstrated between Cysteines replacing F78 and A79 in TMS 2 of SecY and L108 in TMS 3 of SecE. These residues are restricted to a specific helical face of both TMSs and, strikingly, crosslinking re-appeared when the Cys mutations were moved a single α-helical turn, replacing I82 in SecY and S105 in SecE (Figure 9). L108 in TMS 3 of SecE was found to make disulfide bonds with two adjacent positions in TMS 2 of SecY, suggesting some conformational flexibility in this region or in the side-chains of the introduced Cys residues. Previous genetic evidence suggested an interaction between P1 of SecY and P2 of SecE, and we now show that this interaction reflects a close proximity between at least two of the associated transmembrane helices. Interactions of SecY cytoplasmic loop 4 (C4) with SecE C2, and of TMS 7 and TMS 10 of SecY with TMS 3 of SecE have been identified genetically (102,232,249). TMS 2, 7 and 10 of SecY are most conserved, whereas TMS 3 of SecE is the only membrane span that is necessary for the SecE function. Most prlA mutations of SecY that allow the translocation of signal sequence defective preproteins are clustered in P2, TMS 7 and TMS 10 of SecY (102,219). TMS 3 of SecE may therefore be surrounded by the conserved core of the integral membrane domain of the translocase, i.e., TMS 2, TMS 7 and TMS 10 of SecY. Based on a systematic crosslinking study of the signal sequence of a preprotein to the yeast translocase, it has been postulated that the signal sequence and Sss1p/SecE bind to the same or overlapping regions in Sec61p/SecY (594). The same study shows that TMS 2 and TMS 7 of Sec61p can be crosslinked to the signal sequence. Our report extends this postulate and demonstrates that TMS 2 of SecY is indeed in close proximity of TMS 3 of SecE, specifying this interaction to defined residues. Conditions that lead to the membrane insertion of the signal sequence, or
that allow preprotein translocation did, however, not affect the extent of the Cu$^{2+}$-induced crosslinking of SecY(A79C) to Sec(L108C). These conditions also did not affect the crosslinking between other SecE Cys mutants, excluding that SecE is expelled from the translocase by the signal sequence. These data thus suggest a stable interaction between SecY and SecE that persists during translocation.

One of the five examined Cys mutants of TMS 3 of SecE, SecE(L106C), exhibits a Cu$^{2+}$-induced dimerisation, irrespective of its combination with the wild-type, Cys-less or single Cys mutants of SecY. The other four Cys mutants of SecE showed such behavior only when overexpressed in the absence of SecY. SecE(L106C) seems to interact with SecY in a manner that is indistinguishable from the wild-type, which does not dissociate from SecY in the membrane (230). The stability of the SecY-SecE(L106C) interaction was confirmed by purification of the SecYE(L106C)G complex, which after reconstitution showed the same Cu$^{2+}$-induced dimerisation of SecE. The purified SecYE(L106C)G complex provided unequivocal evidence that the crosslinked SecE product indeed consists of a SecE dimer that is associated with the other translocase subunits. Although the oxidised SecYE(L106C)G channel binds SecA and allows it to undergo cycles of ATP binding and hydrolysis, translocation and SecA membrane insertion are severely impaired. Apparently, this SecYEG channel is trapped in a partially

Figure 10. Schematic model for the modulation of the SecYEG translocation channel by the membrane insertion of SecA. Binding of a precursor protein activates the SecYEG-bound SecA, which triggers channel opening of an oligomeric assembly of SecYEG complexes. Subsequent binding of ATP to SecA drives the insertion of a SecA domain together with the signal sequence and amino-terminal mature region of the precursor protein into the translocation channel. This process is accompanied by the inversion of the SecG membrane topology and a re-arrangement of the SecY-bound SecE bringing the L106C residues (indicated by a black stalk) at the periplasmic side of SecE TMS 3 in closer proximity. The latter re-arrangement may be necessary to accommodate the inserted preprotein. For simplicity, a dimeric assembly of the SecYEG complex is shown.
functional state that is no longer able to meet the requirements allowing complete translocation reaction cycles.

The dynamic nature of the SecYEG channel was visualised by the modulation of SecE (L106C) dimerisation by SecA. Membrane-inserted SecA affects the conformation of the SecYEG protein translocation channel in such a manner that it affects the proximity of two SecE TMS 3 helices (See scheme in Figure 10). This was apparent, as stabilisation of the membrane-inserted state of SecA by non-hydrolysable ATP analogues or azide, caused a marked stimulation of the Cu²⁺(phenanthroline)₃⁻-induced dimerisation of SecE (L106C). As both SecE molecules are part of the SecYEG complex, this event may reflect conformational changes within an oligomeric organisation of multiple SecYEG heterotrimers that together form a translocation channel. Such an oligomeric organisation of the protein-conducting channel has been demonstrated by electron microscopy for the homologues eukaryotic Sec61p and the Bacillus subtilis SecYE complex. In addition, the oligomeric organisation of the SecYEG complex explains the paradigm that SecY and SecE do not dissociate in vivo (230) but appear to interact dynamically (299). Our experiments are consistent with an oligomeric assembly of the SecYEG complex where two SecE molecules are in close proximity, but do not reveal the exact stoichiometry of such a complex. As the sites of interactions between SecY and SecE are restricted to specific regions of the two molecules (102,232,249, this study), it is most likely that they interact in a stoichiometric fashion. This is also apparent from their interdependent expression and cellular expression levels (234). We therefore propose that the observed SecE-SecE interaction takes place between two SecYEG subunits within the oligomeric channel.

The modulation of the SecE(L106C) dimerisation by SecA strictly required the presence of proOmpA, although SecA membrane insertion with AMP-PNP is precursor-independent (363). We therefore postulate that interrelated, but separate events underlie the modulation of the translocation channel by preproteins and SecA. Firstly, signal sequence recognition triggers a conformational change in the SecYEG channel and thereby activates or ‘opens’ the translocation channel (See Figure 10: ‘channel opening’). Such a phenomenon has been postulated for both SecYEG (595) and the Sec61p protein-conducting channel (491). Whereas the pore size of the Sec61 channel upon interaction with the ribosome is around 2 nm (474,494), it is opened to 4-6 nm in the presence of ribosome-nascent chain complexes (493). In the E. coli system, the signal sequence recognition may involve a specific conformational state of SecA that triggers opening of the SecYEG channel. Secondly, the active protein translocation channel undergoes conformational changes during the translocation reaction that are elicited through cycles of conformational changes taking place in the SecA molecule. Only with the precursor-activated translocation channel, SecA membrane insertion triggers the intimate contact between two SecE(L106C) molecules causing disulfide-bond formation (Figure 10: ‘membrane insertion’). We propose that SecA membrane insertion results in a subunit re-arrangement or a conformational change of the active SecYEG translocation channel that brings the L106C residues in SecE in a position favorable for disulfide-bond formation (Figure 10).

The modulation of the crosslinking of SecE(L106C) together with the SecG topology inversion (262) and SecA cycling at the cytoplasmic membrane (363) demonstrate that translocase is a highly dynamic protein complex, and indicate a strong relationship
SecY-SecE interactions

The identified contacts between TMS 2 of SecY and TMS 3 of SecE (Figure 9) can be incorporated into a model of the molecular architecture of the SecYEG complex that will serve as a starting point to identify further inter- and intramolecular interactions in order to obtain a low-resolution molecular model of the integral membrane domain of the translocase.

Materials and methods

Materials.

E. coli SecA (549), SecB (77) and proOmpA (562) were purified as described. ProOmpA was iodinated as described for preAmyL (584), and stored frozen in 6 M urea, 50 mM Tris-HCl, pH 7.8. Wild-type and mutant SecYEG complexes were purified as described and reconstituted into liposomes of E. coli phospholipid by detergent dilution (379). Polyclonal antibodies (pAb) raised against purified his-tagged SecY and SecE, and against a synthetic peptide corresponding to a SecG domain were obtained as described previously (379). A stock solution of 80 mM Cu²⁺(phenanthroline)₃ complex was prepared by mixing 120 µl of 0.36 M 1,10-phenanthroline in 50% ethanol with 60 µl of 0.24 M CuSO₄.

Bacterial strains and growth conditions

For all experiments E. coli strain SF100 (570) was used. Cells were grown aerobically at 37 °C on L-broth in the presence of 100 µg/ml ampicillin in a shaking incubator until the end of the logarithmic phase. For the induction of plasmid encoded genes under control of an IPTG inducible promoter, exponentially growing cultures were supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD₆₆₀ of 0.6, and growth was

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Table I: Plasmids used in this study.

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<td>pET630</td>
<td>SecE (W109C) in pET607</td>
<td>W109C(TGT → TGT) ΔClal (ATCGAT → ATCGAC)</td>
</tr>
<tr>
<td>pET636</td>
<td>SecY(F78C) in pET607</td>
<td>F78C (TTT → TGT) Q146Q (CAA → CAG)</td>
</tr>
<tr>
<td>pET637</td>
<td>SecY (A79C) in pET607</td>
<td>A79C (GCT → TGT) Q146Q (CAA → CAG)</td>
</tr>
<tr>
<td>pET638</td>
<td>SecY (L80C) in pET607</td>
<td>L80C (CTG → TGT) Q146Q (CAA → CAG)</td>
</tr>
<tr>
<td>pET639</td>
<td>SecY (G81C) in pET607</td>
<td>G81C (GGG → TGT) Q146Q (CAA → CAG)</td>
</tr>
<tr>
<td>pET640</td>
<td>SecY (I82C) in pET607</td>
<td>I82C (ATC → TGC) Q146Q (CAA → CAG)</td>
</tr>
<tr>
<td>pET301</td>
<td>SecE in pET324</td>
<td></td>
</tr>
<tr>
<td>pET1602</td>
<td>SecE (S105C) in pET324</td>
<td></td>
</tr>
<tr>
<td>pET1603</td>
<td>SecE (L106C) in pET324</td>
<td></td>
</tr>
<tr>
<td>pET1604</td>
<td>SecE (I107C) in pET324</td>
<td></td>
</tr>
<tr>
<td>pET1605</td>
<td>SecE (L108C) in pET324</td>
<td></td>
</tr>
<tr>
<td>pET1606</td>
<td>SecE (W109C) in pET324</td>
<td></td>
</tr>
</tbody>
</table>

Double Cys mutants are not described in the table. Their names are derived from combinations of the names of the single Cys mutants pET636/623 for example overexpresses SecYEG with the mutations F78C and L107C in SechYn and SecE, respectively.
continued for another 2 hrs.

**Plasmid construction**
The vector pET349 (SechYnEG+) allows the overproduction of his-tagged SecYEG under control of the IPTG-inducible trc promoter (202). To facilitate cloning and the introduction of unique Cys mutations in SecY and SecE, the HincII site in SecE was removed from pET349 resulting in pET610 (Table 1). To construct a Cys-less SecY, Cys329 and Cys385 were replaced for serine residues in the secY gene of pET605, resulting in pET608 and pET609, respectively. These mutations were transferred to pET610, yielding pET607 which expresses the Cys-less SecYEG complex with a his-tag at the amino-terminus of SecY. pET607 was used to reintroduce single Cys mutations into SecY and SecE. To facilitate the screening for correct mutants, silent modifications in restriction sites were made. Insertion of single Cys in TMS 3 of SecE was accompanied by the deletion of the Clal site between SecY and SecE (ATCGAT \(\rightarrow\) ATCGAC) and insertion of the single Cys in TMS 2 of SecY was accompanied with the deletion of the Stul site in SecY Q146Q (CAA \(\rightarrow\) CAG). Single Cys mutants of TMS 2 of SecY were combined with the Cys mutants in TMS 3 of SecE by exchange of the SecYE EcoRI/BamHI fragment. Overexpression of single Cys SecE mutants was obtained by cloning the appropriate NcoI/BamHI SecE fragments in pET324. All mutagenesis was done by a two step PCR reaction, and constructs were confirmed by sequence analysis on a Vistra DNA sequencer 725 using the automated Δtaq sequencing kit (Amersham, Buckinghamshire, U.K.). All other DNA techniques followed standard procedures.

**Isolation of inner membrane vesicles**
A rapid membrane isolation procedure was developed to facilitate the analysis of a large number of mutant SecYEG complexes. Liquid nitrogen-frozen cells were quickly thawed at 37 °C, and diluted with an equal volume of 20% glycerol, 50 mM Tris-HCl, pH 8.0 (buffer A). The suspension was subjected to French press treatment (4 times at 8,000 psi), diluted with an equal volume of buffer A, and cleared from debris by centrifugation (10 min at 4,000 x g). Membranes were collected from the supernatant by centrifugation (90 min at 40,000 x g), resuspended in buffer A, loaded on a 4-step sucrose gradient that consisted of 0.3, 0.9, 0.9 and 0.3 ml of a 36, 45, 51 and 54% (w/v) sucrose solution in 50 mM Tris-HCl pH 8.0, respectively. Inner membranes vesicles (IMV) were separated from outer membranes through velocity centrifugation (30 min at 250 000 x g, Beckmann TLA 100.4 rotor), collected from the gradient, and diluted with 5 volumes of buffer A. Purified IMVs were recollected by centrifugation (90 min at 40,000 x g), resuspended in buffer A at 10 mg protein/ml, and stored in liquid nitrogen.

**Crosslinking**
For disulfide bridge formation, vesicles were incubated for 30 min on ice in the presence of 1 mM Cu\(^{2+}\)(phenanthroline)\(_3\) (oxidised) or, as a control, with 10 mM dithiothreitol (DTT) (reduced). Oxidation was terminated with 25 mM neocuproine to protect the unreacted thiols, and the samples were analysed on 10 or 15% SDS-PAGE, followed by western blotting and immunostaining with α-SecY, α-SecE and α-SecG pAbs. SecE(L106C) crosslinking after preincubation was measured with vesicles that had been reduced with 5 mM DTT before dilution in the translocation reaction mixture. Reactions were then incubated as indicated, placed on ice and oxidised with 1 mM Cu\(^{2+}\)(phenanthroline)\(_3\) as described.

**Translocation assays**
Translocation assays were performed in 50 µL buffer B consisting of 50 mM HEPES-KOH,
SecY-SecE interactions

pH 7.5, 30 mM KCl, 5 mM Mg(Ac)₂, and 0.5 mg/ml bovine serum albumine (BSA). Creatine phosphate (10 mM) and creatine kinase (0.5 µg) were added as an ATP-regenerating system. Reaction mixtures furthermore consisted of 1.6 µg of SecB, 1 µg of SecA, 1 µL of ¹²⁵I-labeled denatured proOmpA (1 mg/ml in 6M urea, 50 mM Tris-Cl, pH 7.5) and 10 µg of SecYEG⁺ IMVs or 6.5 µg of SecYEG proteoliposomes that had been pre-incubated for 30 min at 0 °C in the presence of 5 mM DTT or 1 mM Cu²⁺(phenanthroline)₃. Reactions were energised with 2 mM ATP, placed at 37 °C, and at various timepoints samples were taken, chilled on ice and treated with proteinase K (0.1 mg/ml) for 15 min. Reactions were then precipitated (10 min at 20,000 g) with ice cold trichloric acid (10% w/v), acetone-washed and analysed by SDS-PAGE on 12% PAA gels. For SecA membrane insertion assays, reactions were performed with 0.2 µg of ¹²⁵I-labeled SecA and unlabeled proOmpA.

Other analytical techniques

Binding assays were performed as described (216). Translocation ATPase activity of urea-treated IMVs or SecYEG proteoliposomes was measured with proOmpA as substrate (179). SecA membrane insertion assay using ¹²⁵I-SecA were performed as described (363). Protein concentrations were determined by the method of Lowry (564) in the presence of SDS using BSA as a standard. Semi-dry western blotting (Trans-Blot apparatus, Bio-Rad) was performed at 4 mA/cm² of blotting membrane (PVDF, Boehringer) for 45 min, using a buffer consisting of 48 mM Tris, 30 mM glycine and 20% (v/v) methanol, with or without 0.1% (w/v) SDS.

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