The protein-conducting channel SecYEG
Veenendaal, Andreas Kyung Jin

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Mapping the sites of interaction between SecY and SecE by cysteine scanning mutagenesis

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Summary

In Escherichia coli, the SecYEG complex mediates the translocation and membrane integration of proteins. Both genetic and biochemical data indicate interactions of several transmembrane segments (TMSs) of SecY with SecE. By means of cysteine scanning mutagenesis, we have identified intermolecular sites of contact between TMS7 of SecY and TMS3 of SecE. The cross-linking of SecY to SecE demonstrates that these subunits are present in a one-to-one stoichiometry within the SecYEG complex. Sites in TMS3 of SecE involved in SecE dimerization are confined to a specific α-helical interface and occur in an oligomeric SecYEG complex. While cross-linking reversibly inactivates translocation, the contact between TMS7 of SecY and TMS3 of SecE remains unaltered upon insertion of the preprotein into the translocation channel. These data support a model for an oligomeric translocation channel in which pairs of SecYEG complexes contact each other via SecE.
SecY-SecE interaction 2
Introduction

In bacteria, protein translocation and membrane protein insertion is mediated by the translocase. Translocase consists of the SecYEG membrane protein complex, and the peripherally membrane-associated SecA dimer [For review see [1]]. SecA is an ATP-dependent motor protein that drives the stepwise translocation of the precursor protein (preprotein) across the membrane by cycles of ATP binding and hydrolysis [86, 87, 182]. SecY, SecE and SecG are integral membrane proteins that together form a heterotrimeric complex [28, 139] that constitutes a high affinity binding site for SecA [82]. Recent electron microscopic and biochemical studies indicate that the protein conducting channel is comprised of a SecYEG tetramer that is assembled by SecA from monomeric and dimeric SecYEG subcomplexes [160].

The SecYEG complex is a member of a highly conserved protein translocation pathway [41]. It is homologous to the eukaryotic Sec61p complex, which consists of three subunits α, β, and γ that together form the ‘translocon’ of the endoplasmic reticulum (ER) membrane [147]. SecY and SecE are essential subunits of the translocase. SecY comprises ten transmembrane segments (TMSs) (figure 1), whereas SecE is a small membrane protein that in most bacteria contains only a single TMS. In Escherichia coli, SecE contains three TMSs, but only the conserved C-terminal portion including the third TMS is required for activity [59] (figure 1). SecY and SecE form a stable complex in the membrane that does not dissociate in vivo [114]. In the absence of SecE, SecY is degraded by FtsH [112]. Mutations located in the cytoplasmic loop 4 (C4) of SecY [116] and C2 and TMS3 of SecE [64] destabilize the SecY-SecE interaction. Many of the so-called prl mutations (protein localization) that suppress defects in the signal sequence are present in the secY and secE genes. Specific combinations of these mutations in SecY (prlA) and SecE (prlG) result in synthetic lethality, and it has been suggested that this signifies sites of interaction between SecY and SecE [48, 122]. According to this hypothesis, the periplasmic loop 1 (P1) of SecY and P2 of SecE are interacting regions, while TMS3 of SecE interacts with TMS7 and TMS10 of SecY. Recent studies employing cysteine mutagenesis indeed demonstrated that P1 of SecY and P2 of SecE [123], and TMS2 of SecY and TMS3 of SecE [124] are in close proximity. Most of the conserved residues and prlA mutations are clustered in TMSs 2, 7 and 10 of SecY and together with TMS3 of SecE, form the conserved core of the SecYE complex. Strikingly, the regions suggested to interact overlap with the regions that have been implicated in the binding of the signal sequence of the preprotein. TMS2 and TMS7 of Sec61α, the yeast homologue of SecY can be cross-linked to the signal sequence of a preprotein [155]. Cysteine scanning mutagenesis also showed an interaction between two neighboring SecE molecules that is modulated by the SecA and ATP-dependent initiation of preprotein translocation [124]. This observation lends further support for an oligomeric nature of the integral membrane domain of the translocase.

A central question is how the SecYEG complex forms the protein-conducting channel. Therefore, detailed information is required about the molecular architecture of the SecYEG complex. For this purpose, we have initiated a cysteine-scanning mutagenesis approach to probe sites of interaction between SecY and SecE [124]. The method can also be used to detect dynamic changes in subunit interactions. To allow the formation of a disulfide bond, the β-carbons of the two cysteines need to be in close proximity, i.e. 3-4 Å [120]. We have now extended our studies to demonstrate that TMS3 of SecE forms an α-helix, with one face that stably interacts with TMS2 and TMS7 of SecY while the opposite face dynamically interacts with TMS3 of a neighboring SecE molecule that is part of a separate SecYEG complex.
**SecY-SecE interaction 2**

Table I. Plasmids

A synthetic secYEG operon behind the isopropyl-β-D-thiogalactoside-inducible trc promotor was used for the plasmid-derived overexpression of the SecYEG complex. All listed plasmids were constructed via polymerase chain reaction mutagenesis, resulting in the indicated mutations.

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SecY TMS7 mutants in pET607:

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SecE TMS3 mutants in pET607:

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*secY* gene with *Bsp*E (TCCGGT→TCCGGA); *secE* gene with *∆Cla* (ATCGAT→ATCGAC). Double and triple cysteine mutants: the names are combined, e.g. pET2502/613 contains SecE V97C and SecY P276C mutations.

**Experimental procedures**

**Materials**

SecA [27], SecB [189] and proOmpA [190] were purified as described. A stock solution of 80 mM Cu²⁺(phenantroline)₃ was prepared as described previously [124].

**Plasmids**

The plasmids used to overproduce SecYEG are listed in table I. All mutations were introduced by a two-step polymerase chain reaction (PCR) using a template plasmid that allows overexpression of a cysteine-less SecYEG with an amino-terminal (His)$_₆$-tag on SecY [124]. Cysteine mutagenesis was accompanied by the introduction of silent modifications in restriction sites to facilitate the screening for correct mutants. Single cysteine mutations in TMS7 of SecY were introduced together with a *Bsp*E site in SecY [173]. The introduction of single and double cysteine mutations in TMS3 of SecE was accompanied by the deletion of a *Cla* site between SecY and SecE [124]. All mutations were confirmed by complete sequence analysis.

**Bacterial strains, growth conditions and membrane isolation**

Cell growth and isolation of inner membrane vesicles (IMVs) was performed as described previously [124].

**Cross-linking**

For assays of disulfide bridge formation, IMVs (1 mg/ml) were incubated for 30 min at...
37 °C in the presence of 1 mM Cu$^{2+}$(phenantroline)$_3$ (oxidized) or, as a control, with 5 mM dithiothreitol (DTT) (reduced). The oxidation reaction was quenched by the addition of 25 mM neocuproine (Sigma Chem. Co., St. Louis, MO). Oxidized samples were “re-reduced” by the incubation in 100 mM of DTT for 1 h at 37 °C. Samples were analyzed on 12 % SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) or further analyzed by Western blotting onto PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and immunostaining using antibodies against his-tagged SecY or SecE [184].

**Results**

**Construction, expression and activity of single-cysteine mutants of SecE and SecY**

Previously, we have described eight unique cysteine mutations that were introduced in TMS7 of SecY (Val274 to Ser281), covering at least two turns of the putative α-helical structure [173] (Table I). Sequence alignment and hydrophobicity analysis of the family of bacterial SecY proteins predicts the mutations in TMS7 of SecY to be located near the cytosolic membrane interface (figure 1). To investigate possible contacts between TMS3 of SecE and TMS7 of SecY a new set of five unique cysteine mutations was made in TMS3 of SecE covering positions Leu95 to Ala99 (Table I). These are predicted to be at the same membrane depth as the mutants in TMS7 of SecY (figure 1). The five cysteine mutations together with mutation G110C in SecE, located close to the periplasmic membrane interface, were also used to further explore the contact interface with a neighboring TMS3 of SecE.

The single-cysteine SecE mutants were placed into a cysteine-less SecYEG expression
vector [124], and overproduced in *E. coli* strain SF100. IMVs derived from these cells were analyzed for the SecY and SecE expression levels, SecA translocation ATPase activity and proOmpA translocation. The expression levels of the various mutants as analyzed by SDS-PAGE and CBB-staining were found to be identical to that of the overexpressed cysteine-less and wild-type SecYEG complex (data not shown). The proOmpA-stimulated SecA ATPase activity and the translocation of 125I-labeled proOmpA of the mutants were indistinguishable from that of the cysteine-less and wild-type SecYEG complex (data not shown). None of the mutants was capable of translocating the proOmpA variant Δ8-proOmpA (data not shown) which carries a defective signal sequence due to the deletion of Ile8 [199]. This substrate is effectively translocated by PrlA4 IMVs [54]. Taken together, the data demonstrate that the introduction of the cysteines in respective positions of TMS3 of SecE does not alter the activity or specificity of the SecYEG complex.

**SecE TMS3 contacts a neighboring SecE TMS3 at an α-helical interface**

To investigate the contact interface between two TMS3 of SecE, IMVs containing overexpressed SecYEG complex harboring unique cysteine mutations in TMS3 of SecE (L95C, I96C, V97C, A98C, A99C, and G110C) were oxidized with Cu²⁺(phenantroline)₃ and analyzed by SDS-PAGE and immunodetection using an antibody against SecE (figure 2A). Oxidation of the SecYE(A99C)G and SecYE(G110C)G complexes gave rise to a pronounced 28-kDa cross-linking product that is identical to the previously identified SecE-SecE cross-link found for the SecYE(L106C)G complex [124]. The other single-cysteine SecE mutants did not yield a SecE-SecE cross-link.
Modeling of the TMS3 of SecE as an α-helix structure shows that Ala99, Leu106 and Gly110 are confined to the same face of the putative α-helix (figure 7). This result confirms the predicted α-helix structure of TMS3 of SecE, and demonstrates that the interaction between neighboring SecE molecules is confined to a specific side of the α-helix.

To exclude that the formation of the SecE dimers was induced by the high concentration of SecYEG upon overexpression, the oxidation assay was repeated with IMVs containing low amounts of SecYE(L106C)G. A low level of SecYE(L106C)G production was achieved by growing cells harboring plasmid pET627 in the presence of 0.5% glucose and omitting IPTG to prevent induction of the trc promotor activity.

Western Blotting of dilution series showed no more than a 2 to 3-fold increase in the amount of SecE (see also figure 2B) and SecY (data not shown).
shown) as compared to the endogenous level. Overexpression upon induction by IPTG results in at least 250-fold increase in the SecYEG level (data not shown, and see figure 2B). Samples of IMVs derived from cells harboring pET324 (empty control vector) and pET627 (SecYE(L106)G) with or without induction of overexpression by IPTG were analyzed after oxidation by SDS-PAGE and immunodetection using an antibody against his-tagged SecE (figure 2B). Oxidation of IMVs harboring low amounts of SecYE(L106C)G resulted in the efficient cross-linking of the SecE dimer (figure 2B). This demonstrates that the formation of this complex also occurs at levels of SecYEG that are comparable to those found in wild-type membranes.

**SecE TMS3 contacts SecY TMS7**

To identify further contacts between SecE and SecY, the single-cysteine mutations in SecE TMS3 (L95C, I96C, V97C, A98C, A99C) and SecY TMS7 (V274C, I275C, P276C, A277C, I278C, F279C, A280C, and S281C) were co-expressed yielding 40 pairs of cysteine mutants. The activity of the SecY TMS7 mutants have been analyzed previously, and all mutants behave normally except for SecY(I278C)EG that exhibits a *prlA* phenotype [173]. All pairs of cysteine mutants showed normal levels of overexpression (figure 3A; data not shown). IMVs were oxidized with Cu²⁺(phenantroline)₃ and analyzed by SDS-PAGE and CBB-staining (figure 3A) or immunoblotting using antibodies against SecY and SecE (figure 3B; data not shown). For two double-cysteine combinations, a higher molecular mass band was observed upon oxidation at the position of an expected SecY-SecE cross-link. The formation of the cross-link product of SecY(P276C) and SecE(V97C) was very efficient, resulting in a near-to-complete disappearance of monomeric SecY and SecE. This result implies a stoichiometric interaction between SecE and SecY that was previously assumed but not demonstrated experimentally. Cross-linking between SecY(P276C) and E(L95C) was less efficient. Modeling shows that both contacts cannot be confined to the same α-helical face between the corresponding transmembrane segments (figure 7A).

**SecY TMS7 and SecE TMS3 contact interface is α-helical**

To further investigate the structure of the interface between SecY TMS7 and SecE TMS3, additional double-cysteine combinations were constructed of residues that are located deeper in the membrane. Four SecY TMS7 cysteine mutations (F279C, A280C, S281C, and S282C) were combined with two additional SecE TMS3 cysteine mutations (V100C and T101C). The respective SecYEG complexes again exhibit normal overexpression levels and translocation activities (data not shown). Upon the oxidation of the pairs of cysteine mutants, two additional SecY-SecE cross-links could be identified after SDS-PAGE and immunoblotting using antibodies against SecY and SecE (figure 3B). These cross-links occurred between SecY(A280C) and SecE(V100C) or SecE(T101C), but the efficiency was less as compared to the SecY(P276C)-SecE(V97C) combination. Modeling of TMS7 of SecY and TMS3 of SecE as α-helical segments shows that these newly identified contacts are confined to the same helical interface as the SecY(P276C)-SecE(V97C) cross-link (figure 7). Since the cross-link between SecY(P276C) and SecE(L95C) cannot be modeled into this α-helical interface, we assume that SecE Leu95 is located outside the membranous environment in a non α-helical structure (figure 7b).

**Interaction between SecY and SecE within an oligomeric SecYEG complex**

Since the SecY(P276C)-SecE(V97C) and intermolecular SecE(L106C) cross-links are very strong, a triple-cysteine mutant of the SecYEG complex was constructed to determine if such contacts occur within an oligomeric SecYEG complex. IMVs prepared from cells expressing the SecY(P276C)E(V97C, L106C)G showed normal levels of overproduction (data not shown) and activity (figure 5). Immunodetection of oxidized samples showed
the presence of strong SecY-(SecE)₂ and weaker (SecY)₂-(SecE)₂ cross-link products (figure 4). Similar but less efficient cross-link products could be demonstrated for a SecY(P276C)E(V97C)G mutant (data not shown). These data therefore suggest that the identified SecE-SecE cross-links are at a contact interface between two separate SecYEG complexes. In all samples, a band is observed that stains with the antibody against SecE at the position of SecY-(SecE)₂ (figure 4). However, this band is unrelated to these proteins as it is also present in the reduced samples and it does not stain with the antibody against SecY. Furthermore, in all samples a diffuse protein band was detected that stains with the SecY antibody and that migrates with a molecular mass that is in between that of the SecY-(SecE)₂ and (SecY)₂-(SecE)₂ cross-linking products (figure 4). Since this band was observed also with the cysteine-less SecYEG complex, albeit weaker, we assume that it represents an aggregated SecY dimer whose formation is stimulated by the oxidation.

SecY-SecE and SecE-SecE cross-linking reversibly inactivate the translocase

IMVs harboring overexpressed cysteine-less SecYEG, SecYE(L106C)G, SecY(P276C)E(V97C)G and SecY(P276C)E(V97C, L106C)G were assayed for the effect of cross-linking on the translocation activity. IMVs were either reduced in the presence of DTT or oxidized with Cu²⁺(phenantroline)₃. Oxidized samples were “re-reduced” by the incubation in DTT, and the activity was assayed by the translocation of ¹²⁵I-proOmpA (figure 5). The activity of the cysteine-less SecYEG was not affected by the oxidation-reduction conditions. Under oxidizing conditions, however, translocation of proOmpA proceeded only to the I₂₉ intermediate due to the presence of a disulfide bond in the C-terminal end of proOmpA [124]. All three cysteine mutant SecYEG complexes displayed normal activity under reduced condition, but were nearly completely inactive under oxidizing conditions. With all mutants, substantial activity could be recovered after re-reduction of the samples.
The interhelical SecY TMS7-SecE TMS3 contact is retained during translocation

Using the thiol-mediated contact between neighboring SecE molecules as a molecular ruler, we have shown previously that during the initiation of preprotein translocation, the intermolecular SecE contact is enhanced [124]. To investigate the dynamics of the SecY-SecE contact, the same technique was employed using the SecY(P276C)E(V97C)G complex. IMVs were pre-incubated in DTT, incubated under various translocation conditions and subsequently oxidized with Cu²⁺(phenanthroline)₃ to probe for the efficiency of SecY-SecE cross-linking. Because formation of the SecY-SecE contact is already very efficient under non-translocating conditions, a less-than-optimal condition of oxidation (on ice) was applied to be able to detect changes in the cross-linking efficiency. Translocation conditions were induced by the addition of SecA and the preprotein proOmpA in the presence of ATP, AMP-PNP, or ATP in the presence of the SecA ATPase inhibitor azide [90]. None of these conditions, however, resulted in a significant change in the efficiency of the SecY-SecE cross-linking (figure 6). Therefore, it is concluded that TMS7 of SecY and TMS3 of SecE remain in close proximity during protein translocation.
Discussion

Cysteine-scanning mutagenesis is a technique that can be used to map the sites of contacts between the helices in membrane proteins. We have used this technique to obtain more detailed information about the interaction of the two essential components SecY and SecE of the protein-conducting channel of *E. coli*. This study provides experimental evidence that TMS3 of SecE forms an α-helix with one face contacting a neighboring SecE molecule whereas the other face contacts TMS7 (this paper) and TMS2 [124] of SecY.

Previously, we have identified position Leu106 of TMS3 of SecE as a site of contact with a neighboring SecE molecule [124]. In that study, five unique cysteine substitutions were introduced near to the periplasmic membrane interface of TMS3 of SecE (S105C-W109C) in order to cover a complete turn of an α-helix. To determine if this contact interface extends along the entire putative α-helical structure, six additional cysteine mutants were made close to the cytosolic (L95C-A99C) and periplasmic (G110C) membrane interface. In this screen, we now have identified two additional amino acid residues in TMS3 of SecE that map at the interface between two SecE molecules, i.e., Ala99 and Gly110. Modeling of TMS3 of SecE as an α-helical structure demonstrates that these residues are strictly confined to a specific side of the α-helix (figure 7). Interestingly, the cross-linking efficiency for the L106C mutation is significantly higher than for A99C and G110C. This could mean that the two contacting transmembrane segments are tilted or twisted relative to each other, with an optimal point of contact around Leu106.

By combining a set of single cysteine mutants in TMS7 of SecY (V274C-S282C) [173] with mutants in TMS3 of SecE (L95C-T101C), several sites of contact between SecY and SecE could be detected. In particular, SecY(P276C) to SecE(V97C) could be efficiently cross-linked (figure 3 and 4). The same position of SecY also cross-linked to SecE(L95C) albeit with low efficiency (figure 3A). SecY(A280C) was found to contact both SecE(V100C) and SecE(T101C) (figure 4). The identified sites of contact fit with an α-helical contact interface, except for the SecY(P276C)-SecE(L95C) interaction (figure 7). Considering the low efficiency of cross-linking of the latter cysteine pair, and the uncertainty in predicting the borders of the TMSs, it seems likely that Leu95 of SecE is located outside the membrane plane and is not part of the α-helical structure of TMS 3. This is illustrated in figure 7B.

The cross-linking of SecY(P276C) with SecE(V97C) was nearly complete leaving little non-reacted monomeric SecY and SecE upon oxidation. This implies that there is only one SecE molecule per SecY subunit in the SecYEG complex. In this respect, their interdependent stability [112,114], similar levels of overexpression [174] and well-defined regions of interaction [116,122-124] are consistent with the experimentally determined SecY-SecE stoichiometry.

The cysteine-scanning mutagenesis ([124], this paper) now provides a detailed image of the molecular environment of SecE TMS3 (figure 7). Herein, TMS3 of SecE is surrounded by another TMS3 of SecE and TMS2 and TMS7 of SecY. In the current arrangement, there is ample space for at least one other SecE contacting helix. Based on the predictions made using the synthetic lethality of combinations of prlA and prlG mutants [122], TMS 10 of SecY seems a likely candidate. It is of interest to note that the amino acid residue positions that cause the synthetic lethality of prlA208 (SecY TMS7: I278N) with prlG1 (SecE TMS3: L108R) do not map at the SecY TMS7 - SecE TMS3 contact interface (figure 7). The same holds true for all of the prlA mutations that map in TMS2 and TMS7 of SecY. None of these amino acid residues face TMS3 of SecE, but instead point in the opposite direction (figure 7). Strikingly, these mutations lead to a destabilization of the SecY-SecE interaction [171], in particular when PrlA and PrlG mutants are combined [122].

Biochemical and electron microscopic studies [159,160] suggest that the SecYEG complex oligomerizes to form a protein...
conducting channel. The structure consists of a tetrameric SecYEG complex with an outer diameter of 10.5-12 nm and a central pore-like opening [160]. The shape of the tetrameric SecYEG complex resembles that of the ribosome-bound purified eukaryotic Sec61p complex visualized by cryo-electron microscopy which is a ring-like structure, 5–6 nm high and ~9 nm wide [149,150]. The cylindrical pore of ~2 nm in diameter extends throughout the protein complex perpendicular to the plane of the membrane, and aligns with the protein exit tunnel of the ribosome [149]. A recent chemical cross-linking and immunoprecipitation study failed to demonstrate an oligomeric SecYEG complex, and it was suggested that the SecYEG complex functions as a monomer [165]. It was argued that the formation of an oligomeric SecYEG complex is an artifact of high overproduction levels and purified proteins. Our specific cysteine-mutagenesis assay demonstrates that the oligomeric SecYEG complex can also be formed at SecYEG levels comparable to that of wild-type. Yahr and Wickner [165] used the non-specific cross-linker formaldehyde in their

Figure 7. Schematic representation showing the identified sites of interaction between TMS2 and TMS7 of SecY and TMS3 of SecE, and the identified sites of interaction between TMS3 of neighboring SecE molecules. (A) Top view. TMSs are schematically drawn as helices. Cysteine-substituted residues involved in the cross-linking are shown (closed circles) and connected by a line with the interacting amino acid residues. Sites of prlA mutations are depicted as open circles. (B) Side view. Interacting cysteine-substituted residues are indicated in black boxes, and sites of known prlA mutations are depicted in thicker outlined boxes. The sites of interaction are shown by connecting lines. The weak cross-link between Leu95 of SecE and Pro276 of SecY is indicated by a dotted line.

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study. SecY is, however, very unstable in the presence of low concentrations of organic solvents such as ethanol and formaldehyde, and readily denatures. This may explain the failure to detect SecY-SecY and SecY-SecA cross-links [134]. Interestingly, a recent paper on the projection structure of the SecYEG complex indicates that the molecule is arranged as a dimer in the crystal lattice [161]. Moreover, the same study shows by analytical ultracentrifugation, a monomer-dimer-tetramer equilibrium of the SecYEG complex. Also, by blue native PAGE, the presence of oligomeric SecYEG complexes is evident (J. de Keyzer, unpublished results). Taken together, these studies strongly suggest that the SecYEG complex is able to assemble into oligomeric structures.

We have combined the strong SecY(P276C)-SecE(V97C) and SecE(L106C)-SecE(L106C) contacts by means of a triple-cysteine mutant. Oxidation of the SecY(P276C)SecE(V97C,L106C)G complex clearly demonstrates the formation of products that correspond to SecY-(SecE)2 and (SecY)2-(SecE)2 cross-links. This unequivocally demonstrates that the SecE-SecE interaction takes place at a contact interface between two neighboring SecYEG complexes. Another cysteine scanning mutagenesis study demonstrates that two SecG molecules are in close proximity [71]. It should be stressed that disulfide cross-linking is a very sensitive technique that detects sites of interaction only when they are in close proximity, i.e., within a distance of 3-4 Å [120]. Taken together, these data collaborate with the electron microscopy studies that demonstrate that the SecYEG complex can oligomerize into a large complex [160].

The formation of thiol-stabilized SecY-SecE and SecE-SecE contacts reversibly inactivates the activity of the translocase (figure 5) [124]. Apparently, flexibility within and between SecYEG complexes is an essential requirement for the translocation mechanism. This may signify conformational changes or dynamic subunit interactions during the translocation reaction, as previously shown for the SecE-SecE contact [124]. By blocking SecA membrane de-insertion, a significant enhancement of the SecE-SecE cross-linking efficiency was observed. This phenomenon strictly required the presence of a preprotein showing its relation to the translocation process. On the other hand, the same conditions do not result in an alteration of the SecY-SecE cross-linking as shown for the interaction between SecE TMS3 with SecY TMS2 [124] and SecY TMS7 (figure 6, this study). A cross-linking study with the yeast Sec61α, a SecY homolog, demonstrates the signal sequence of an inserting preprotein contacts TMS7 and TMS2, while no contact could be detected with Sec61γ [155]. This has led to the hypothesis that Sec61γ (i.e., TMS3 of SecE) might function as a kind of mock signal sequence that is displaced from Sec61α (i.e., SecY) upon the insertion of the signal sequence of a preprotein [155]. This mechanism is not plausible for the SecYEG complex as the interaction between TMS2 and TMS7 of SecY with TMS3 of SecE stably persists during translocation. It seems more likely that the signal sequence contacts the helical faces of TMS2 and TMS7 that point away from TMS3 of SecE. Remarkably, these faces correspond to the sites of the prlA mutations (figure 7).

The hypothesis that SecYEG functions as monomer [165] requires a large re-arrangement of the SecYEG helices in order to open a translocation pore that can accommodate the inserting SecA molecule and preprotein [200] while shielding SecA from contact with the phospholipids phase [201,202]. Our studies do not support such large helical re-arrangements, as both TMS2 and TMS7 of SecY remain in close proximity to TMS3 of SecE during translocation. Rather, our studies indicate the recruitment of multiple SecYEG complexes much akin to a ‘rigid body’ assembly event. To further test this model, future biochemical experiments should be directed at the mapping of the intramolecular contacts between the helices within the SecY protein.
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SecYEG oligomerization probed by Fluorescence Resonance Energy Transfer

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Summary

The heterotrimeric SecYEG complex constitutes the protein-conducting channel in bacteria. We have purified and fluorescently labeled single cysteine mutants of SecE to study the quaternary structure of SecYEG reconstituted into proteoliposomes by means of fluorescence resonance energy transfer (FRET). The data show that SecYEG oligomerizes in a lipid environment and that exchange of SecYEG subunits can occur between oligomers.
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Introduction

Protein translocation in bacteria is mediated by the translocase that consists of the heterotrimeric membrane protein complex SecYEG [28,139] and the membrane associated ATPase SecA [3]. The latter drives the stepwise translocation of proteins [86,87] while SecYEG constitutes an evolutionary conserved protein-conducting channel [13,40,203]. Various studies on the channel organization have revealed oligomeric forms of SecYEG [71,124,125,130,149-151,157,159-164] although it has also been proposed that the channel is formed by a single SecYEG molecule [128,165]. In Escherichia coli, SecYEG exists as monomeric, dimeric and tetrameric species. The dimer interface between neighboring SecYEG complexes is formed by transmembrane segment 3 (TMS3) of SecE in which three sites of contact have been identified by cysteine scanning mutagenesis [124-126]. In this work, we have used positions in and close to the dimer interface to conduct fluorescence resonance energy transfer (FRET) experiments to provide insight in the quaternary form of SecYEG reconstituted into proteoliposomes.

Experimental procedures

Materials

SecA [27] and proOmpA [190] were purified as described previously. Oregon Green® 488 maleimide, Texas Red® maleimide and tris-(2-carboxyethyl)phosphine (TCEP) were purchased from Molecular Probes (Leiden, The Netherlands). Stock solutions of 40 mM of Oregon Green and Texas Red were prepared in dimethylformamide (DMF) and dimethylsulfoxide (DMSO), respectively. SOURCE™ 30Q column material and HiTrap™ Chelating HP columns were purchased from Amersham Biosciences (Freiburg, Germany).

Plasmids, cell cultures and membrane vesicle isolation

The plasmids used to overproduce histagged SecYEG are pET607, pET2501 and pET2523. They encode cysteine-less SecYEG, SecYE(I96C)G and SecYE(S120C)G, respectively. pET607 [124] and pET2501 [125] were constructed previously, pET2523 was constructed in a similar way as pET2501 and its cysteine mutation (secE A120C (TCC→TGT)) was confirmed by sequence analysis. Cells were grown and inside-out inner membrane vesicles (IMVs) harboring overproduced SecYEG were isolated as described previously [124].

Purification, cysteine labeling and membrane reconstitution of SecYEG

SecYEG was purified essentially as described in [204]. In short, IMVs preparations were solubilized in 2% dodecyl maltoside (DDM) and SecYEG was purified by anion exchange chromatography (via SOURCE 30Q column material), followed by affinity chromatography (via a Ni²⁺-charged HiTrap Chelating HP column). The cysteine-directed fluorescent dye labeling was performed in between the two purification steps. For this, eluted fractions harboring semi-purified SecYEG from the anion exchange chromatography, were pooled and incubated with a two-fold molar excess of TCEP over protein for 15 min at 4 ºC to reduce the cysteine residues. Subsequently, a ten-fold molar excess of Oregon Green or Texas Red dye over protein was added. Labeling was carried out at a pH of 8.0 for 60 min at 4 ºC. The reaction was quenched by the addition of a ten-fold molar excess of reduced glutathione over dye for 15 min at 4 ºC. The following second purification step removed unbound dye from the samples. The labeling specificity was analyzed by 15% SDS-PAGE, followed by visualization of fluorescence on a Roche Lumi-Imager F1. Subsequently, the gel was stained with Coomassie Brilliant Blue (CBB) to evaluate the purification steps. Finally, SecYEG was reconstituted into liposomes, extracted from E. coli lipids, by rapid dilution as described before...
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[184]. The proteoliposomes were recovered by ultracentrifugation and resuspended in buffer A (50 mM Tris-HCl pH 8.0; 50 mM KCl).

Spectrofluorometry

All measurements were performed on a SLM Aminco-Bowman Series 2 Luminescence spectrometer (SLM Aminco, Urbana, IL) with excitation and emission slit widths set to 4 nm. Oregon Green fluorescence was excited at 480 nm and emission scans were recorded as indicated in the figures. Proteoliposomes were diluted in the cuvette with buffer A prior to the measurements. Emission scans of tryptophan fluorescence (excited at 280 nm) were recorded to determine the relative protein concentration in the proteoliposomes, and the concentration was corrected if necessary.

Miscellaneous methods

ProOmpA stimulated SecA ATPase activity was determined as described [132]. Fusion of the proteoliposomes was induced by 3 cycles of freezing in liquid nitrogen and thawing at room temperature, followed by dis-aggregation by sonication for 10 seconds in a bath sonicator. Protein concentrations were determined with the DC Protein Assay (Bio-Rad Laboratories, Hercules, Canada) using bovine serum albumin (BSA) as a standard.

Results

Functional reconstitution of cysteine labeled SecYEG into liposomes

For fluorescence measurements, proteoliposomes were used containing SecYEG with a fluorescent probe derivatized to a unique cysteine residue at position Ile-96 or Ser-120 in SecE. For this purpose, IMVs harboring overproduced cys-less SecYEG, SecYE(I96C)G or SecYE(S120C)G were solubilized in 2% DDM and SecYEG was purified in a two-step purification assay using the his6-tag at the amino-terminus of SecY. Cysteine specific labeling was carried out with maleimide derivatives of Oregon Green or Texas Red, and the labeled SecYEG complex was repurified by Ni-NTA chromatography to remove unbound probe. The final product was analyzed by Coomassie-stained SDS-PAGE for its purity (data not shown) and by *in gel* fluorescence imaging to assess the cysteine labeling (figure 1). The cysteine in SecYE(I96C)G could be labeled specifically under conditions that allow only weak labeling of the other proteins (e.g. SecY) or of the cys-less SecYEG. A similar result was obtained for SecYE(S120C)G (data not shown). The efficiency of labeling of the indicated SecE cysteine mutants was estimated by calculating the probe to protein molar ratio as determined by absorption measurements and was in the range of 70-90% (data not shown).

Purified and labeled SecYEG was reconstituted into liposomes and tested for its ability to support proOmpA stimulated SecA ATPase activity. The results show that the presence of the dye in SecE does not affect the translocation ATPase activity (figure 2).
Energy transfer reveals SecYEG oligomerization

As a first step, tryptophan emission scans were recorded to determine and normalize the relative protein concentration of the samples in the cuvette. Fluorescence measurements show that the Oregon Green or Texas Red dyes do not interfere with the tryptophan emission (data not shown). Donor (Oregon Green) and acceptor (Texas Red) labeled SecYEG were mixed in a 1:1 ratio either before or after reconstitution into liposomes. In the first case, donor and acceptor fluorophores co-localize within the same liposomes, while in the latter case, donor and acceptor fluorophores are localized in separated liposomes. Fluorescence emission scans were recorded with the excitation wavelength set to 480 nm to excite the Oregon Green fluorophore. A significantly lower fluorescence around 520 nm, which corresponds to the expected Oregon Green emission maximum, was observed for both SecYE(I96C)G and SecYE(S120C)G when the donor and acceptor fluorophores are co-localized as compared to conditions when donor and acceptor are present in separate liposomes (figure 3). This clearly indicates the occurrence of energy transfer. For SecYE(I96C)G, the decrease in Oregon Green emission is accompanied by an increase in fluorescence around 610 nm representing the Texas Red emission (figure 3A). This effect was not observed for SecYE(S120C)G (figure 3B). In another FRET experiment, Oregon Green labeled SecYEG proteoliposomes were mixed with Texas Red labeled SecYEG proteoliposomes in a 1:1 ratio and subsequently subjected to freeze/thaw and sonication treatment. The emission scan reveals that this treatment also results in lower Oregon Green emission fluorescence, indicative of energy transfer (figure 4).

Discussion

The SecYEG complex constitutes the protein-conducting channel in bacteria. Various studies have demonstrated the existence of oligomeric forms of SecYEG [63,71,124,125,130,149-151,157,159-164]. In this work, we have conducted FRET experiments to determine if SecYEG exists in an oligomeric state when reconstituted into liposomes. A similar study has been done on the SecYE complex from Thermus thermophilus HB8 in which oligomerization of SecYE in proteoliposomes was demonstrated by FRET [163]. Energy transfer comes from the
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Figure 3. FRET analysis of SecYEG reconstituted into proteoliposomes. Fluorescence spectra of Oregon Green (donor) and Texas Red (acceptor) labeled SecYEG proteoliposomes. A, SecYE(I96C)G. B, SecYE(S120C)G. Spectra of 1:1 mixtures of Oregon Green and Texas Red labeled SecYEG before (dotted line) and after (solid line) reconstitution into proteoliposomes. The excitation wavelength was set to 480 nm.

absorption of the excited-state energy from one fluorophore (the donor) by another fluorophore (the acceptor). This phenomenon is reflected by a decrease in emission fluorescence of the donor (since less photons are emitted) and an increase in emission fluorescence of the acceptor (that is excited by the absorbed energy from the donor). The prerequisite for energy transfer is a spectral overlap of the emission fluorescence the donor with the excitation fluorescence of the acceptor. The efficiency of energy transfer depends on the level of spectral overlap, the distance between the two fluorophores and their relative orientation in space. We have chosen Oregon Green and Texas Red as the donor-acceptor couple, since their overlapping spectra make them suitable candidates for use in FRET analyses. The Förster radius ($R_o$), which defines the distance at which the energy transfer is 50% efficient, has not been determined for the Oregon Green-Texas Red couple. However, the typical value for $R_o$ is ~50 Å for donor-
acceptor couples with characteristics and spectra similar to Oregon Green and Texas Red, such as the fluorescein-rhodamine pair [205, 206].

SecE was used for fluorescent labeling since TMS3 of SecE is known to form the dimer interface between neighboring SecYEG complexes [124, 125]. Specific positions in SecE can be studied by site-directed labeling of cysteine-substituted residues, but Ser-120 was selected for its position in the periplasmic loop 1 that is accessible for maleimides from the solution. This loop connects to TMS3 and thus is close to the dimer interface. Furthermore, a cysteine-directed cross-linking study suggested a possible SecE dimer cross-link formation at the cysteine-substituted Ser-120 [123]. We have also targeted a residue in TMS3 of SecE that is localized close to the cytosolic face of the membrane. Initially, Ala-99 was chosen as it represents one of the sites of contact in the SecE dimer interface [125], which also senses a conformational change upon ATP binding (Chapter 5). However, this membrane embedded position was not readily accessible to fluorophores (data not shown). As an alternative, Ile-96 was targeted since this position is close to Ala-99 while still accessible for fluorophores. Importantly, the labeled SecYEG complexes are fully functional as they support proOmpA stimulated SecA ATPase activity (figure 2).

Energy transfer was observed that appeared as a significant loss in donor (Oregon Green) emission fluorescence when populations of donor and acceptor fluorophore labeled SecYEG complexes are co-localized in the same liposome environment (figure 3). This effect was seen for both I96C and S120C labeled SecE. High concentrations of donor and acceptor labeled proteins in the liposomes could lead to random transient contacts and thus possibly result in energy transfer. However, the typical protein to lipid molar ratio for SecYEG reconstituted proteoliposomes is ~1:4000 and under these conditions energy transfer by non-specific contacts can be ruled out [163]. We can therefore conclude that the observed energy transfer signifies the association of SecYEG complexes into oligomers in proteoliposomes.

Energy transfer could also be induced when donor and acceptor labeled SecYEG, originally in separate liposome populations, are co-localized by liposome fusion as a result of...
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freeze/thaw treatment (figure 4). This suggests a dynamic exchange of subunits between oligomers of SecYEG in the liposome environment. Interestingly, our finding seems to contradict with the FRET data for the SecYE from *T. thermophilus* that suggest no apparent exchange of subunits [163]. Since Mori and co-workers have performed FRET experiments on labeled SecY instead of SecE, a remote possibility is that exchange occurs between SecE but not between SecY subunits. However, SecY and SecE do not dissociate in vivo [114], which must imply that complete SecYE(G) complexes are exchanged between oligomers. Possibly the higher physiological temperature or the lack of a SecG homologue may cause a significant slower exchange rate of subunits for the SecYE complex from the thermophilic bacterium *T. thermophilus*. Also, the FRET analysis were done on *T. thermophilus* SecYE complex reconstituted into E. coli liposomes and the subunit exchange rate may be affected in such a heterologous system.

For FRET experiments with labeled SecYE(I96C)G, the observed energy transfer is accompanied by Texas Red emission fluorescence (figure 3a). This was not the case for experiments done with labeled SecYE(S120C)G (figure 3b). A longer distance between the donor and acceptor dyes or suboptimal relative orientation of the dyes may explain the apparent lack of acceptor emission for position Ser-120. Minimal acceptor emission fluorescence can also be noticed in other FRET studies [163].

Conformational changes in the protein-conducting channel at specific positions in the SecE dimer interface were observed in cysteine-directed cross-linking studies under conditions that result in a translocation-arrested state by blocking the SecA deinsertion step or by the addition of nucleotides ([124], chapter 5). However, no noticeable change in FRET could be detected in initial experiments using SecYEG proteoliposomes to which similar conditions were applied (data not shown). Moreover, nucleotides were found to greatly affect the fluorescence emission of Oregon Green, thus hampering the fluorescence measurement. Possibly, the use of a different donor-acceptor couple may be required to study conformational changes in the SecYEG complex by FRET analysis.

Our results show that SecYEG forms oligomers in proteoliposomes. This conclusion is in agreement with other structural studies on the protein-conducting channel. Furthermore, SecYEG subunits can be exchanged between oligomers. Our data provides a basis for future FRET studies on the quaternary structure of the protein-conducting channel.
The core of the bacterial translocase harbors a tilted transmembrane segment 3 of SecE

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Summary

The bacterial translocase mediates the translocation and membrane integration of proteins. The integral membrane proteins SecY and SecE are conserved core subunits of the translocase. Previous cysteine scanning studies showed that the transmembrane segment (TMS) 3 of SecE contacts TMS 2 and 7 of SecY, and TMS 3 of another SecE. We now demonstrate that SecE also contacts TMS 10 of SecY. Combining all available cysteine scanning mutagenesis data, a three-dimensional model has been built in which the positions of the helices that form the central core of the bacterial translocase are mapped. Remarkably, this model reveals that TMS 3 of SecE is strongly tilted relative to SecY.