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Functional and structural analysis of the Escherichia coli translocase
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Chapter 5

SecYEG assembles into a tetramer to form the active protein translocation channel

Translocase mediates preprotein translocation across the *Escherichia coli* inner membrane. It consists of the SecYEG integral membrane protein complex and the peripheral ATPase SecA. Here we show by functional assays, negative-stain electron microscopy and mass measurements with the scanning transmission microscope that SecA recruits SecYEG complexes to form the active translocation channel. The active assembly of SecYEG has a side length of 10.5 nm and exhibits an approximately 5 nm central cavity. The mass and structure of this SecYEG as well as the subunit stoichiometry of SecA and SecY in a soluble translocase-precursor complex reveal that translocase consists of the SecA homodimer and four SecYEG complexes.

INTRODUCTION

*Escherichia coli* translocase consists in its minimal form of three integral membrane proteins termed SecY, SecE and SecG, and a peripheral ATPase SecA (Brundage et al., 1990; Hanada et al., 1994). SecY and SecE form a stable stoichiometric complex in the cytoplasmic membrane that does not dissociate *in vivo* (Matsuyama et al., 1990; Joly et al., 1994). SecYE is purified from the cytoplasmic membrane as a soluble complex together with the SecG protein. SecG strongly stimulates the *in vitro* translocase activity (Nishiyama et al., 1993) and therefore the SecYEG complex is used for the reconstitution of the translocation reaction in proteoliposomes (Hanada et al., 1994; Douville et al., 1995; Van der Does et al., 1998). SecA is a homodimeric protein that serves both as a receptor for precursor proteins and as an ATP-driven molecular motor during the translocation reaction (Economou, 1998; Driessen et al., 1998). SecA binds to the cytoplasmic membrane with a low affinity for phospholipids and a high affinity for the SecYEG complex (Hendrick and Wickner, 1990; Hartl et al., 1991). It binds to SecYEG, at least partially, via a direct interaction with SecY (Snyders et al., 1997, Manting et al., 1997). Upon binding to SecYEG, SecA is activated for precursor-stimulated cycles of ATP-binding and -hydrolysis (Lill et al., 1990). This process permits the stepwise movement of a translocating polypeptide chain across the membrane (Schiebel et al., 1991) by a two-stroke reaction (Van der Wolk et al., 1997). Translocation is further stimulated by the presence of a proton-motive force, which ensures unidirectionality of the translocation reaction and facilitates the SecA reaction cycle (Driessen, 1992; Nishiyama et al., 1999).

SecA is a highly dynamic protein. Calorimetric studies of the nucleotide-modulated structural changes of the soluble SecA molecule demonstrate that it exists in a compact, ADP-bound state and an extended, ATP-bound state (Den Blaauwen et al., 1996). During translocation, SecA inserts into the cytoplasmic membrane with both a 30 kDa carboxy-terminal domain and a 65 kDa amino-terminal domain (Economou et al., 1994; Price et al., 1996; Eichler and Wickner, 1997). SecYEG appears to form a large transmembrane structure that accommodates at least a part of SecA together with a translocating precursor protein, shielding both from phospholipids (Joly and Wickner, 1993; Eichler et al., 1997; van Voorst et al., 1998). The *Bacillus subtilis* SecYE complex and the homologues eukaryotic Sec61p complex have been visualised by electron microscopy as quasi-pentagonal structures with a diameter of 8.5 nm and a central cavity with a diameter of 1.5-2 nm (Hanein et al., 1996; Meyer et al., 1999). Beckmann et al. (1998) have reported the three-dimensional structure of the ribosome-bound Sec61p at 2.6 nm resolution. It is a 4 nm thick disk with a diameter of 9.5 nm containing a 1.5-3.5 nm wide central pore.

In this study, we demonstrate that upon solubilization, a majority of *E. coli* SecYEG form
dimers that have a length of 8.5 nm, a width of 6.5 nm and a weakly stained central indentation. This SecYEG dimer resembles the *B. subtilis* SecYE complex described by Meyer *et al.* (1999), but does not represent the active translocation channel. Membrane insertion of SecA triggers the assembly of SecYEG into a larger complex consisting of four SecYEG subunits, that has a width of 10.5 nm and a central stain-filled depression of about 5 nm width. To confirm the presence of tetrameric SecYEG in active translocase, a translocation intermediate of the preprotein proOmpA was used to stabilise translocase in detergent solution. This soluble translocase-precursor complex contains four SecY molecules per SecA dimer, as determined by quantitative immunoprecipitation and corroborated by mass measurements. We propose that SecA recruits and assembles four SecYEG complexes to form the active translocase. The SecYEG tetramer is sufficiently large to accommodate a central cavity or pore of 4-6 nm size, that has been observed with the Sec61p complex under translocating conditions (Hamman *et al.*, 1997).

**RESULTS**

SecYEG is solubilised as a dimer. Translocase was studied in a purified system using proteoliposomes reconstituted with SecYEG (Van der Does *et al.*, 1998). First, SecYEG was resolubilised in dodecyl maltoside without prior incubation with the other components of the translocation reaction. Excess phospholipids were removed from the soluble SecYEG complex by anion exchange chromatography, without a detectable loss of the SecYEG activity (data not shown; see Materials and Methods). The complex was then visualised using negative-stain electron microscopy (EM). In uranyl-formate stained preparations, the SecYEG complex was visible as an elongated structure with a length of 8-9 nm and central stain-filled indentation (Figure 1). Single particle analysis and averaging of the 8 nm structures resulted in classes of structures that resembled each other in shape and size (Figure 1, right inserts). Smaller particles were discerned as well, but their structural features were less distinct. Single particle analysis and averaging of the 8 nm structures resulted in classes of structures that resembled each other in shape and size (Figure 1, right inserts). The analysis of the small particles yielded an average with 6.8 nm length and 4.5 nm width (top right inset in Figure 1). The structure of the 8 nm *E. coli* SecYEG complex is similar in

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**Fig. 1. Structural analysis of soluble SecYEG.** Electron microscopy of negatively stained SecYEG complexes solubilised in dodecyl maltoside reveals particles of two distinct sizes (overview, scale bar = 100 nm). While particles with a size of ~5 nm lack distinct structural features, the larger particles of 8-9 nm are elongated, sometimes having a central stain-filled pit (bottom inserts, scale bar = 10 nm). Single particle analysis and class averaging yields projection maps with a width of 6.7 nm, a length of 8.7 nm, and a more or less pronounced 2 nm wide stain-filled indentation with all three major classes, containing about 450 particles each (right inserts, scale bar = 5 nm). A small size reduction from the top average to the bottom average indicates that top views and bottom views were recorded. The major class average of the smaller particles is shown in the top right inset and has a width of 4.5 nm and a length of 6.8 nm. It is close to half an elongated particle.
Fig. 2. Formation of large SecYEG structures after incubation with membrane-inserted SecA. SecYEG proteoliposomes were incubated with SecA and AMP-PNP, solubilised and subjected to anion-exchange chromatography. The fraction containing SecYEG was visualised by negative-stain electron microscopy revealing a heterogeneous size distribution (overview, scale bar = 100 nm). A significant fraction of particles had a size of 10-12 nm, a 4-6 nm central stain-filled cavity and either a squarish or elongated shape (bottom inserts, scale bar = 10 nm). Major class averages (200-400 particles) were generated by single particle analysis (right inserts, scale bar = 5 nm). The size variation of the tetrameric projections indicates that top views and bottom views were recorded.

Membrane inserted SecA triggers tetramerization of SecYEG. In the next experiment, SecYEG proteoliposomes were incubated with SecA in the presence of AMP-PNP. This non-hydrolysable ATP analogue triggers membrane-insertion of the SecA protein even in the absence of preprotein (Economou et al., 1994). Proteoliposomes were then collected by centrifugation, solubilised in dodecyl maltoside solution and subjected to anion exchange chromatography. The His-tagged SecYEG complex elutes in the very beginning of the salt gradient (Van der Does et al., 1998) and was easily separated from SecA and excess phospholipids. After incubation with membrane inserted SecA, a significant fraction of SecYEG had assembled in 10-12 nm sized structures that often exhibited a prominent central stain-filled depression (Figure 2). Incubation of SecYEG proteoliposomes with SecA in the absence of AMP-PNP did not result in any change in shape or size of SecYEG, as observed with B. subtilis SecYE (Meyer et al., 1999). Due to the bi-directional reconstitution of SecYEG into proteoliposomes (Van der Does et al., 1998), approximately 50 % of the total SecYEG was inactive. Therefore, an inhomogeneous particle distribution emerged, with a major fraction of dimeric SecYEG. The larger particles comprised around 30 % of the particles and often exhibited a square shape and a stain-filled central cavity. Class averages of these square-shaped structures had a side length of 10.5 - 12 nm and a 5 nm wide central stain-filled depression (Figure 2, right inserts). The size and the four-fold symmetry of these structures suggest that SecYEG had assembled into a tetrameric structure upon interaction with membrane-inserted SecA. As with the dimeric SecYEG, the major class averages of the SecYEG...
tetramer resembled each other in size and shape and appeared to represent top or bottom views. The size difference in the class averages however suggests a slightly asymmetrical particle, with a 10.5 nm projection (upper two inserts) and a 12 nm projection (second insert from bottom). Minor structural classes contained oval-shaped particles that may represent tilted projections (Figure 2, lower right insert).

**Formation of a soluble translocase-precursor complex.** A stable translocation intermediate was prepared in SecYEG proteoliposomes complemented with SecA and ATP. The precursor of outer membrane protein A, proOmpA, efficiently translocates in this system (Brundage et al., 1990; Figure 3A, lanes 1-4). Under oxidising conditions, however, proOmpA translocates into the SecYEG proteoliposomes as an intermediate with an apparent molecular mass on SDS-PAGE of 31 kDa (Figure 3A, lanes 6-9). This translocation intermediate, termed I_{31}, resembles the processed I_{29} intermediate formed in inner membrane vesicles due to a disulphide bond between the unique cysteine residues C290 and C302 of proOmpA (Tani et al., 1990; Schiebel et al., 1991). The I_{29} intermediate is associated with SecA and is unstable at 37 °C upon depletion of ATP, reduction of the disulphide bond, or the addition of antibodies against SecA, but is completely stable at low temperature (Schiebel et al., 1991).

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**Fig. 3. Characterization of the I_{31} intermediate.** (A) Translocation of proOmpA (lanes 1-4) or translocation intermediate I_{31} (lanes 5-8). Reconstituted SecYEG (10 µg/ml) was mixed with SecA (60 µg/ml) and proOmpA (20 µg/ml) in the presence or absence of 10 mM DTT. Reactions were energised with 2 mM of ATP, incubated at 37 °C for the indicated time, chilled on ice, and digested with proteaseK (0.1 mg/ml) for 15 min. Accumulation of translocated preprotein was analysed by western blotting using monoclonal antibodies against proOmpA. (B) Densitometrical quantitation of the data in (A). (C) I_{31} blocks active translocation sites. Proteoliposomes that had undergone translocation of I_{31} (lane 1), proOmpA (lane 2), or no translocation (lane 3) were tested for their ability to support a second round of translocation. Reactions were performed for 20 min in the absence of DTT similar as in (A), except for that proOmpA was replaced by 35S-labelled single-cysteine proOmpA (C290S). (D) I_{31} does not support formation of the 30 kDa SecA fragment. Translocations with were performed with ^125I-labelled SecA and in the presence or absence of 10 mM DTT, proOmpA, 2 mM AMP-PNP, or 2 mM ATP, as indicated. After 20 min incubation at 37 °C, or with AMP-PNP at 4 °C, reactions were digested with proteaseK. The 30 kDa SecA fragment was visualised using autoradiography and quantitated by densitometry. The averages and error margins of two experiments are shown as arbitrary densitometric units.
SecYEG oligomers

I$_{31}$ reached its maximum level within 15 min of incubation at 37 °C, while the translocation reaction in the presence of DTT continued nearly linearly up to at least 20 min (Figure 3B). To confirm that I$_{31}$ occupied all active translocation sites, proteoliposomes bearing I$_{31}$ and proteoliposomes that had undergone complete translocation cycles were harvested, and tested for a second round of translocation. SecYEG proteoliposomes that had not been preincubated with proOmpA were used as controls. For this round of translocation, we used $^{35}$S-methionine labelled proOmpA(C290S), a site-directed single cysteine mutant that is unable to form an intramolecular disulphide bond and translocates completely under both reducing and oxidising conditions. Proteoliposomes that had accumulated I$_{31}$ were inactive for proOmpA translocation (Figure 3C, lane 1), whereas those that underwent full translocation were nearly as active as control proteoliposomes not incubated with preprotein (lane 2 and 3). Since I$_{31}$ quantitatively occupies the translocation sites and the accumulation level of fully translocated proOmpA exceeds that of I$_{31}$ (Figure 3B), SecYEG proteoliposomes must allow multiple rounds of translocation. This is supported by their activity after their re-isolation from a previous translocation reaction. Membrane insertion of SecA in the presence of proteoliposomes was assayed using $^{125}$I-labelled protein. As expected, a proteolytically stable 30 kDa fragment was either formed during translocation, or with AMP-PNP. In contrast, I$_{31}$-bound SecA yielded only background levels of the 30 kDa fragment, similar to a control experiment were ATP was omitted. This indicates that I$_{31}$ retains SecA in a deinserted state (Figure 3D).

Fig. 4. Immunoprecipitation of soluble translocase-precursor complexes. Translocation reactions were incubated for 20 min in the presence or absence of DTT, 2 mM ATP, 2 mM AMP-PNP, or 2 mM ATP together with 10 mM NaN$_3$, as indicated. Solubilised proteoliposomes were incubated with proteinA-sepharose beads coated with antiserum against OmpA (top panels), SecYE (bottom panels), SecA (lane 7), or no antiserum (control, lane 8) and precipitated. Precipitates were eluted and analysed by western blotting using monoclonal antibodies against SecA or proOmpA as indicated.

The requirements for a solubilised translocase-precursor complex were established using immunoprecipitation. SecYEG proteoliposomes were incubated with SecA and proOmpA under various conditions, as described below. They were then collected and after solubilization, the interactions between the SecYEG complex, SecA and proOmpA were determined by co-immunoprecipitation (Figure 4). AMP-PNP (Economou et al., 1994) or the ATPase inhibitor sodium azide (Van der Wolk et al., 1997) enforces SecA membrane insertion. However, neither condition gave rise to a translocase-precursor complex that is stable in micellar solution, as only minute amounts of SecA were immunoprecipitated with anti-SecYE or anti-OmpA serum (lanes 1 and 2). After incubation under conditions that resulted in the completion of translocation, some proOmpA but no SecA remained associated with the SecYEG complex upon solubilization (lane 5). This may represent an incomplete release of proOmpA after translocation, due to the absence of the accessory translocase subunit SecDF (Matsuyama et al., 1993) or the proton-motive force (Geller, 1990). A marked increase in the amount of immunoprecipitable proOmpA was observed when the translocation reaction was performed in the absence of DTT to yield I$_{31}$ (lane 6). In addition, SecA co-immunoprecipitated with SecYE and proOmpA, whereas in control samples
Fig. 5. The SecY:SecA ratio in I$_{31}$-bound translocase. Translocase was immunoprecipitated via I$_{31}$ using NHS-sepharose coated with IgG against OmpA. Samples incubated without proOmpA were used as negative controls to verify the specificity of the immunoprecipitation. The amounts of SecA and SecY in the immunoprecipitate were determined by densitometry, using purified SecA (2.6 µM) and SecY (5.1 µM) as standards. Dilutions of the immunoprecipitate (lane 4 and 5) were compared with dilutions of the protein standards (lane 1 and 2) that gave similar intensities after immunostaining, taking the average as the outcome of one experiment. Three immunoprecipitates were in this manner compared with two independent dilution series of the protein standards, yielding a total of six experiments in which the SecY:SecA ratio was determined as 2.0 ± 0.3.

lacking proOmpA or ATP no immunoprecipitation of SecA was observed (lanes 3 and 4). In the reciprocal experiment proOmpA was immunoprecipitated by anti-SecA (lane 7). These data establish that the translocation intermediate I$_{31}$ is sufficiently stable to allow its solubilization.

Translocase contains four SecY molecules per SecA dimer. The purification of a translocation intermediate via antibodies against the precursor offered the possibility to determine the amount of SecA and SecY in translocase by quantitative immunoblotting. First, SecY and SecA concentration standards were prepared. Hexa-histidine tagged SecY was purified from the soluble SecYEG complex via Ni$^{2+}$-NTA chromatography (Manting et al., 1997). Amino acid analysis of this SecY sample determined its concentration as 5.05 (± 0.05) µM, on the basis of 10 amino acids in a duplo analysis. A SecA standard with a concentration of 2.58 (± 0.03) µM was used as a comparison. To obtain purified translocase, we immunoprecipitated the translocase-I$_{31}$ complex with antibodies against OmpA. The amounts of SecA and SecY associated with the translocation intermediate was then analysed by quantitative western blotting, using dilution series of SecA and SecY as standards (Figure 5). The SecY:SecA molar ratio in the precipitated samples was determined to be 2.0 ± 0.3 (n = 6) by densitometry on films from chemoluminescent blots. As SecA is a dimeric molecule (Akita et al., 1991; Driessen, 1993), the SecY:SecA ratio confirms the tetrameric organisation of SecY in translocase.

Structural analysis of the soluble translocase. Proteoliposomes that had accumulated I$_{31}$ were collected by ultracentrifugation, solubilised in dodecyl maltoside, and subjected to sucrose gradient centrifugation. All translocase subunits (SecA, SecY, SecE, and SecG) cofractionationed with I$_{31}$ (Figure 6A, fraction 4), while excess phospholipids migrated to the top and insoluble material to the bottom of the gradient (fraction 10). When complete reaction mixtures were incubated in the presence of DTT, or when ATP was omitted, proOmpA appeared to form aggregates, as it was retrieved mainly in the bottom gradient fractions. The comigration of all translocase subunits in density gradient centrifugation was specific for dodecyl maltoside. When the translocase-I$_{31}$ complex was solubilised in n-octyl-$\beta$-D-glucopyranoside, SecE and SecG dissociated from SecY and migrated to fractions with a lower density. This is consistent with the finding that with this detergent the stability of the SecYEG complex is dependent on the presence of excess phospholipids (Driessen and Wickner, 1990; Manting et al., 1997). The density of the SecYEG complex and of translocase-I$_{31}$ was similar in gradients with dodecyl maltoside, as SecYEG migrated to similar fractions in the gradients irrespective of the formation of I$_{31}$ (data not shown). Negatively-stained gradient fractions containing translocase-I$_{31}$ revealed particles with a pronounced size and shape heterogeneity, due to the presence of SecYEG dimers and a lack of orientation of the larger particles (Figure 6B). Nevertheless, square shaped particles were found that
Fig. 6. Fractionation and negative-stain electron microscopy of preparations containing translocase-I$_{31}$. (A) Solubilised proteoliposomes that had accumulated I$_{31}$ were loaded on a 10-45 % (w/v) sucrose gradient containing 0.03 % (w/v) of dodecyl maltoside. After equilibration centrifugation, fractions were separated from top to bottom. The fraction containing all translocase components and proOmpA (*) was separated from excess lipids (fractions 1-3; not shown) and unsolubilised or aggregated material (fraction 10). (B) Negatively stained preparations of the sucrose gradient fraction (*) demonstrates a highly heterogeneous particle distribution containing predominant large particles with a size of 10-15 nm (overview; scale bar = 100 nm). A selection of large particles is shown in the bottom inserts (scale bar = 10 nm), and class averages (100-300 particles) in the right inserts (scale bar = 5 nm). A major class of particles (top right insert) resembles the SecYEG structures observed in Figure 2.

Mass analysis of solubilised SecYEG and translocase-I$_{31}$ Scanning transmission electron microscopy (STEM; Müller et al., 1992) was employed to determine the particle masses in all three cases described above. Samples were freeze-dried after adsorption to thin carbon film and extensive washing by double distilled water. Particle masses were evaluated from dark-field micrographs recorded at a dose of typically 300 electrons/nm$^2$. Figure 7 shows a striking upshift of the mass values observed with solubilised SecYEG (A), SecA- and AMP-PNP- treated SecYEG (B), and the translocase-I$_{31}$ complexes (C). The histogram in Figure 7D shows the mass histogram of all small particles present in the translocase-I$_{31}$ preparation. Distinct peaks emerged reproducibly in the mass histograms, as summarised in Table 1. Although the corresponding Gaussian profiles in Figures 7A-C had the same position within the error limits, their standard deviation varied between 32 and 57 kDa, because the background signal was different in different preparations. To interpret these masses, they were compared with the particle masses estimated from the average particle projections, and masses calculated from the assumed stoichiometry of the protein complexes. Soluble SecYEG that had not been incubated with translocation ligands was present in equal amounts as monomers and dimers, demonstrating that the majority (around 65 %) of SecYEG is organised as a dimer (Figure 7A). The minor shoulders on both sides of this histogram indicated two further particle types, but the corresponding mass values cannot be related to distinct oligomers. Importantly, the STEM analysis confirms the tetrameric assembly of the SecYEG that was pre-incubated with SecA and AMP-PNP (Figure 7B). These particles, with a mass of around 420 kDa, were also observed in sucrose gradient fractions prepared from proteoliposomes that had been incubated with proOmpA and ATP under oxidising conditions (Figure 7C). Two other classes of large particles were observed in these preparations. Particles with a mass of 686 kDa are

had a size and shape similar to the tetrameric SecYEG, as documented by the selected particles in the bottom insert of Figure 6, and the class average (top right). The latter compares favourably to the largest tetrameric structure found in SecA/AMP-PNP induced SecYEG tetramers (Figure 2, right inserts).
consistent with the presence of translocase containing the I_{31} translocation intermediate. Another particle mass of 531 kDa represents a complex that was not observed with the samples containing purified SecYEG (Figure 7C). As SecA efficiently binds to soluble SecYEG (Van der Does et al., 1998), we propose that these complexes exist of SecA together with the precursor and unassembled, dimeric SecYEG. Minor peaks required to fit the histograms in Figure 7B-D represent approximately 10% of the measurements and were not assigned.

**Fig. 7.** Mass analysis. The scanning transmission electron microscope (STEM) allows mass analysis of heterogeneous particle mixtures. Unstained preparations prepared by freeze-drying were recorded and evaluated as described (Müller and Engel, 1998). (A) Solubilised SecYEG reveals two major peaks (at 176 and 268 kDa) and two minor peaks at the left and right sides of the histogram (B) SecYEG after incubation with SecA and AMP-PNP demonstrates a striking shift of the particle mass. The three major peaks are centred at 183 kDa, 289 kDa, and 421 kDa. The peak at 580 kDa includes about 10% of all particles measured. (C) Sucrose gradient fraction from solubilised proteoliposomes after the accumulation of the I_{31} translocation intermediate reveals a further shift towards larger complexes. The histogram includes particles larger than 10 nm. The three major peaks are centred at 416 kDa, 531 kDa, and 686 kDa, the minor peaks at 276 kDa and 900 kDa. (D) Mass analysis of the same preparation, but of particles smaller than 10 nm. The major Gauss peak is located at 200 kDa, the minor peak at 330 kDa.

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1Calculated from the molecular masses of the indicated proteins. 2Estimated mass of a dodecyl maltoside micelle incorporating the indicated protein mass. 3Calculated masses of the SecYEG dimer and tetramer were determined by their surface observed in negative-stain EM after single particle analysis (349 nm for the dimer and 582 nm for the tetramer), a height of 6 nm, and a density of 1.35 g/cm³, with no correction for the putative pore. ND = not determined.

Table 1. Size and interpretation of the reoccuring peaks in STEM mass analysis.
DISCUSSION
This study demonstrates that *E. coli* translocase consist of the dimeric SecA ATPase and a channel structure composed of four SecYEG complexes. A remarkable enzymological event underlies formation of the SecYEG tetramer: SecYEG complexes are recruited by the SecA protein and assembled upon SecA membrane insertion. The tetrameric organisation of active SecYEG elucidates on how the complex provides a structure that allows ATP-driven membrane insertion of SecA domains and the concomitant translocation of preproteins. The recruitment of additional subunits also explains the plasticity observed with the protein conducting channel in the mammalian endoplasmic reticulum (ER) membrane, which increases its pore size during translocation (Hamman *et al.*, 1997, 1998). It is conceivable that SecA covers the pore in SecYEG, thereby preventing the aspecific passage of macromolecules and ions through the translocation channel.

We have used proteoliposomes reconstituted with SecYEG as a minimal system to study SecA- and ATP-driven translocation. SecG is a specific subunit of the bacterial translocase, and its function appears to be related to SecA. The membrane insertion and deinsertion of SecA is accompanied by topology inversions of the highly hydrophobic SecG, which facilitate the SecA reaction cycle (Nishiyama *et al.*, 1996). Our studies do not include SecD, SecF and the uncharacterised YajC protein, all of which form a complex that interacts with SecYE (Sagara *et al.*, 1994; Duong and Wickner, 1997). SecG and SecDF(YajC) have apparent complementary functions, but SecYEG is the most abundant SecYE-containing complex present in the cytoplasmic membrane (Pogliano and Beckwith, 1994; Duong and Wickner, 1997). Purified SecYEG suffices to reconstitute translocation sites that allow multiple rounds of translocation mediated by SecA and ATP (Figure 3B; Bassilana and Wickner, 1993). Finally, Duong and Wickner (1998) have shown that this complement of translocase subunits also permits the integration of hydrophobic transmembrane segments into the lipid bilayer.

Solubilised SecYEG exists as monomers and dimers, the latter representing a majority having a mass of approximately 280 kDa, including detergent. This value is the average of three independent STEM measurements and the mass estimated from the size of negatively stained particles (Table 1). We suspect that part of the SecYEG dimers have dissociated during purification. Alternatively, SecYEG may be present in the membrane in an equilibrium between its monomeric and dimeric form. Upon interaction with SecA and AMP-PNP, a substantial amount (30 %) of SecYEG had assembled into tetramers, with a measured mass of 420 kDa (two independent experiments) and a calculated mass of 477 kDa (Table 1). The latter value is an overestimate, because the central indentation in the SecYEG tetramers has not been accounted for. These particles were also observed in the STEM analysis of solubilised proteoliposomes that were preincubated with oxidised proOmpA and ATP. In addition, the proteoliposomes harboured translocate-precursor complexes with a mass of 686 kDa and smaller, 530 kDa complexes consisting of SecA and dimeric SecYEG. The latter particle may also be accounted for by SecYEG tetramers and monomeric SecA, but this is unlikely as SecA functions as a dimer and does not dissociate during translocation (Driessen, 1993). In our calculations of the protein masses (Table 1) we presume a stoichiometric organisation of SecY, SecE and SecG within each SecYEG subunit, making it a 75 kDa protein complex. SecY and SecE form a stable complex that does not dissociate in the cytoplasmic membrane (Joly *et al.*, 1994). From their interdependent stability and similar expression levels in the cytoplasmic membrane (Matsuyama *et al.*, 1990, Pogliano and Beckwith, 1994), as well as their defined regions of interaction (Baba *et al.*, 1994; Flower *et al.*, 1995) it is apparent that they interact stoichiometrically. SecG is somewhat loosely associated with the SecYE complex (Joly *et al.*, 1994), and may therefore be present in sub- or super-stoichiometrical amounts to SecYE. The number of SecG molecules and the amount of detergent bound to SecYEG are unknown variables in the interpretation of the STEM mass analysis. Therefore, the mass data need to be compared to the projection maps of negatively stained particles.
The soluble *E. coli* SecYEG dimer was visible in negative-stain EM mainly as a two-fold symmetric, elongated particle, but smaller particles were present as well. Averages of both particle types were calculated and compared. The smaller particle (top right inset in Figure 1) had the dimensions (4.5 nm x 6.8 nm) of half the larger particle (6.7 nm x 8.7 nm; right inset in Figure 1), consistent with the mass measurements. The central position of their stain-filled depression in the larger particle indicates that the structures represent a top or bottom view of the SecYEG complex. The resolution obtained by single particle analysis and class averaging approximates 2 nm, and is insufficient to allow a more detailed analysis of the structural organisation and subunit assembly within the SecYEG dimer. The reported structure of *B. subtilis* SecYE resembles the SecYEG dimer, but has a semi-pentagonal appearance (Meyer et al., 1999). Although also in *B. subtilis* translocation is enhanced by SecG (Van Wely et al., 1999; unpublished data), this subunit is apparently not required for formation of the SecYE dimer. This is consistent with data obtained in *E. coli* which indicate that SecYE is sufficient to support translocation, albeit inefficiently (Duong and Wickner, 1997).

The active conformation of the preprotein conducting channel is not the SecYEG dimer, but is represented by a larger structure with a four-fold symmetry, consisting of a SecYEG tetramer. This square shaped particle had a side length of 10.5 nm (Figure 2). Firstly, it was formed upon interaction with membrane-inserted SecA, and not with SecA without activation with AMP-PNP. Secondly, it was present in proteoliposomes that had been incubated with complete translocation reactions. Thirdly, biochemical analysis of the SecY:SecA ratio in a soluble translocase-precursor complex demonstrated that SecY is organised as a tetramer. We propose a two-step model for the formation of translocase in which the SecA dimer first binds two SecYEG dimers, thereby bringing them together, and subsequently enforces their stable assembly into a functional channel upon membrane insertion. Speculatively, SecG and SecDF influence formation of this active channel by promoting the stability of the tetramer. The assembly of translocase via a complex between SecA and dimeric SecYEG is supported by the putative formation under translocation conditions of such protein complexes. The ability of SecA to bind unassembled SecYEG is also apparent from results obtained in detergent solution.

The eukaryotic Sec61p complex of the ER membrane is homologues to SecYEG (Hartmann et al., 1994; Matlack et al., 1998). Purified Sec61p has a closed ring structure and a central pore or indentation with a diameter of 1.5-2 nm, and these structures are also observed in the ER membrane (Hanein et al., 1996). When bound to the ribosome, the central cavity of Sec61p aligns the putative nascent chain-conducting channel in the large ribosomal subunit (Beckmann et al., 1998). The size of the protein conducting channel in the mammalian ER membrane is dramatically larger (4-6 nm) during translocation than the putative pore observed in structural analysis of Sec61p in the absence of nascent chains (Hamman et al., 1997). It was therefore suggested that the Sec61p structure does not represent the active protein conducting channel. Indeed, biophysical studies with microsomal membranes confirm the presence of two distinct populations of Sec61p complexes, with a pore size of either 0.9-1.5 nm or 4-6 nm. The latter is formed only upon incubation with ribosome-nascent chain complexes and collapses after release of the nascent polypeptide (Hamman et al., 1998). Our results with the purified and reconstituted SecYEG channel support a model in which the large ‘active’ channels are formed via recruitment and assembly of small subcomplexes.

Both the dimeric and tetrameric SecYEG complexes will be deleterious to the integrity of cytoplasmic membrane as a chemo-osmotic barrier. In the ER membrane, the lumenal side of the Sec61p channel is closed by the BiP ATPase. BiP remains bound to Sec61p after ribosomal targeting and channel opening, but is released when translocating nascent chains have a reached a length of 70 amino acids or more (Hamman et al., 1998). In analogy, the SecA molecule may be involved in closing the pore of the SecYEG complex, either by binding at the cytosolic side, through the insertion of specific domains, or both. In addition to SecA, the SecDF complex may contribute to the maintenance of the proton-motive force during translocation (Arkowitz and Wickner, 1994). The SecA membrane topology appears complex and its integral membrane state is
not dependent on translocation (Kim et al., 1994; Van der Does et al., 1996; Chen et al., 1996; Ramamurthy and Oliver, 1997). Topology studies on SecA may be obscured because the SecA protein is part of a large transmembrane structure. It may be accessible for chemical probes or proteases added from the periplasmic side of the membrane via the proteinaceous channel constituted by SecYEG. The determination of its crystal structure will elucidate more structural aspects of SecA. Although it did not yield immediate structural information, the stabilisation of translocase via a translocation intermediate is an important advance towards its large scale purification and crystallisation. In addition, it will be important to establish the thermodynamics of the translocation reaction and the surface topography of the SecYEG-bound SecA during the various stages of the translocation reaction. Finally, a major question lies in the structural and mechanistical features of the protein conducting channel during the integration of membrane proteins.

MATERIALS AND METHODS

Materials. ProOmpA (Crooke et al., 1988), SecB (Weiss et al., 1988), and SecA (Cabelli et al., 1988) were purified as previously described. A plasmid encoding (C290S)proOmpA was constructed by PCR starting from plasmid pET149 (Van der Wolk et al., 1997) using the mutagenesis primer 5'-GGCAACACCTGTGACAACGTG-3'. The resulting plasmid pET503 was used for the synthesis of 35S-labeled (C290S)proOmpA with an in vitro transcription/translation reaction and immunopurified as described (Van der Wolk et al., 1997). Hexa-histidine tagged SecYEG was purified and reconstituted into proteoliposomes as described by Van der Does et al. (1998). Rabbit polyclonal antisera against SecY, SecE, OmpA, SecA and SecB were raised against the purified proteins at the animal facility of the Department of Chemistry, University of Groningen, The Netherlands. The antisera against SecY or SecE were raised against the His6-tagged proteins, and contained specific cross-reactivity with His6-tags on other proteins. Monoclonals against OmpA were raised and selected by Prof. Dr. L. de Leij, Academic Hospital Groningen. SecA was detected by a mixture of monoclonals (oligoclonal) (Den Blaauwen et al., 1997). Protein samples were analysed by SDS-PAGE using 12-15 % acrylamide gels, followed by western blotting or silver staining (Bio-Rad, Hercules, CA, USA). Western blots were developed as films using chemoluminescence (Tropix, Bedford, MA, USA). For densitometry a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and SigmaScan/Image Software (Jandel Corp., San Rafael, CA) were used. Protein A- or NHS-sepharose beads were from Pharmacia (Uppsala, Sweden), dodecyl maltoside and octyl glucoside from E. coli phospholipids from Avanti polar lipids (Alabaster, AL, USA).

Translocation reactions. Translocation reactions were performed with 6.0 µg of SecA, 2.0 µg of urea-denatured proOmpA, 1.0 µg of reconstituted SecYEG (8-10 µl of proteoliposomes) and 2 mM ATP in 100 µl translocation buffer consisting of 50 mM Hepes-KOH, pH 7.6, 100 mM KCl, and 5 mM MgAc. For oxidation or reduction of proOmpA, complete reaction mixtures were preincubated for 10 min on ice in the absence or presence of 10 mM DTT. Reactions were started by placing them at 37 °C and stopped by chilling on ice, followed by digestion with protease K, TCA precipitation and a washing step with ice-cold acetone (Van der Wolk et al., 1997). For densitometrical quantitation of translocation, bands representing accumulated proOmpA or I11 were compared with 5 % and 10 % of a reaction mixture that was not digested with proteaseK as a standard. For a second round of translocation, proteoliposomes were collected by centrifugation (Beckman Airfuge, 30 psig, 10 min) and resuspended in ice-cold translocation buffer. Translocation reactions were then performed as described above, except for the proOmpA, which was replaced by 2 µl of urea-denatured 35S-labelled (C290S)proOmpA.

Immunoprecipitations. Antibodies (20 µl) were mixed with 10 µl protein A-agarose suspension in a total volume of 200 µl with buffer S, consisting of 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 20 % (w/v) glycerol and 0.05 % (w/v) dodecyl maltoside. Mixtures were incubated for 1.5 h at 4 °C and the beads were then washed with 0.5 ml buffer S (Eppendorf centrifuge, 14 000 rpm, 3 min). Isolated proteoliposomes from two translocation reactions were solubilised with 2 % (w/v) dodecyl maltoside in buffer S (200 µl) and mixed with the proteinA-bound antibodies for 1 h at 4 °C. Beads were then washed extensively with buffer S and bound proteins were eluted with SDS-sample buffer (10 min, 50 °C). Samples were analysed on western-blotting using mouse monoclonals as primary antibodies to avoid cross-reaction of the secondary antibody with the rabbit IgG used for the precipitation. Alternatively, purified IgG was covalently coupled to NHS-sepharose, according to the manufacturers recommendations. Immuno-precipitations were carried out
as described above with 10 µl of NHS-coupled IgG sepharose. Bound proteins were eluted with SDS-sample buffer in the absence of reducing agents, to avoid dissociation of the IgG molecules.

For quantitative immunoblotting, purified SecA in 0.1 % (w/v) SDS was prepared by buffer exchange using a PD10 gel filtration column (Pharmacia). His-tagged SecY was purified from 0.5 mg of soluble SecYEG complex by Ni²⁺-affinity chromatography (Manting et al., 1997) and dialysed against water. Precipitated protein was collected by centrifugation (5 min, 14 krpm, Eppendorf) and solubilised in 0.1 % (w/v) SDS (10 min, 50 °C). Non-solubilised material was removed by centrifugation (10 min, 14 krpm, Eppendorf). Amino acid concentrations of the SecY and SecA standards were determined in duplo, using RNAse as a standard, by Eurosequence, Groningen. Using a dilution range of 1 to 1/20 000 of the SecA and SecY standards, the protein contents in immunoprecipitates of translocase-I31 complexes were estimated by immunoblotting. Next, the appropriate dilutions were used to densitometrically determine the amounts of SecA and SecY in the immunoprecipitates from films of chemoluminescent blots.

Isolation of SecYEG complexes. Either proteoliposomes containing purified SecYEG (50 µl, 0.4 mg/ml in 50 mM Tris-HCl, 50 mM KCl), or reaction mixtures (500 µl) containing SecYEG proteoliposomes (20 µg), SecA (120 µg) and 2 mM AMP-PNP in translocation buffer, were solubilised on ice in a total volume of 2 ml 2 % (w/v) dodecyl maltoside, 10 mM Tris-HCl, pH 8.0, 10 mM KCl and 15 % (w/v) sucrose. The sample was injected on a miniQ anion-exchanger mounted in a Smartsystem (Pharmacia) equilibrated at 6 °C with a buffer containing 10 mM Tris-HCl pH 8.0, 15 % sucrose, 10 mM KCl and 0.03 % (w/v) dodecyl maltoside. The column was eluted with a linear gradient of 10 mM to 1 M KCl in the same buffer and 75 µl fractions were collected. The protein content of these fractions were assayed by SDS-PAGE and appropriate fractions were used for electron microscopy. Activity of the purified SecYEG complex was confirmed by reconstitution in E.coli phospholipids via rapid dilution (Van der Does et al., 1998) and measuring the proOmpA stimulated SecA ATPase activity.

To isolate translocase-precursor complexes, proteoliposomes yielding the I31 translocation intermediate were harvested from a total of 2 ml translocation reactions performed in the absence of DTT. Proteoliposomes were collected by ultracentrifugation (120 000 g, 30 min), solubilised in 250 µl of buffer S and loaded on 10 ml linear sucrose gradients ranging from 10-45 % (w/v) in 50 mM Tris-HCl, pH 8.0, 50 mM KCl and 0.03 % (w/v) dodecyl maltoside. After equilibration centrifugation (16 h, 120 000 g, 4 °C), gradients were fractionated as 1 ml samples by pipetting from the top.

Electron microscopy. Fractions from anion-exchange chromatography or sucrose gradients were absorbed to carbon films rendered hydrophilic by glow discharge. Grids were washed briefly with distilled water and stained with saturated uranyl formate. Images were taken in a Hitachi H-7000 transmission electron microscope at 100 kV and a magnification of 50 000 x. Suitable films were scanned at 50 lines/mm (corresponding to a pixel size of 0.4 nm) using a Leafscan 45. Particles were automatically selected based on their size by calculating the cross-correlation function with a ring-shaped reference of either 7 nm or 10 nm diameter, or were selected interactively, using the SEMPER image processing system (Saxton et al. 1979). Multivariate statistical classification (Frank et al., 1988) was achieved on data sets containing over 3000 particles prior to angular alignment and cluster averages were calculated to allow for picking an unbiased reference. Aligning ~3000 particles from each set of experimental conditions using the respective reference laterally and angularly, and subsequent classification yielded a final set of cluster averages from which class averages containing closely related structures were calculated. Typically, the class averages shown included 50-60 % of all selected particles.

Scanning transmission electron microscopic mass analysis of detergent solubilised particles was carried out as described previously (Müller and Engel, 1998). Elastic dark images of unstained complexes were recorded using a VG-HB5 STEM at electron doses of typically 300 electrons/nm² and 80 kV acceleration voltage. The particle mass was evaluated by measuring the number of electrons elastically scattered by a circular region enclosing the particle and subtracting the background contribution due to the thin carbon film using the IMPSYS software (Müller et al., 1992). Gaussian curves were fitted to the mass histogram peaks by a Marquart algorithm (Bevington, 1962).

REFERENCES


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