The SecYEG preprotein translocation channel is a conformationally dynamic and dimeric structure

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***Escherichia coli* preprotein translocase comprises a membrane-embedded trimeric complex of SecY, SecE and SecG. Previous studies have shown that this complex forms ring-like assemblies, which are thought to represent the preprotein translocation channel across the membrane. We have analyzed the functional state and the quaternary structure of the SecYEG translocase by employing cross-linking and blue native gel electrophoresis. The results show that the SecYEG monomer is a highly dynamic structure, spontaneously and reversibly associating into dimers. SecG-dependent tetramers and higher order SecYEG multimers can also exist in the membrane, but these structures form at high SecYEG concentration or upon overproduction of the complex only. The translocation process does not affect the oligomeric state of the translocase and arrested preproteins can be trapped with SecYEG or SecYE dimers. Dissociation of the dimer into a monomer by detergent induces release of the trapped preprotein. These results provide direct evidence that preproteins cross the bacterial membrane, associated with a translocation channel formed by a dimer of SecYEG.**

Keywords: blue native gel electrophoresis/membrane protein oligomerisation/preprotein translocase/SecYEGA/translocation channel

Introduction

Preproteins containing an N-terminal classical apolar leader peptide cross the membrane by means of a multi-subunit translocase. In *Escherichia coli*, the minimal preprotein translocase comprises a membrane-embedded complex of SecY and SecE and a peripheral membrane domain, SecA (Danese and Silhavy, 1998; Economou, 1998; Driessen *et al.*, 2001). The SecYE complex copurifies from the cytoplasmic membrane with the SecG protein (SecYEG complex; Brundage *et al.*, 1992). In addition, the SecYEG complex has an affinity for the heterotrimeric SecDFyajC complex and the YidC protein (Duong and Wickner, 1997a; Scotti *et al.*, 2000). The SecYE complex forms the heart of the translocation machinery conserved throughout biology (Hartmann *et al.*, 1994; Eichler, 2000). SecY and SecE are respective

homologues of the Sec61 α and Sec61 γ subunits of the Sec61p heterotrimeric complex of the endoplasmic reticulum (ER), while SecG does not share any obvious sequence identity with the third subunit, Sec61 β (Rapoport *et al.*, 1996).

Biochemical analysis of preprotein translocation has provided us with a better understanding of the bacterial translocation cycle. Newly synthesized proteins destined for translocation are directed by their signal sequence and shuttled via the chaperone SecB to the ATPase, SecA (Hartl *et al.*, 1990). SecA is peripherally associated with the cytoplasmic membrane, having a high affinity for the SecYEG complex and acidic lipids (Lill *et al.*, 1990; Hendrick and Wickner, 1991). Preprotein binding triggers the motion of SecA through an ATP-dependent, repetitive and complex membrane insertion cycle (Economou and Wickner, 1994; Eichler and Wickner, 1997). SecA is thought to feed sequential segments of the polypeptide into the translocase, thus promoting the stepwise movement of the polypeptide chain across the membrane (Schiebel *et al.*, 1991). Neither SecDFyajC nor SecG are essential for translocation, but serve to facilitate the SecYE function. The SecG subunit stimulates SecA membrane insertion and thus increases translocation efficiency (Nishiyama *et al.*, 1996). The SecDFyajC domain induces translocation arrest, providing time for the formation of translocation intermediates (Duong and Wickner, 1997b). Translocation is then driven by the proton-motive force, as SecA dissociates from the preprotein (Schiebel *et al.*, 1991; Driessen, 1992).

There are several lines of evidence that the heterotrimeric Sec61p and SecYEG complexes form a protein-conducting channel across the ER and bacterial inner membranes, respectively. Electrophysiological experiments have shown that translocation-dependent ion-conducting pores exist in the ER and *E. coli* membrane (Simon and Blobel, 1991, 1992). Cross-linking studies demonstrated that SecY/Sec61 α lines the path of the translocating preprotein (Joly *et al.*, 1994; Mothes *et al.*, 1994), and fluorescence analysis using various probes to measure the accessibility of the channel interior estimated a diameter of 9–15 Å for the ribosome-free Sec61p complex (Hamman *et al.*, 1998). Furthermore, electron microscopy revealed that all the Sec61p, SecYEG and SecYE complexes are organized into ring-like assemblies, both in detergent solution and in membranes (Hanein *et al.*, 1996; Meyer *et al.*, 1999; Manting *et al.*, 2000). In yeast, the pore-like structure formed by the Sec61p ring is aligned with the polypeptide exit channel of the ribosome, suggesting that these openings form the conductive channel for translocating polypeptides (Beckmann *et al.*, 1997; Ménétret *et al.*, 2000).

The large size of the ring structures (~85 Å in diameter) means that both Sec61p and SecYE/G complexes should

be composed of oligomers. Analysis by scanning transmission electron microscopy, sucrose gradient fractionations and analytical ultracentrifugation estimated that these assemblies are formed by 2–4 copies of the SecYEG monomers (Hanein *et al.*, 1996; Meyer *et al.*, 1999; Manting *et al.*, 2000; Collinson *et al.*, 2001). However, none of these studies have addressed whether these oligomers actually constitute the active protein-conducting channel. The structure of the crystalline SecYEG in phospholipid bilayers shows arrangements of dimers (Collinson *et al.*, 2001). In contrast, cross-linking experiments on an active translocation machinery did not identify any SecYEG oligomers, suggesting its action as a monomer (Yahr and Wickner, 2000). A fluorescence collisional quenching experiment on the Sec61p complex demonstrated significant fluctuations of the pore diameter, depending on the translocation status (Hamman *et al.*, 1998). Dramatic and irreversible changes in the SecYEG structure were also seen by electron microscopy after incubation with SecA and nucleotides (Manting *et al.*, 2000). These structural changes may reflect the assembly of the oligomeric translocase and/or a major rearrangement of the Sec subunits within the oligomer. Thus, a comprehensive view of the Sec complex in relation to its dynamic oligomeric behaviour and its function as a translocation channel requires further biochemical analysis.

With this goal in mind, we have analyzed the architecture of the SecYEG oligomers; in particular, the stability, conformational dynamics and function of the various oligomeric forms of SecYEG. Our findings reveal that the SecYEG complex can exist in multimeric associations but only the dimeric SecYEG complex is stably engaged with a translocating preprotein.

Results

Oligomeric forms of the purified SecYEG complex

The SecYEG complex was purified in the presence of dodecyl- β -D-maltopyranoside (DDM) from membranes enriched for His₆-tagged SecE, SecY and SecG, as has been previously described (Collinson *et al.*, 2001). The SecY subunit migrates with an apparent molecular weight (M_r) of 35 kDa, while SecE_{his} and SecG have similar migration properties and are not well resolved by SDS-PAGE (Figure 1A).

The oligomeric assembled state of the SecYEG complex was evaluated by blue native gel electrophoresis (BN-PAGE; Figure 1C; Schägger and von Jagow, 1991). In order to avoid aggregation artefacts that often arise when handling high concentrations of membrane protein solutions (see below), only very dilute radiochemical amounts of the SecYEG complex were analyzed. All Sec subunits were radio-iodinated (Figure 1B), retaining the translocation activity of the preparation after reconstitution into proteoliposomes (data not shown). Upon application on BN-PAGE, the SecYEG complex neither aggregated nor denatured. The complex migrated with two distinct M_r of ~130 and ~270 kDa; the relative proportions of these two isoforms depending on the detergent concentration (Figure 1C, lanes 1 and 3). The same two bands can also be detected by immunostaining of similar concentrations of non-radiolabelled SecYEG

complex (Figure 5A, left panel). When the detergent was diluted below the critical micellar concentration ($CMC_{DDM} \approx 0.01\%$), as expected, the sample aggregated and failed to enter the gel (Figure 1C, lane 2). In the presence of SDS, the Sec complex fully disassembled and SecY migrated with an apparent M_r of 66 kDa (lane 4). Thermal treatment led to the progressive dissociation of the complex into single subunits, as expected (Figure 1C, lane 5; Brundage *et al.*, 1992). The calculated molecular mass of SecYEG complex containing one of each subunit was ~75 kDa and the apparent M_r of SecY according to BN-PAGE was 66 kDa. Therefore, the identity of the visualized ~130 and ~270 kDa forms required further investigation.

The SecYEG complex spontaneously forms dimers, which readily dissociate into monomers

Cross-linking agents were incubated with the SecYEG complex to determine the composition of the two observed oligomeric forms. Formaldehyde, glutaraldehyde and amine-reactive cross-linkers, with spacer arms of variable length, were all used in this study. The results obtained were typified by disuccinimidyl suberate (DSS; Figure 2) and all other cross-linkers yielded the same result. Covalent complexes of SecY to His₆-SecE, to SecG, and to both, were detected (Figure 2A). The cross-linking pattern was independent of the detergent concentration (Figure 2A, left and right panels) and the oligomeric state of the SecYEG complex, which depended on the former (Figure 1C). The optimal concentration of DSS for efficient cross-linking was determined (~0.68 mM DSS; Figure 2A, left panel), but still only intra-complex cross-links were detected. This is consistent with a previous observation showing the absence of inter-complex cross-links in SecYEG-enriched membranes (Yahr and Wickner, 2000).

Next, we compared the M_r of the native and cross-linked SecYEG complexes on BN-PAGE (Figure 2B). In the absence of SDS, the electrophoretic mobility of the cross-linked SecYEG complex (Figure 2B, lane 11) was the same as the 130 kDa native complex (Figure 2B, lane 1 or 12, lower band). We thus conclude that the 130 kDa form is a monomer of SecYEG, while the 270 kDa form corresponds to a SecYEG dimer (Figure 2B, labelled M and D). We noted that in the presence of SDS, the cross-linked SecYEG complex (lane 7, upper band) migrated slightly faster than in the absence of SDS (lane 11). Similarly, upon a moderate incubation at 37°C (lane 2), the heat-treated but undissociated SecYEG complex (Figure 2B, labelled YEG) also had a higher mobility than the native SecYEG monomer (see below also). Surprisingly, this analysis also revealed that the cross-link between the Sec subunits (Figure 2B, lanes 3–7) resulted in a concomitant dissociation of the SecYEG dimer into monomers (Figure 2B, lanes 8–12). The active group of the cross-linker may attack amino acid side chains involved in dimer stabilization, thereby promoting monomer formation. This may explain why the results here and elsewhere (Manting *et al.*, 1997; Yahr and Wickner, 2000) fail to detect the inter-subunit cross-links expected from an assembly of multimers of SecYEG.

Fig. 1. BN- and SDS-PAGE analysis of the purified SecYEG complex. (A) Coomassie Blue staining of the purified SecYEG complex and (B) autoradiography of the ¹²⁵I-labelled SecYEG complex (~30 000 c.p.m., ~20 ng) separated by 17% SDS-PAGE. (C) Autoradiography of the [¹²⁵I]SecYEG complex analyzed by linear gradient BN-PAGE (6–15%). The radiolabelled SecYEG complex was diluted in TSG buffer containing the indicated amount of detergent and incubated on ice or at 37°C for 5 min before loading on to the gel. The high molecular weight markers are ¹²⁵I-labelled ferritin (440 and 880 kDa), catalase (232 kDa) and BSA (66 and 132 kDa). The aggregates of SecYEG proteins remain in the loading area of the gel (top).

The SecYEG monomers associate reversibly to form dimers

The SecYEG dimer is a labile structure that progressively dissociates into monomers at elevated detergent concentrations (Figure 3A, lanes 1–5). Since the initial step of our SecYEG purification involves an extraction by high concentrations of DDM, the dissociation of the SecYEG dimer into monomers is likely to be reversible. Indeed, progressive dilution of the high detergent-monomerized SecYEG promotes reassociation of the SecYEG into dimers (Figure 3A, lanes 5–10). Once the detergent was diluted beyond the CMC, as expected, the complex aggregated (Figure 3A, lanes 11 and 12). In contrast, the heat-treated SecYEG complex (Figure 3B, lane 2) showed a dramatic reduction in the ability to re-form dimers, following the same procedure (Figure 3B, lanes 3–7). The small amount of re-forming dimers probably arose from the SecYEG monomers (Figure 3B, M), which survived the heat treatment. Again aggregation results upon further dilution of the detergent (Figure 3B, lane 8). Thus, the heat-treated SecYEG complex may have undergone an irreversible conformational change preventing dimer re-formation. We conclude that the capacity of the SecYEG monomers to associate into dimers is a specific property of the native SecYEG complex.

Fig. 2. Cross-linking analysis of the purified SecYEG complex. (A) Separation of the DSS-linked SecYEG proteins by 10% SDS-PAGE. The [¹²⁵I]SecYEG complex was prepared in CL buffer (50 mM HEPES–KOH pH 8.0, 50 mM KCl) with 0.02 or 0.2% DDM. The DSS reagent was dissolved in dimethylsulfoxide (DMSO) and incubated with the SecYEG complex at the indicated final concentration. After 30 min at room temperature, the cross-linker was quenched by Tris–HCl pH 8.0 (50 mM final) and the cross-linked products were dissolved in Laemmli sample buffer, followed by gel electrophoresis and autoradiography. (B) Separation of the DSS-linked SecYEG complex by 6–15% BN-PAGE. The [¹²⁵I]SecYEG complex was prepared in CL buffer with 0.02% DDM and incubated with the indicated concentration of DSS, as described above. The cross-linked products were dissolved in BN-sample buffer with or without 0.05% SDS. For reference, the native and heat-treated SecYEG complexes were loaded on the same gel (lanes 1 and 2). Incubation with DMSO does not induce the dissociation of the SecYEG dimers (lane 12).

Oligomeric isoforms of SecYEG complex in membranes

There is a possibility that the SecYEG structure in the membrane is different to that of the detergent solubilized form. Inner membrane vesicles (IMVs) were prepared from an *E. coli* strain overexpressing HA-tagged SecY_{HA}G or SecY_{HA} complexes (Duong and Wickner, 1997a). Immediately following solubilization of membranes in DDM, the extract was analyzed by BN-PAGE and the Sec complex immuno-decorated with anti-SecG or anti-HA antibodies (Figure 4A). From SecY_{HA}G-enriched membranes extracts, monomers and dimers were identified (Figure 4A, lane 1, M and D), consistent with the observations made from the purified complex. The SecYEG complex crudely extracted from membranes also exhibited the detergent concentration-dependent equilibrium between monomeric and dimeric forms (Figure 4A, lane 1 compared with lanes 2–4). However, there was a notable difference between the crude and purified SecYEG forms; a higher *M_r* complex was detected by both SecG and HA antibodies (Figure 4A, T). This form probably represents a tetrameric association of SecY_{HA}G

Fig. 3. Dynamic dimeric association of the SecYEG complex. (A) Reversible dissociation of the native SecYEG dimers. To vary the amount of detergent while keeping the SecYEG concentration constant, a stock solution of [125 I]SecYEG complex ($\sim 1.5 \times 10^6$ c.p.m./ μ g) was first prepared in TSG buffer with 0.02 or 0.2% DDM. Aliquots ($\sim 30\,000$ c.p.m.) were then diluted on ice to the desired DDM concentration and analyzed by BN-PAGE and autoradiography. (B) Irreversible dissociation of the heat-treated SecYEG complex. The [125 I]SecYEG complex was prepared in TSG buffer with 0.2% DDM and incubated for 5 min at 37°C (lane 2). The heat-treated SecYEG complex was then diluted on ice to the desired DDM concentration and analyzed by BN-PAGE and autoradiography.

monomers, rather than an association with other translocase components, since anti-SecA, -SecF or -YidC antibodies failed to cross-react with this complex (data not shown). From SecY_{HA}-enriched membrane extracts, SecY_{HA} monomers and dimers were also identified (Figure 4A, lane 5). The SecY_{HA} complex also displayed an association equilibrium with a reversible dependence on the detergent concentration (Figure 4A, lane 5 compared with lanes 6–8). Surprisingly, no tetrameric SecY_{HA} was detected in these membrane extracts.

One explanation could be that SecG is responsible for tetramer formation. SecG facilitates *in vitro* preprotein translocation (Nishiyama *et al.*, 1996); it is conceivable that the SecG-dependent tetramerization is an important component of the translocation cycle. However, the observed SecYEG tetramer is far more stable and less dynamic than the dimer component, and remained approximately constant through fluctuations in detergent concentration (Figure 4A, lanes 1–4). Furthermore, the SecYEG tetramers were more stable during a moderate heat-treatment, relative to the dimeric structures (Figure 4B, left panel). According to previous observations (Duong and Wickner, 1999), the SecYEG monomers and dimers containing the *prlA4* mutation into SecY

Fig. 4. The oligomeric form of the SecYEG complex in the membranes. (A) Immunodetection of the Sec complexes in membranes enriched for the SecY_{HA}G or SecY_{HA} proteins. IMVs ($\sim 10\,\mu$ g) were solubilized on ice in TSG buffer with the indicated detergent concentration. Protein aliquots ($\sim 0.2\,\mu$ g for SecY_{HA}G and $\sim 0.1\,\mu$ g for SecY_{HA} IMVs) were analyzed by BN-PAGE and immunostained with anti-HA IgG. For reference, ~ 20 ng of the purified SecY_{his}G complex was loaded on the same gel and immunostained with anti-SecG IgG (left panel). (B) Thermal stability of the oligomeric SecYEG complexes. IMVs ($\sim 10\,\mu$ g) enriched for the SecY_{HA}G or SecY₄E_{HA}G complexes were solubilized with 0.05% DDM and incubated at the indicated temperature for 5 min. Aliquots were then analyzed by BN-PAGE and immunostained with anti-HA IgG. (C) Oligomeric state of SecYEG during translocation. The translocation assays were performed in 100 μ l as described in Materials and methods using proteoliposomes (0.35 μ g of SecYEG proteins) or IMVs ($\sim 10\,\mu$ g of proteins), then solubilized using the indicated concentrations of DDM. Aliquots ($\sim 0.2\,\mu$ g of proteins) were analyzed by BN-PAGE and immunostained with anti-SecG IgG.

(labelled SecEY₄G) dissociate more readily than the wild-type complex during incubations at elevated temperature (Figure 4B, compare right and left panels), but conversely, the tetrameric SecEY₄G seemed to be heat stabilized. Since studies of the *prl* mutants have shown that an increase in translocase activity correlates with a decrease in SecYEG associations (Duong and Wickner, 1999), the

higher stability of the SecYEG tetramer seems to contradict this observation.

Translocation partners and ligands may be important for the monomer–dimer–tetramer equilibrium. The purified SecYEG complex was reconstituted into proteoliposomes and subjected to translocation reactions (Figure 4C, left panel). Although this SecYEG preparation was active for preprotein translocation (Collinson *et al.*, 2001 and data not shown), the oligomeric distribution of SecYEG remained unchanged after incubation with the translocation ligands and co-factors (Figure 4C, left panel). This was apparent when the Sec complex was extracted from the membrane with two different tetramer-compatible detergent concentrations. The same observation was also made from both SecYEG- and SecYE-enriched IMVs (Figure 4C, right panel). Thus, AMP–PNP-driven SecA membrane insertion and preprotein translocation does not seem to change the overall distribution of the oligomeric forms of the SecYEG complex.

The SecYEG complex forms higher oligomers at elevated protein concentration

Since the SecYEG tetramers were only seen in SecYEG-enriched IMVs, they may be a result of unnaturally high concentrations of these proteins in the membrane. Indeed, forms corresponding to SecYEG octamers could also be detected in these IMVs (Figure 4A, band above the 880 kDa marker) and similar multimers appeared in proteoliposomes only when reconstituted at elevated SecYEG concentrations (data not shown). Furthermore, SecYEG tetramers and higher order oligomers were visualized when increasing concentrations of the purified SecYEG complex were loaded on BN–PAGE (Figure 5A). This propensity for multimerization has also been observed by analytical ultracentrifugation and octamers were seen in solutions of concentrated SecYEG preparations (Collinson *et al.*, 2001). Finally, wild-type IMVs (i.e. without the plasmid overproducing SecYEG) probed with SecG antibodies revealed SecYEG monomers and dimers only (Figure 5B). Once again, the translocation process did not affect the oligomerization state and tetramers were not detected in these wild-type membranes (Figure 5C). Clearly, the SecYEG complex has the property to self-associate into higher order oligomers at high protein concentrations, in solution or membranes.

A translocating preprotein is engaged with the dimeric SecYEG complex

Given the inherent capacity of the SecYEG complex to form multimers, the question of the stoichiometry of the preprotein channel can be addressed only by trapping the translocase in its functional form, i.e. with a substrate engaged in a translocation assembly. To create a stable SecYEG–preprotein complex, bovine pancreatic trypsin inhibitor (BPTI) was attached via a thiol-reducible cross-linker to a C-terminal cysteine residue of ¹²⁵I-labelled proOmpA. The globular structure of BPTI arrested translocation of proOmpA, thereby yielding a translocation intermediate with only a 26 kDa domain capable of translocating across the membrane (I₂₆; Schiebel *et al.*, 1991; Figure 6A, lane 5). The arrested intermediate completed translocation into the IMVs only after release of BPTI from proOmpA [+dithiothreitol (DTT), lane 4].

The I₂₆ intermediate was also generated with the SecYE_{HA}-enriched IMVs (lane 6), but with a lower efficiency. In the absence of ATP or at 4°C, proOmpA–BPTI was not translocated and was fully digested by proteinase K (Figure 6A, lanes 1–3).

As expected, only conditions that lead to a stably-arrested translocation intermediate allow anti-SecG-driven co-immunoprecipitation of a complex between SecYEG and proOmpA (Figure 6B, lanes 1–5). This precipitation requires SecA and hydrolysis of ATP, thus these proOmpA immunoprecipitates represent authentic translocation intermediates. Anti-SecG antibodies did not promote immunoprecipitation of proOmpA arrested within the SecYE-enriched, but SecG-depleted, membranes (Figure 6B, lane 6). Furthermore, extraction of this complex from the membrane with an increased concentration of DDM or incubation of the extract at 37°C, led to its complete dissociation (Figure 6B, lanes 7 and 8). Therefore, the SecYEG–proOmpA complex is quite an unstable structure in detergent solution. Accordingly, a previous report has shown that the stability of the SecYEG–preprotein complex also depends upon the nature of the detergent employed (Yahr and Wickner, 2000).

The same detergent extracts used for immunoprecipitation (Figure 6B) were analyzed by BN–PAGE (Figure 6C). Experiments with radioactive proOmpA–BPTI arrested in the SecYEG-enriched IMVs (or SecYEG reconstituted proteoliposomes, data not shown) identified a significant fraction of the molecule with a *M_r* slightly above that of the purified SecYEG dimer (Figure 6C, lane 5 compared with lane EYG). This form appeared only in conditions that led to the formation of a translocation intermediate and a stable preprotein–SecYEG complex (Figure 6C compared with A and B, lanes 1–5), yet disappeared when the SecYEG–preprotein complex was dissociated at 37°C (Figure 6B and C, lane 7). As expected, a band with a slightly lower *M_r* was obtained when proOmpA–BPTI was arrested in IMVs enriched for SecYE, but lacking SecG (Figure 6C, lane 6). Furthermore, solubilization of the membranes with an elevated detergent concentration that leads to the dissociation of SecYEG dimer into monomers, induced the concomitant disappearance of this band (Figure 6C, lane 8 and see below). In contrast to the reversible association previously seen for the SecYEG dimers, proOmpA was unable to reassociate with SecYEG following detergent dilution (Figure 6B and C, lane 9).

Finally, IMVs bearing the arrested and radioactive proOmpA–BPTI were solubilized with an increasing detergent concentration (Figure 7). Near the CMC of the detergent, solubilization was inefficient and membrane proteins hardly entered into the gel (Figure 7A, lanes 1 and 2). At slightly higher concentrations, membrane solubilization was achieved and allowed the detection of the (SecYEG)₂–preprotein complex (Figure 7A, lanes 4–8). During the progressive disappearance of the (SecYEG)₂–preprotein complex, there was a concomitant dissociation of SecYEG dimer into monomers (Figure 7A compared with B). In contrast, the tetrameric form seen upon overproduction of SecYEG remained largely unaffected by the detergent concentrations. Therefore, these results show that an authentic translocation intermediate was engaged with the dimeric form of the SecYEG translocase.

Fig. 5. Multimerisation of the SecYEG complex. (A) Nucleation of the purified SecYEG complex. A solution of purified SecYEG (12 mg/ml) was diluted to the desired protein concentration in TSG buffer-0.1% DDM. Aliquots were analyzed by BN-PAGE (gel 4–15% left panel; and 4–12% right panel) and stained with Coomassie Blue. The SecYEG complex was also incubated with 0.05% SDS where indicated on the figure. (B) Immunodetection of the SecYEG complex in wild-type membranes. IMVs (~10 µg) were solubilized in TSG buffer with 0.2% DDM. Aliquots (1 µg of protein for the wild-type membranes) were diluted four times in TSG buffer containing the indicated concentration of DDM, then analyzed by BN-PAGE and immunostaining with anti-SecG IgG. (C) Oligomeric state of SecYEG during translocation in wild-type IMVs. The translocation assays were performed in 100 µl as described in Materials and methods using wild-type IMVs (~10 µg), then solubilized on ice using the indicated concentrations of DDM. Aliquots were analyzed by BN-PAGE and immunostained with anti-SecG IgG.

Discussion

This study provides direct evidence for the translocating protein crossing the membrane associated with an oligomeric SecYEG structure. We have identified this oligomeric structure as a SecYEG dimer. In support of our biochemical findings, a recent 3D structure of SecYEG calculated from membrane-embedded complexes also revealed a dimeric association of SecYEG with a possible channel structure (C.Breyton, W.Haase, T.A.Rapoport, W.Kühlbrandt and I.Collinson, manuscript submitted).

Fig. 6. The active preprotein translocation channel is comprised of a dimeric SecYEG complex. (A) Creation of trapped translocation intermediate. [¹²⁵I]proOmpA-BPTI was incubated with SecYEG- or SecYE-enriched IMVs in 100 µl of TL buffer, as described in Materials and methods. Addition of 2 mM DTT after 10 min of incubation allowed proOmpA to complete translocation across the membrane (lane 4). Translocation reactions were treated with proteinase K (1 mg/ml, 15 min on ice), then TCA-precipitated and analyzed by 12% SDS-PAGE and autoradiography. (B) Immunoprecipitation of a SecYEG-proOmpA complex. The translocation reactions described in (A) were scaled up to 250 µl, then layered over an equal volume of 0.2 M sucrose and centrifuged (100 000 g, 10 min, 4°C). Membranes were resuspended in half-volume TSG buffer (125 µl) and solubilized with 0.1% DDM (30 min, 4°C). The same reaction loaded in lane 5 was also incubated further with either 0.1% DDM for 5 min at 37°C (lane 7), 0.4% DDM for 30 min at 4°C (lane 8) or 0.4% DDM for 30 min at 4°C, then diluted four times to 0.1% DDM (lane 9). After centrifugation (142 000 g, 30 min, 4°C), the SecYEG complex was immunoprecipitated with anti-SecG protein A-Sepharose beads. Co-immunoprecipitated proOmpA was monitored by SDS-PAGE and autoradiography. (C) Molecular weight of the SecYEG-proOmpA complex. Aliquots of the detergent extracts prepared in (B) were directly loaded on BN-PAGE and analyzed by autoradiography. For the *M_r* reference, the purified [¹²⁵I]SecYEG complex in TSG-0.02% DDM (lane EYG) and [¹²⁵I]proOmpA-BPTI (left lane) were loaded on the same gel.

The other major finding is that the SecYEG complex is a dynamic structure reversibly associating into a dimer. We propose that the lability of the dimer is an important feature of the functional translocase.

The oligomeric states and active form of the SecYEG translocase
Immunoprecipitation experiments and chemical cross-linkers have shown that the purified SecYEG complex is a

Fig. 7. The dissociation of the SecYEG dimer promotes the release of the trapped translocation intermediate. Radiochemical [125 I]proOmpA–BPTI (~50 000 c.p.m.) was incubated with SecYEG-enriched IMVs (10 μ g) in 100 μ l of TL buffer in the presence or the absence of ATP, as described in Materials and methods, then layered over an equal volume of 0.2 M sucrose and centrifuged (100 000 g, 10 min, 4°C). Membranes were resuspended in half-volume TSG buffer and solubilized with the indicated concentration of DDM (30 min, 4°C). (A) Aliquots (~2 μ g proteins) were loaded on BN–PAGE and analyzed by autoradiography or (B) by western blotting (~0.4 μ g aliquots), followed by immunostaining with anti-SecG antibodies.

stoichiometric association between SecY, SecE and SecG (Collinson *et al.*, 2001; Figure 2). Unexpectedly, cross-linkers were also found to induce the dissociation of the SecYEG dimer, thereby rendering these agents useless in an analysis of the oligomeric structure of the translocase. On BN–PAGE, the SecYEG complex neither aggregated nor denatured and migrated as a dimer–monomer mixture with an apparent M_r of ~270 and ~130 kDa. The size of this dimeric SecYEG complex correlates well with one of the masses of the observed ring structures, estimated by scanning transmission electron microscopy (268 kDa; Manting *et al.*, 2000). Studies by sucrose gradient centrifugation of the SecYE complex of *Bacillus subtilis* generated a mass of ~210 kDa (Meyer *et al.*, 1999); possibly smaller because of the smaller SecE and missing SecG subunits. Strikingly, all these dimeric structures were also seen as oligomeric rings in these electron microscopy studies.

Beside the SecYEG dimers, higher oligomeric forms were detected here, as well as elsewhere (Manting *et al.*, 2000; Collinson *et al.*, 2001). However, we found these structures only at high SecYEG concentrations, either in solution or in SecYEG-enriched membranes, but not in extracts from wild-type membranes. In addition, only SecYEG monomers and dimers were found when an equivalent amount of the purified SecYEG complex was loaded onto BN–PAGE (Figure 4A, left panel). Therefore, the multimerization of SecYEG probably occurred during incorporation of a large number of complexes into the bacterial membrane, and not following the extraction of the SecYEG complex with the detergent. Altogether, these findings open the possibility that these multimers are not representing the active translocation channel but are related to the self-oligomerization of SecYEG at high concentrations, as previously proposed (Yahr and Wickner, 2000). It is nonetheless interesting that the formation of these multimers depends on the SecG subunit. Of course, it is tempting to speculate that SecG facilitates translocation via an increased tetramerisation of

the translocase. However, our data do not support this hypothesis, since we were unable to detect changes in the distribution of these oligomers in actively translocating IMVs or proteoliposomes. Furthermore, the SecYE complex is the only essential part of the translocation machinery (Duong and Wickner, 1997a) and both the dimerization of the SecYE complex and the channel-like structures observed by electron microscopy are independent of the SecG subunit (Figure 4A; Meyer *et al.*, 1999). In addition, SecG is dispensable for cell viability and *in vivo* deletion of *secG* diminishes preprotein translocation only slightly (Hanada *et al.*, 1996; Flower *et al.*, 2000). Thus, these SecG-stabilized multimers that appear only upon overproduction may not play a role in translocation *per se*, but reflect an unknown property of SecG in the normal architecture and regulation of the translocation apparatus.

To establish whether oligomeric SecYEG functions during translocation, it was necessary to entrap the translocase in a functional state. Experiments intended to saturate the SecYEG-enriched membranes with a translocation intermediate suggested that a fraction of the SecYEG complex is inactive (Yahr and Wickner, 2000). Therefore, simply measuring the ratio of a SecYEG–SecA complex may not be sufficient to reveal the stoichiometry of the active translocase (Manting *et al.*, 2000). In the conditions used in this study, we were unable to detect a stable association of SecYEG–SecA. A transient association of SecA is more likely, when its dynamic role in the translocation cycle is considered. We could, however, entrap a translocation intermediate with the dimeric SecYEG complex. One possibility is that translocation arrest of a preprotein promotes dissociation of the SecYEG tetramer. However, since the tetramer is quite a stable structure, we view this contingency as unlikely. In contrast, a pronounced dissociation of the SecYEG dimers into monomers occurred at an increased detergent concentration and crucially, a concomitant loss of the proOmpA–SecYEG complex. Therefore, our results show that the dimeric SecYEG complex bears an authentic translocation intermediate, but we do not exclude the possibility of different and unseen oligomeric forms, stable only transiently or in phospholipid bilayers and associated with other translocase subunits such as SecDFyajC or YidC.

The preprotein translocation channel is a conformationally dynamic structure

Assembly of dimers into tetramers was observed by electron microscopy and an equilibrium between monomers, dimers and tetramers was identified by analytical ultracentrifugation (Manting *et al.*, 2000; Collinson *et al.*, 2001). In the latter study, the low levels of detected dimer were probably due to the high concentrations of detergent and the presence of tetramers due to the high concentrations of protein (Figure 4A). However, this oligomeric variability we see in solution may not exist in the *E. coli* inner membrane. A number of experimental observations suggest that the SecYEG complex does not change its oligomeric state during translocation and exists as a preformed dimeric structure: (i) the solubilization of the IMVs with a low amount of detergent revealed only low levels of monomeric SecYEG or SecYE complexes (Figure 4A); (ii) the reconstitution of the translocase into

liposomes involved detergent dilution, which in turn induced the dimerization of SecYEG monomers (Figure 3A); (iii) only this dimeric complex was formed after resolubilisation of the SecYEG–proteoliposomes (Figure 4C); and (iv) no modification in the relative distribution between monomers and dimers was detected in actively translocating SecYEG–proteoliposomes and IMVs (Figures 4C and 5C). It is thus possible that a rapid and spontaneous association of the SecYEG dimers also occur during the biogenesis of the Sec complex in the *E. coli* membrane. Furthermore, previous studies have shown that the individual SecYE subunits do not exchange between one complex and another during catalytic translocation, *in vivo* (Joly *et al.*, 1994) or *in vitro* (Yahr and Wickner, 2000), suggesting that the bacterial channel may never completely disassemble. In eukaryotes, translocation is mostly co-translational and ribosomes may initiate the assembly of Sec61p oligomers, but not their maintenance, since these oligomers are still present after stripping of the ribosomes from membranes (Hanein *et al.*, 1996). In *E. coli*, *de novo* assembly of the translocation channel from separate SecYEG units for each round of translocation may not be efficient for this rapidly growing organism.

The lability of the SecYEG dimers we saw in solution may reflect a fundamental property of the translocase. Indeed, the translocation channel must accommodate and shield parts of the dimeric SecA protein during translocation (Economou and Wickner, 1994; Eichler and Wickner, 1997), and at the same time allow the transport of partially folded polypeptide chains across the membrane (Tani *et al.*, 1990). It seems reasonable that a channel constituted by two loosely associated monomers would be able to perform these functions. The importance of a flexible SecYEG dimer is also compatible with observations made on the *prl* mutants, showing that an increase in the SecYEG complex lability enhances the translocase functions (van der Wolk *et al.*, 1998; Duong and Wickner, 1999). Finally, the plasticity of the SecYEG dimers may provide a mean for the biogenesis of integral membrane proteins. Photocross-linking experiments support the idea that the translocation channel opens laterally towards the lipid bilayer (Martoglio *et al.*, 1995) and that a transmembrane segment is arrested within the translocase before it partitions spontaneously into the lipid phase of the membrane (Duong and Wickner, 1998; Heinrich *et al.*, 2000). Liberation of the hydrophobic preprotein segment into the surrounding lipid bilayer may be facilitated by the partial dissociation of the SecYEG dimers into the plane of the membrane.

Materials and methods

Materials

SecA (Hendrick and Wickner, 1991), SecB (Hartl *et al.*, 1990), proOmpA and proOmpA–BPTI were purified as described (Schiebel *et al.*, 1991; Duong and Wickner, 1997b). IMVs were prepared from *E. coli* strain BL21 (*hsdS*, *ompT*[−], *gal*) or BL425 (*hsdS*, *ompT*[−], *gal*, *secG::kan*) carrying plasmids encoding for the SecY_{His}G, SecY_{EHA}G, SecY_{4EHA}G or SecY_{EHA} complexes. These plasmids have been previously described (Duong and Wickner, 1997a, 1999; Collinson *et al.*, 2001). DDM was purchased from ICN Biomedicals. Water-soluble and insoluble cross-linker reagents with spacer arms of different lengths (DSG, DMS, BS³,

DSS, EGS, formaldehyde and glutaraldehyde) were purchased from Pierce.

Isolation and purification of the SecYEG complex

Purification of the SecY_{His}G complex was achieved by Ni²⁺-chelating chromatography, according to the procedure described by Collinson *et al.* (2001). The membranes were initially solubilized with 1% detergent, and the purified SecYEG complex was stored in TSG buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM DTT) and CMC amounts of DDM. Reconstitution of the SecYEG complex into liposomes was as previously described (Collinson *et al.*, 2001). Immunoprecipitation of the SecY_{EHA}G complex was performed for 60 min at 4°C with 100 µl of anti-SecG protein A–Sepharose beads (Duong and Wickner, 1997a). Beads were washed three times with 1 ml of TSG buffer–0.05% DDM and proteins were eluted with Laemmli sample buffer.

Radiolabelling

¹²⁵I-labelling of the Sec complex was performed with an iodogen-coated tube (Pierce) containing 30 µg of purified SecYEG (Collinson *et al.*, 2001) and 100 µCi of [¹²⁵I]Na. After 5 min on ice, the iodinated proteins were desalted with a G25 spin-column (Bio-Rad) equilibrated in TSG buffer and CMC levels of detergent. The ¹²⁵I-labelled SecYEG complex (~1.5 × 10⁶ c.p.m./µg) was stored at −80°C and used within one month. ¹²⁵I-labelling of proOmpA–BPTI has been described elsewhere (Duong and Wickner, 1997b).

Translocation assay

Translocation assays were performed in 100 µl of TL buffer (50 mM Tris–HCl pH 7.9, 50 mM KCl, 5 mM MgCl₂, ± 1 mM DTT) containing 2.0 µg of SecB, 4.0 µg of SecA, 10 µg of bovine serum albumin (BSA), 6 µg of proOmpA or ~50 000 c.p.m. of [¹²⁵I]proOmpA–BPTI (~5 × 10⁵ c.p.m./µg) and an ATP regenerating system (5 mM creatine phosphate, 10 µg/ml creatine kinase). The translocation reaction was initiated by addition of 10 µl of SecYEG proteoliposomes (~0.35 µg of SecYEG proteins) or 10 µl of IMVs (~10 µg of membrane proteins) and incubated for 15 min at 37°C with the indicated nucleotides. Translocation reactions were stopped with potato apyrase (grade VIII, Sigma) and chilled on ice before further treatment (Figures 6 and 7).

Analytical methods

Performance of linear gradient blue native gels and electrophoretic conditions were as described by Schagger and von Jagow (1991). The molecular weight markers were from AP Biotech. Electrophoresis of BN gels was as described (Schagger, 2001), but we used a wet-electroblotter and immunoblots were visualized using the ECL reagents (AP Biotech). Autoradiography of ¹²⁵I-labelled polypeptides was performed at −80°C with intensifying screens. Protein concentrations were determined using the Bradford reagent (Bio-Rad) and BCA assay (Pierce).

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References

- Beckmann,R., Bubeck,D., Grassucci,R., Penczek,P., Verschoor,A., Blobel,G. and Frank,J. (1997) Alignment of conduits for the nascent polypeptide chain in the ribosome–Sec61 complex. *Science*, **278**, 2123–2126.
- Brundage,L., Fimmel,C.J., Mizushima,S. and Wickner,W. (1992) SecY, SecE and band 1 form the membrane-embedded domain of *Escherichia coli* preprotein translocase. *J. Biol. Chem.*, **267**, 4166–4170.
- Collinson,I., Breyton,C., Duong,F., Tziatzios,C., Schubert,D., Or,E., Rapoport,T. and Kuhlbrandt,W. (2001) Projection structure and oligomeric properties of a bacterial core protein translocase. *EMBO J.*, **20**, 2462–2471.
- Danese,P.N. and Silhavy,T.J. (1998) Targeting and assembly of

- periplasmic and outer-membrane proteins in *Escherichia coli*. *Annu. Rev. Genet.*, **32**, 59–94.
- Driessen, A.J.M. (1992) Precursor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive force. *EMBO J.*, **11**, 847–853.
- Driessen, A.J.M., Manting, E.H. and van der Does, C. (2001) The structural basis of protein targeting and translocation in bacteria. *Nature Struct. Biol.*, **8**, 492–498.
- Duong, F. and Wickner, W. (1997a) Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. *EMBO J.*, **16**, 2756–2768.
- Duong, F. and Wickner, W. (1997b) The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. *EMBO J.*, **16**, 4871–4879.
- Duong, F. and Wickner, W. (1998) Sec-dependent membrane protein biogenesis: SecYEG, preprotein hydrophobicity and translocation kinetics control the stop-transfer function. *EMBO J.*, **17**, 696–705.
- Duong, F. and Wickner, W. (1999) The PrlA and PrlG phenotypes are caused by a loosened association among the translocase SecYEG subunits. *EMBO J.*, **18**, 3263–3270.
- Economou, A. (1998) Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. *Mol. Microbiol.*, **27**, 511–518.
- Economou, A. and Wickner, W. (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell*, **78**, 835–843.
- Eichler, J. (2000) Archaeal protein translocation crossing membranes in the third domain of life. *Eur. J. Biochem.*, **267**, 3402–3412.
- Eichler, J. and Wickner, W. (1997) Both an N-terminal 65-kDa domain and a C-terminal 30-kDa domain of SecA cycle into the membrane at SecYEG during translocation. *Proc. Natl Acad. Sci. USA*, **94**, 5574–5581.
- Flower, A.M., Hines, L.L. and Pfennig, P.L. (2000) SecG is an auxiliary component of the protein export apparatus of *Escherichia coli*. *Mol. Gen. Genet.*, **263**, 131–136.
- Hamman, B.D., Hendershot, L.M. and Johnson, A.E. (1998) BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation. *Cell*, **92**, 747–758.
- Hanada, M., Nishiyama, K. and Tokuda, H. (1996) SecG plays a critical role in protein translocation in the absence of the proton motive force as well as at low temperature. *FEBS Lett.*, **381**, 25–28.
- Hanein, D., Matlack, K.E., Jungnickel, B., Plath, K., Kalies, K.U., Miller, K.R., Rapoport, T.A. and Akey, C.W. (1996) Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. *Cell*, **87**, 721–732.
- Hartl, F.U., Lecker, S., Schiebel, E., Hendrick, J.P. and Wickner, W. (1990) The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E.coli* plasma membrane. *Cell*, **63**, 269–279.
- Hartmann, E., Sommer, T., Prehn, S., Gorlich, D., Jentsch, S. and Rapoport, T.A. (1994) Evolutionary conservation of components of the protein translocation complex. *Nature*, **367**, 654–657.
- Heinrich, S.U., Mothes, W., Brunner, J. and Rapoport, T.A. (2000) The Sec61p complex mediates the integration of a membrane protein by allowing lipid partitioning of the transmembrane domain. *Cell*, **102**, 233–244.
- Hendrick, J.P. and Wickner, W. (1991) SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. *J. Biol. Chem.*, **266**, 24596–24600.
- Joly, J., Leonard, M.R. and Wickner, W.T. (1994) Subunit dynamics in *Escherichia coli* preprotein translocase. *Proc. Natl Acad. Sci. USA*, **91**, 4703–4707.
- Lill, R., Dowhan, W. and Wickner, W. (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell*, **60**, 259–269.
- Manting, E.H., van der Does, C. and Driessen, A.J. (1997) *In vivo* cross-linking of the SecA and SecY subunits of the *Escherichia coli* preprotein translocase. *J. Bacteriol.*, **179**, 5699–5704.
- Manting, E.H., van Der Does, C., Remigy, H., Engel, A. and Driessen, A.J. (2000) SecYEG assembles into a tetramer to form the active protein translocation channel. *EMBO J.*, **19**, 852–861.
- Martoglio, B., Hofmann, M.W., Brunner, J. and Dobberstein, B. (1995) The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer. *Cell*, **81**, 207–214.
- Ménétre, J.-F., Neuhofer, A., Morgan, D.G., Plath, K., Radermacher, M., Rapoport, T.A. and Akey, C.W. (2000) The structure of ribosome-channel complexes engaged in protein translocation. *Mol. Cell.*, **6**, 1219–1232.
- Meyer, T.H., Ménétre, J.-F., Breitling, R., Miller, K.R., Akey, C.W. and Rapoport, T.A. (1999) The bacterial SecY/E translocation complex forms channel-like structures similar to those of the eukaryotic sec61p complex. *J. Mol. Biol.*, **285**, 1789–1800.
- Mothes, W., Prehn, S. and Rapoport, T.A. (1994) Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. *EMBO J.*, **13**, 3973–3982.
- Nishiyama, K., Suzuki, T. and Tokuda, H. (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. *Cell*, **85**, 71–81.
- Rapoport, T.A., Jungnickel, B. and Kutay, U. (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.*, **65**, 271–303.
- Schägger, H. (2001) Blue-native gels to isolate protein complexes from mitochondria. *Methods Cell Biol.*, **65**, 231–244.
- Schägger, H. and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.*, **199**, 223–231.
- Schiebel, E., Driessen, A.J.M., Hartl, F.-U. and Wickner, W. (1991) $\Delta\mu_{\text{H}^+}$ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell*, **64**, 927–939.
- Scotti, P.A., Urbanus, M.L., Brunner, J., de Gier, J.W., von Heijne, G., van der Does, C., Driessen, A.J., Oudega, B. and Lührink, J. (2000) YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. *EMBO J.*, **19**, 542–549.
- Simon, S.M. and Blobel, G. (1991) A protein-conducting channel in the endoplasmic reticulum. *Cell*, **65**, 371–380.
- Simon, S.M. and Blobel, G. (1992) Signal peptides open protein-conducting channels in *E.coli*. *Cell*, **69**, 677–684.
- Tani, K., Tokuda, H. and Mizushima, S. (1990) Translocation of ProOmpA possessing an intramolecular disulfide bridge into membrane vesicles of *Escherichia coli*. Effect of membrane energization. *J. Biol. Chem.*, **265**, 17341–17347.
- van der Wolk, J.P., Fekkes, P., Boersma, A., Huie, J.L., Silhavy, T.J. and Driessen, A.J.M. (1998) PrlA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA–SecY interaction during the initiation of translocation. *EMBO J.*, **17**, 3631–3639.
- Yahr, T.L. and Wickner, W.T. (2000) Evaluating the oligomeric state of SecYEG in preprotein translocase. *EMBO J.*, **19**, 4393–4401.

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