Studies on the bacterial protein conducting pore
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SecYEG purification

Interaction between SecA and SecYEG in micellar solution and formation of the membrane-inserted state

Chris van der Does, Erik H. Manting, Andreas Kaufmann, Marco Lutz, and Arnold J. M. Driessen

Summary

Preprotein translocation in *Escherichia coli* is mediated by the translocase with SecA as peripheral ATPase and SecY, SecE and SecG as membrane domain. To facilitate largescale purification of the SecYEG heterotrimer, SecY was fused at its amino-terminus to a hexa-histidine tag and co-overexpressed with SecE and SecG. The presence of the his-tag allowed purification of homogeneously pure SecYEG complex by a single anion-exchange chromatographic step starting from octylglucoside-solubilised inner membranes. Endogenous levels of SecD and SecF co-purified with the SecYEG protein. Purified SecYEG complex retained a native-like, $\alpha$-helical conformation in octylglucoside and in micellar solution bound SecA with high affinity. In the presence of the nonhydrolysable nucleotide analogue adenosine 5’-(β,γ-imidotriphosphate), octylglucoside-solubilised SecYEG is nearly as effective as the reconstituted enzyme in inducing the formation of a proteinase K-protected 30 kDa fragment of $^{125}$I-labeled SecA, while SecYEG is proteolysed to fragments smaller than 6 kDa. These data demonstrate that the 30 kDa SecA fragment is neither protected by the lipid phase nor by SecYEG, but rather indicate that it represents a SecYEG- and nucleotide-induced stable conformational state of a SecA domain.

Introduction

The last decade has seen a major advance in the study of bacterial protein translocation (for reviews see (557,558)). The components involved in the translocation reaction have been genetically identified, biochemically purified, and the translocation reaction as it proceeds across the inner membrane of *Escherichia coli* has been reconstituted in liposomes using purified components (226,261). The general secretion pathway in *E. coli* consists of a cytoplasmic chaperone, SecB (69), a peripherally membrane-associated ATPase, SecA (549), and five inner membrane proteins, i.e., SecY (209), SecE (235), SecG (255), SecD and SecF (275,559). The minimal requirements for preprotein translocation across the inner membrane are met by SecA, SecY and SecE which together with SecG form the *translocase* (226). SecG enhances the fidelity of the reconstituted SecYE protein translocation reaction at least 20-fold(259), while *secG* null strains are cold-sensitive for growth (255). SecD and SecF are integral membrane proteins that expose large hydrophilic domains to the periplasmic space (275,277,559). *SecD* and *secF* null strains are also cold-sensitive (277). SecY, SecE and SecG can be co-immunoprecipitated, isolated and purified as a
stable heterotrimeric complex (226, 274). Genetic evidence suggests that SecY and SecE are dissociable subunits (300), but biochemical evidence suggests that they interact stably once assembled in the membrane (230). Together SecY and SecE suffice to constitute a high-affinity membrane binding site for SecA (79, 274). Recent evidence suggests that the proteins encoded by the secD operon, i.e., SecD, SecF, and YajC, also form a heterotrimeric complex, that associates with the SecYEG complex to form a large hexameric integral membrane protein domain (SecYEGDFYajC) which is stable in the mild detergent digitonin (274). Stabilization by overexpression (270, 289) experiments indicate that SecG and SecE interact with SecY, while SecF interacts with SecD and SecY. YajC is a dispensable protein which has no known function in protein export (275). When present on a high copy plasmid, it suppresses the SecYd mutation and causes impaired growth of the SecY39 mutant at 42°C (290). SecD and SecF have been implicated in the maintenance of proton motive force across the cytoplasmic membrane (287), in the release of translocated proteins into the periplasmic space (281), and in the stabilization of membrane-inserted SecA (274, 285). Overexpression of SecD and SecF restores the translocation of preproteins with a defective signal peptide (277). SecA is a dissociable subunit of the translocase and exists both in free cytosolic forms and as membrane bound forms (547). SecA interacts in a nonsaturable manner with acidic phospholipids (315), and binds with high affinity to the SecY subunit of the SecYEG complex (204, 205, 270, 274) SecYEG-bound SecA exhibits a high affinity for the binary SecB:preprotein complex, and recognises the preprotein by interacting with both the signal sequence and mature domain of the preprotein (315, 569). SecA is a preprotein-stimulated ATPase (179), which is activated for ADP:ATP exchange in the presence of acidic phospholipids and SecYEG (179, 226). Binding of ATP elicits a conformational change (178) that releases SecB into the cytosol (113) and promotes insertion of a SecA carboxyl-terminal domain into the membrane (206, 285, 363). At the same time, limited translocation of SecA-bound preprotein polypeptide segments occurs (343). SecA releases the bound preprotein upon the hydrolysis of ATP (343), concomitantly with the de-insertion of the SecA domain from the membrane (363). Repeated cycles of ATP binding and hydrolysis coupled to the membrane insertion and de-insertion of SecA at the SecYEG complex ultimately allow the stepwise translocation of the preprotein across the membrane (343, 347, 367). The proton motive force stimulates the rate of translocation (340).

The preprotein translocation reaction across the inner membrane of E. coli shares many characteristics with that of the eukaryotic endoplasmic reticulum (for review see ref (21)). The quaternary organisation of the mammalian and yeast endoplasmic reticulum translocase largely resembles that of the SecYEG complex of E. coli. The mammalian Sec61p complex consists of three subunits, i.e., Sec61α, Sec61β and Sec61γ. Sec61α and Sec61γ are homologous to the bacterial SecY and SecE, respectively, while Sec61β has a similar organisation as SecG, but is not homologous. The mammalian Sec61p complex is found associated with the TRAM protein has no known homologues in bacteria. In S. cerevisiae, also a posttranslational translocase has been identified, which in addition to Sec61 (Sec61α in mammals), Sbh1 (Sec61β in mammals), Sss1 (Sec61γ in mammals), involves Sec62, Sec63, Sec71 and Sec72 (409). S. cerevisiae does not contain a SecA homolog, but luminal BiP (Hsp70) couples the hydrolysis of ATP to the translocation of preproteins into the ER lumen.
These complexes have been purified to homogeneity (409,414), and investigated by High-resolution electron microscopy showing ringlike structures suggesting that the membrane domain consists of three to four Sec61p trimers with a central pore (474). The translocation pore of the mammalian Sec61p complex may have a diameter of 40-60 Å during protein translocation (490). A low-resolution structure of the bacterial SecYEG complex is not yet available.

The SecYEG complex can be purified from wild-type cells (226), or as separate, isolated subunits from overproducing strains (227). To facilitate studies on the structure and function of the bacterial translocase, we have developed a rapid purification protocol that permits isolation of milligram amounts of functional SecYEG protein using membranes derived from an E. coli strain that overexpresses the SecYEG complex with a his-tag on SecY.

The catalytic and functional properties of the SecYEG complex have been analysed in micellar solution in the absence of compartmentalisation. The solubilised complex binds stoichiometrical amounts of SecA with high affinity and, more strikingly, supports the nucleotide-induced formation of a 30 kDa proteinase protected fragment of SecA that is thought to be membrane-integrated. Since the SecYEG complex is degraded under these conditions, it appears that this SecA fragment is neither protected by the lipid bilayer nor by a transmembrane shell of SecYEG protein, but rather represents a stable conformation of a SecA domain induced by its interactions with SecYEG and nucleotides.

Results

Purification of functional SecYEG complex

To simplify the purification of the SecYEG complex from an overproducing strain (202), affinity purification of the complex with histagged SecY or SecE on Ni²⁺-NTA columns was tried. For this purpose, vectors were used that express the secYEG genes in tandem with a hexa-histidine tag and a cleavable enterokinase site at either the amino- or carboxyl terminus of SecY (Table 1, 205). The his-tagged SecY protein can be co-overexpressed to high levels together with SecE and SecG as shown by CBB-staining of a SDS-PAGE gel loaded with SechYnEG⁺ IMVs (Figure 1A). We have previously shown that the his-tag does not interfere with the level of SecYEG overexpression, the proOmpA-stimulated SecA translocation ATPase and 35S-proOmpA translocation into IMVs (205). Ni²⁺-affinity chromatography with Triton X-100, octylglucoside, or dodecylmaltoside solubilised IMVs resulted only in the purification of histagged SecY or SecE protein with almost no detectable co-purification of the other components of the SecYEG complex. Moreover, E. coli phospholipids could not be included in the elution buffer as they interfered with the binding of the histagged proteins to the Ni²⁺-NTA resin. Previously, it was shown that phospholipids are needed to maintain the

Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
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<tbody>
<tr>
<td>pET324</td>
<td>pTRC99A-derived vector containing lacZα behind the trc promotor</td>
</tr>
<tr>
<td>pET302</td>
<td>pET324-derived vector containing a His-tag and an enterokinase site</td>
</tr>
<tr>
<td>pET340</td>
<td>pET324 with secYEG under control of the trc promotor.</td>
</tr>
<tr>
<td>pET320</td>
<td>pET302 containing secYEG behind trc promotor with N-terminally His-tagged SecE</td>
</tr>
<tr>
<td>pET349</td>
<td>pET302 containing secYEG behind trc promotor with N-terminally His-tagged SecY</td>
</tr>
<tr>
<td>pET512</td>
<td>pET340 containing C-terminally His-tagged SecY</td>
</tr>
</tbody>
</table>
SecYEG complex in an active state during solubilisation and reconstitution (225). Therefore, the conventional method for purification of the SecYEG complex was attempted which is based on octylglucoside solubilisation of urea-treated IMVs followed by three consecutive chromatographic steps (226). Since the urea-extraction reduced the efficiency of solubilisation, this step was eliminated and octylglucoside-extracted proteins were directly loaded on a DEAE column. In contrast to the native complex, the SecYEG heterotrimer with amino-terminally his-tagged SecY binds only weakly to the column and elutes at a KCl concentration of about 10 mM in an essentially pure form (Figure 1B). Similar results were obtained with carboxyl-terminally his-tagged SecY (data not shown). In contrast, wild type SecYEG complex elutes at a KCl concentration of about 50-60 mM along with most of the other membrane protein (226). For convenience, the purified complex harbouring the amino-terminally his-tagged SecY is termed SechYnEG in the further sections.

The identity of the protein bands in the purified fractions was verified by the use of pAbs directed against the his-tagged SecY and SecE proteins, a pAb raised against a synthetic peptide corresponding to a SecG domain, and by total amino acid analysis of the proteins excised from the gel. In addition, minor amounts of two other proteins were present co-eluting with the SechYnEG protein. These proteins, with apparent molecular masses of 54

Table 2. Purification of SecYEG protein. The percentage of recovery of SecA translocation ATPase is indicated in parenthesis.

<table>
<thead>
<tr>
<th>Protein Sample</th>
<th>SecA translocation ATPase mg</th>
<th>Total activity mmol P/min</th>
<th>Specific activity nmol P/min/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>SecYEG membranes</td>
<td>60 (100%)</td>
<td>7860 (100%)</td>
<td>131</td>
</tr>
<tr>
<td>DEAE pool</td>
<td>15 (25%)</td>
<td>8118 (103%)</td>
<td>580</td>
</tr>
</tbody>
</table>
and 27 kDa reacted with α-SecD and SecF antibodies, respectively. Immunoblot analysis further showed that the purified SecYnEG was devoid from YajC and leader peptidase (Lep), that were recovered in the flow-through and bulk protein fractions, respectively (data not shown).

Column fractions were reconstituted by rapid dilution in liposomes composed of E. coli lipids, and analysed for the proOmpA stimulated SecA translocation ATPase activity. Only the fractions containing SecYnEG protein were active in this assay (Figure 1B). Purification resulted in an almost 5-fold increase in the specific SecA ATPase activity, and apparently without loss of activity (Table 2). When analysed at the same SecY content, proteoliposomes reconstituted with SecYnEG were nearly as active in the ATP- and SecA-dependent translocation of 35S-proOmpA as non-urea-treated SecYnEG+ IMVs (Figure 2). The use of a his-tagged SecY, therefore, allows for a rapid purification of large quantities of functional SecYnEG complex with only a minimal loss of activity.

**Stability of the SecYEG complex**

To facilitate further biochemical and biophysical research, the stability of the detergent-solubilised SecYnEG was compared with the reconstituted enzyme, and with SecYnEG+ IMVs. For this purpose, the SecA translocation ATPase activity was measured after incubation under different sets of conditions. SecYnEG+ IMVs appeared highly stable, and hardly any inactivation occurred after a three day incubation at 37°C (Figure 3). Reconstituted SecYnEG complex was stable on ice and when stored at -20°C or below, but lost its activity when incubated for longer periods of time at 25 and 37°C. Finally, the purified SecYnEG complex in octylglucoside was stable only at 4°C and below, and rapidly inactivated at 37°C (t1/2 = 15-20 min) (Figure 3). For stability, it was necessary that the glycerol concentration was kept at 40% (v/v), and that at least 0.2 mg/ml of E. coli lipids are present. Under these conditions, it was possible to store the purified complex for at least 7 months at -20°C without loss of activity.

**Octylglucoside-solubilised SecYEG is in a near-native, α-helical conformation**

The influence of octylglucoside on the secondary structure of SecYnEG was analysed by circular dichroism (CD) spectroscopy. The
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spectrum of reconstituted SechYnEG is typical for an α-helical protein (Figure 4A). Computational deconvolution of the CD spectrum revealed an α-helical content of at least 83% indicating that the protein is largely in a non-denatured state. Although some loss of secondary structure seemed to occur, the SechYnEG complex remained mainly α-helical (72%) when solubilised in octylglucoside. The CD spectrum of SecA (Figure 4B) points at a high amount of β-structure (55% β-sheet, 18% β-turn, and 18% α-helical). Octylglucoside (1.25%) hardly altered the spectrum (48% β-sheet, 16% β-turn and 21% α-helical). These data indicate that both SechYnEG and SecA maintain a native-like secondary structure when present in detergent solution.

**Octylglucoside-solubilised SecYEG binds SecA with high affinity**

To determine the orientation of reconstituted SechYnEG, the accessibility of the enterokinase recognition site at the amino-terminus of SecY to externally added enterokinase was analysed in the absence and presence of octylglucoside. In the absence of octylglucoside, enterokinase removes about 50% of the his-tags from the reconstituted SecY protein (Figure 5, compare lane 1 and 2), and completely when solubilised by octylglucoside (lane 4). Immunoblot analysis of the samples using a mAb directed against the his-tag confirmed that enterokinase indeed removes the his-tag from SecY (See Figure 6B). The data suggest that SechYnEG is reconstituted in a scrambled orientation.

Next, the ability of SecA to protect the his-tagged SecY of SechYnEG proteoliposomes against enterokinase cleavage was determined. Experiments were performed at 25°C where the SechYnEG complex is reasonably stable in detergent solution when incubated for only short periods of time, i.e., 30 min to 1 hr (Figure 3C). When the amount of BSA in the buffer was gradually replaced by SecA, protection of the

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**Figure 4.** Circular dichroism spectra of SechYnEG (A) and SecA (B) in the absence (closed lines) and presence (dashed lines) of octylglucoside. Shown spectra were corrected for the lipid and detergent contributions.

**Figure 5.** Orientation of SecY in reconstituted SechYnEG proteoliposomes. The accessibility of the introduced proteolytic enterokinase site at the amino-terminus of SecY towards purified enterokinase was used to probe the orientation of SecY in the reconstituted SechYnEG proteoliposomes. SechYnEG proteoliposomes were incubated in the absence (lane 1 and 2) and presence of 1.25% octylglucoside (OG) (lane 3 and 4) and as indicated, treated with enterokinase as described in the Experimental Procedure section. Incubations were at 25 °C in a buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM CaCl₂. After 1 hr, samples were analysed by 12% SDS-PAGE and CBB staining. The positions of SecE, SecG, his-tagged (hisSecY) and enterokinase-cleaved SecY (SecY) are indicated by an arrow.
his-tagged SecY against proteolysis by enterokinase occurred (Figure 6A and B). Strikingly, the same protective effect of SecA was observed when the experiments were performed in the presence of octylglucoside to solubilise the SechYnEG complex (lanes C and D). In the absence of SecA, enterokinase completely removed the his-tag from SecY. On the other hand, full protection against proteolysis was obtained when SecA monomer (~ 0.95 µM) was added in a slight excess relative to SecY (~ 0.7 µM). The protective effect of SecA on reconstituted and detergent solubilised his-tagged SecY was not influenced by nucleotides (ATP, AMP-PNP, or ADP) and/or proOmpA. In contrast to the reconstituted enzyme, the solubilised SechYnEG did not support SecA translocation ATPase activity (data not shown). These data demonstrate that SechYnEG maintains the ability to bind SecA with high affinity when present in detergent solution.

SecYEG dependent and nucleotide-induced formation of the SecA membrane inserted state in detergent solution

At 37°C, in the presence of ATP and preprotein, the SecYEG-bound SecA undergoes a conformational change resulting in the formation of a proteinase inaccessible 30 kDa carboxy-terminal domain (206,285,363). It has been suggested that this fragment corresponds to a membrane-inserted domain of SecA. The 30 kDa SecA fragment can also be formed without preprotein at 4°C in the presence of nonhydrolysable ATP, i.e., AMP-PNP. To determine whether the octylglucoside solubilised SechYnEG supports the formation of this membrane-inserted state, SecA was radiolabeled with 125I, added to purified SechYnEG either present in octylglucoside micellar solution or reconstituted in proteoliposomes, and treated with proteinase K (up to 1 mg/ml). In the presence of AMP-PNP, the solubilised SechYnEG supported the formation of the 30 kDa SecA fragment as effectively as proteoliposomes reconstituted with SechYnEG, and about halve as active as urea-treated SechYnEG IMVs (Figure 7A). In the absence of AMP-PNP or SechYnEG, no 30 kDa SecA fragment was formed. In contrast to octylglucoside, Triton X-100 did not support the formation of the 30 kDa fragment (Figure 7B). Our data demonstrate that octylglucoside-solubilised SechYnEG supports the formation of the 125I labeled 30 kDa fragment of SecA which implies that the 30 kDa fragment is not protected by the lipid phase.

To establish whether the 30 kDa SecA domain is protected by SechYnEG, samples were also analysed for remaining SechYnEG complex. In the absence of SecA and AMP-PNP, already at a low proteinase K
concentration (i.e., 10 µg/ml) both the reconstituted (Figure 8A) and the solubilised SecYnEG (data not shown) was rapidly degraded to fragments of 6 kDa and smaller than could be detected by silver staining. Similar results were obtained by CBB staining (data not shown), and are in accordance with previous observations that SecY is highly susceptible to proteolysis (79). Since the staining assay could not be used for proteolysis experiments in which SecA was included, two other approaches were followed, i.e., immunoblot analysis of the SecY using a pAb directed against the purified SecY protein (Figure 8B), and autoradiography of 125I-labeled SecYnEG complex (Figure 8C). At the concentration of proteinase K (i.e., 1 mg/ml) used to form the 30 kDa SecA fragment, immunoblotting with the α-SecY IgG revealed that SecA was unable to prevent proteolysis of SecY, both in the absence and presence of AMP-PNP, either with the SecYnEG complex reconstituted into proteoliposomes (Figure 8B) or solubilised in octylglucoside (data not shown). Although the pAb presumably recognises multiple epitopes on SecY, these are likely confined to the exposed loops of SecY that are most readily proteolysed. As an independent assay, reconstituted SecYnEG was labeled with the nonspecific lipophilic photoaffinity probe 3-(trifluoromethyl)-3-(m-iodophenyl)-diazirine (TID) added in 125I-labeled form. 125I-TID readily labeled the SecY,
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SecE and SecG proteins (Figure 8C, lane 1). Since only radiochemical amounts of $^{125}$I-TID were used for labeling, photo crosslinking had little effect on the SecA translocation ATPase of the SecYEG proteoliposomes (data not shown), indicating that the enzyme retained most of its activity. Reconstituted or solubilised $^{125}$I-labeled SecYEG was completely digested by proteinase K (i.e., 1 mg/ml) for 15 min at 4°C, separated on a 16% tricine gel and analysed by Western blotting using a pAb directed against the purified SecY protein (α-SecY IgG). The position of hisSecY and an immunoreactive amino-terminal fragment of SecY are indicated. (C) $^{125}$I-TID labeled SecYEG proteoliposomes (10 µg/mL) were incubated for 30 min at 4°C in translocation buffer D in the absence (lanes 1 - 4) and presence (lanes 5 - 8) of 1.25% octylglucoside. Samples (lanes 2 and 6) were supplemented with 20 µg/mL SecA (lanes 3 and 7), or both with SecA and 1.25 mM AMP-PNP (lanes 4 and 8). As indicated, samples were treated with proteinase K (1 mg/mL) for 15 min at 4°C, solubilized in SDS-sample buffer, separated on 16% tricine gels and analysed by autoradiography. The positions of the his-tagged SecY(hisSecY), SecEG, phospholipids (PL), and proteolytic fragments are indicated. Molecular mass standards are marked.

Figure 8. SecA and AMP-PNP do not protect SecYEG against proteolysis by proteinase K. (A) SecYEG proteoliposomes (100 µg/mL) in 50 mM KCl, 50 mM Tris-HCl, pH 8.0, were incubated for 15 min at 4°C in the absence (lane 2) or presence (lane 3) of proteinase K (10 µg/mL). Samples were separated on a 16% tricine gel and analysed by silver staining. As a control, the amount of proteinase K present in the assay was silver-stained (lanes 1). The position of hisSecY, SecE and SecG are indicated. Additional polypeptide bands in lane 2 are proteolytic degradation products of his-tagged SecY. (B) SecYEG proteoliposomes (10 µg/mL) (lane 1) were incubated for 30 min in the translocation buffer D without any additions (lane 2), with 1.25 mM AMP-PNP (lane 3), 20 µg/mL SecA (lane 4), or both with SecA and AMP-PNP (lane 5). Samples were subsequently incubated with proteinase K (1 mg/mL) for 15 min at 4°C, separated on a 16% tricine gel and analysed by Western blotting using a pAb directed against the purified SecY protein (α-SecY IgG). The position of hisSecY and an immunoreactive amino-terminal fragment of SecY are indicated. (C) $^{125}$I-TID labeled SecYEG proteoliposomes (10 µg/mL) were incubated for 30 min at 4°C in translocation buffer D in the absence (lanes 1 - 4) and presence (lanes 5 - 8) of 1.25% octylglucoside. Samples (lanes 2 and 6) were supplemented with 20 µg/mL SecA (lanes 3 and 7), or both with SecA and 1.25 mM AMP-PNP (lanes 4 and 8). As indicated, samples were treated with proteinase K (1 mg/mL) for 15 min at 4°C, solubilized in SDS-sample buffer, separated on 16% tricine gels and analysed by autoradiography. The positions of the his-tagged SecY(hisSecY), SecEG, phospholipids (PL), and proteolytic fragments are indicated. Molecular mass standards are marked.
formation of the 30 kDa SecA fragment, SecYEG is proteolysed to fragments of 6 kDa and smaller.

Discussion

In this paper a simple method is described for the rapid isolation of large quantities (about 5 mg per litre of cells) of homogeneously pure SecYEG protein using IMVs derived from *E. coli* cells overexpressing this complex with a hexa-histidine tagged SecY. The method is based on the weak anion-exchange column binding characteristics of the octylglucoside-solubilised SecYnEG complex. The his-tag on SecY causes a dramatic shift in the elution profile of the SecYEG complex such that it elutes in a region that is essentially free from contaminants. Wild type SecYEG complex elutes from the DEAE column together with the bulk protein fractions (226), and even when overproduced, more than one chromatographic step is needed to purify the complex (548). Due to the distribution of charges, the sequence bearing the hexa-histidine tag and the enterokinase site will have a net negative charge at pH 8.0. Therefore, tighter instead of weaker binding to the anion exchange resin was expected. The reason for this aberrant chromatographic behaviour is unclear. On the other hand, protocols based on Ni$^{2+}$-affinity column chromatography failed to purify the SecYnEG protein as a complex, and instead only the his-tagged protein is retained by the column (205, this paper). It thus appears that the subunit interaction cannot be maintained using Ni$^{2+}$-affinity column chromatography, which is either due to the absence of phospholipids, extensive washing procedures, or both.

The presence of a his-tag does not affect the activity of the SecYEG protein. Translocation and SecA translocation ATPase activities of SecYnEG$^+$ IMVs are identical to that of SecYEG$^+$ IMVs (205). Also the specificity of the SecYEG complex appears not to be affected. Unlike proteoliposomes reconstituted with purified SecYnEG containing a *prlA* mutation, SecYnEG proteoliposomes and IMVs are unable to translocate a signal sequence mutant of proOmpA with a deletion of Ile at position 8 (A.K. and C.v.d.D., unpublished results).

As judged from the recovery of SecA translocation ATPase activity, slightly more than 100% of the total activity observed with SecYnEG$^+$ IMVs was recovered after purification. An increase in SecA translocation ATPase activity was noted during the purification of the SecYEG complex from wild-type IMVs (226). When the mixed orientation of the reconstituted SecYnEG is taken into account, and assuming that the wrongly oriented molecules do not contribute to the SecA translocation ATPase activity, an almost 2-fold increase in total activity is predicted. These data are normalised to the activity observed with urea-treated IMVs. Urea-treatment largely inactivates the SecA bound to the translocation sites (179), and in particular with SecYnEG$^+$ IMVs, a major part of the SecA protein remains bound to the membranes (202,547). It may well be that this SecA blocks translocation sites that are recovered when the SecA is dissociated from SecYEG during solubilisation and purification. Alternatively, it may be that urea-treatment results in a partial inactivation of the SecYEG complex. In this respect, we have noted that SecA re-addition to urea-treated IMVs does not completely restore the translocation activity to the level observed with native membranes. Taken together it appears that our purification method allows for the isolation of pure SecYEG complex with a high recovery of activity.

Circular dichroism analysis of the secondary structure of the SecYnEG reconstituted into liposomes suggests that the complex is in a largely $\alpha$-helical conformation.
The complex encompasses 15 transmembrane segments (TMS) that are connected by cytosolic and periplasmic loops. A high α-helical content is thus consistent with the predicted secondary structure of the SecYEG, and readily accounts for the presence of 15 α-helical TMS. There is only a small loss of secondary structure when the protein is incubated with octylglucoside or dodecyl-maltoside (C.v.d.D., unpublished data), suggesting that the complex retains a near native-like secondary structure when present in detergent solution. The amino-terminally his-tag on SecY can readily be removed by enterokinase, while SecA protects the SechYN against cleavage when the SechYN is either reconstituted in proteoliposomes or present in detergent solution. The latter is remarkable as the translocation and lipid ATPase activity of SecA is (reversibly) inactivated by detergent (315). In detergent solution approximately stoichiometric amounts of the SecA monomer relative to SecY protein are needed for complete protection against enterokinase digestion. A one-to-one SecA - SecY interaction has been reported in wild-type IMVs (79). The SechYN complex (0.7 µM) was used at a concentration far above the binding affinity (30 nM) for SecA. Therefore, it is not possible to derive a K_4 value from these proteolysis experiments. Since only a slight excess of SecA is needed relative to SechYN complex, it is evident that the interaction must be of high affinity. SecA also binds with high affinity to amino-terminal fragments of SecY on blotting membranes (204).

Octylglucoside-solubilised SechYN not only binds SecA, but in the presence of the nonhydrolysable ATP analogue AMP-PNP it also supports the formation of the 30 kDa fragment of 125I-labeled SecA. The 30 kDa fragment corresponds to a carboxyl-terminal domain of SecA (206), and is thought to insert into the membrane upon binding of AMP-PNP (285,363). We now show that this reaction can be carried out at 4°C under conditions that the octylglucoside-solubilised SechYN is stable. At 37°C, the complex rapidly aggregates in detergent solution, and therefore it was not possible to analyse the formation 30 kDa SecA fragment under “translocating” conditions, i.e., in the presence of ATP and preprotein. Previously, solubilisation of the membranes with Triton X-100 was used as a control to demonstrate that the 30 kDa proteolytic fragment is a membrane-integrated SecA domain (363), i.e., in the presence of Triton X-100, the 30 kDa fragment is readily digested by proteinase K. Our studies show that, in contrast to octylglucoside, Triton X-100 is unable to stabilise the 30 kDa fragment. Triton X-100 is a more potent detergent in comparison to octylglucoside due to its lower critical micellar concentration. It may be destructive for the SecA - SecYEG interaction, or disrupt the SecA conformation. Freezing and thawing of the solubilised SechYN complex in the presence of proteinase K did not result in a loss of the 30 kDa 125I-SecA fragment (C.v.d.D., unpublished results). Recent studies suggest that the membrane-inserted form of SecA is not exposed to the lipid phase since it is inaccessible to lipid-embedded photoaffinity crosslinkers (324). This has led to the suggestion that the 30 kDa fragment entirely penetrates the membrane and exposes domains to the periplasmic membrane face, or alternatively, that it is shielded from lipids by the SecYEG complex (324,364). Our data with the SecYEG complex in octylglucoside solution supports the finding that the 30 kDa SecA fragment is not protected against proteinase digestion by the lipid membrane, but also excludes the possibility that it is exposed to a different compartment, i.e., the periplasmic membrane face of IMVs. The latter seems also unlikely on the basis of the observation that AMP-PNP and SecYEG-bound SecA is
accessible from the cytosolic membrane face for
binding towards a mAb recognising an epitope
which is part of the 30 kDa SecA fragment
(378). Most importantly, the conditions used to
form the SecA fragment result in the complete
digestion of the SecYEG complex, yielding
degradation products that are smaller than 6
kDa. Therefore, it appears that the SecA
fragment is also not proteinase-protected by a
belt of SecYEG helices. Our data suggests that
only small fragments of SecY suffice to
maintain the SecA domain in a protease-
resistant conformation, or, alternatively that the
formation of the 30 kDa proteolytic fragment is
irreversible. In this respect, SecA has been
shown to bind to SecY fragments that cover
only the 107 amino-terminal residues of the
protein (204). It should be emphasized that the
30 kDa fragment is not unique for the SecYEG-
bound form of SecA and albeit less stable, it can
also be formed in solution (206). This is an
indication that the 30 kDa fragment might
resemble a conformational state rather than
being proteinase-inaccessible. Taken together,
we conclude that the 30 kDa fragment
represents a stable conformation of a SecA
domain that is induced by its interaction with
nucleotides and further stabilised by the
SecYEG complex.

Our data do not argue with the
hypothesis that SecA drives preprotein
translocation across the membrane by the co-
insertion of a SecA domain and bound
preprotein (363), but demonstrate that any
evidence for membrane insertion based on the
formation of a protease-resistant conformation
has to be taken with caution. Recently, it has
been shown that SecA is also proteolysed into a
stable amino-terminal 65 kDa fragment under
exactly the same conditions that result in the
formation of the 30 kDa fragment, i.e., in the
presence of ATP and preprotein or AMP-PNP
alone (364) or at low proteinase concentration
in solution (206). Both fragments cover more
that 90% of the molecular mass of SecA, which
is a 102 kDa protein. The 65 kDa fragment
bears both of the nucleotide binding sites of
SecA and this part must evidently be accessible
from the cytosol. Since the evidence that the
65 kDa fragment is membrane-inserted is also
based on proteinase protection experiments,
while it is also not photoaffinity labeled from
the lipid phase (364), its exact localization
requires further investigation. In this respect,
recent studies on the membrane topology of
monocysteine SecA variants indicate that
amino-terminal, central and carboxy-terminal
regions of SecA are periplasmically accessible
to biotin maleimid (129). Biotine maleimide
labeling can be blocked by prior treatment with
a membrane-impermeable Cysteine-reactive
reagent, suggesting that the labeling does not
occur within the membrane bilayer. These
regions therefore either span the membrane or
are located within a channel which is
periplasmically accessible to molecules of the
size of biotin maleimide. These data confirm
previous studies on the periplasmic accessibility
of the carboxy-terminal (202) and other regions
(284) of SecA to trypsin. The extreme carboxy-
terminal part of SecA contains the SecB binding
domain (113), and releases the SecB upon the
initiation of preprotein translocation by binding
of ATP to SecA (113). Since other regions of
the carboxyl-terminal part of SecA interact with
SecY (204), it seems that the carboxyl-terminus
fulfills a critical role in anchoring of SecA to
the SecYEG heterotrimer during a translocation
cycle. Alternatively to the membrane insertion
hypothesis, polypeptide segments may also be
translocated across the membrane by a
mechanism that involves only conformational
changes (and mechanical movements) of the
SecA domains (178), while they are anchored at
the SecYEG complex (129,202).

Summarising, the SecYEG complex can
now be purified in large quantities in a
functional form, and retains many of its
catalytic and functional properties in micellar solution. This provides the unique opportunity to biochemists and biophysicists to study the mechanism of preprotein translocation in the absence of compartmentalisation.

Materials and methods

Materials

E. coli SecA (549), SecB (77), and proOmpA (562) were purified as described. 35S-labeled proOmpA was synthesised by in vitro transcription/translation, affinity-purified (136), and stored frozen in 6 M urea and 50 mM Tris-HCl, pH 7.8. pAbs were raised against synthetic peptides conjugated to bovine serum albumin (BSA) that correspond to domains of SecG (H2N-APAKTEQTQP-COOH) (Research Genetics Inc., Huntsville, AL) (α-SecG IgG), YajC (H2N-YRPQQKRTKEHKKLMDSS-COOH), SecD (H2N-KEELSNQRTVQAIDEGYRG-COOH) (α-SecD IgG) and SecF (H2N-MAQEYTVEQLNHGRKC-COOH) (α-SecF IgG) (Neosystem Laboratoire, Strasbourg, France). The pAb against leader peptidase (α-Lep IgG) was a generous gift of W. Wickner (Dartmouth College, Hanover, NH). E. coli lipids (Avanti Polar lipids, Inc., Brimingham, AL) were washed with aceton and ether and stored frozen in 20% sucrose, 50 mM Tris-HCl, pH 8.0, and frozen as nuggets in liquid nitrogen.

Plasmid construction

The vector pET340 (SecYEG+) allows the overproduction of SecYEG under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible trc promoter (202), and contains an ampicillin resistance gene and colE1 origin. The construction of plasmids overproducing the SecYEG complex with a hexa-histidine tag at the amino-terminus of SecY (pET349; SechYnEG+), and SecE (pET320; SecYhEnG+), and carboxy-terminus of SecY (pET512; SechYcEG+) is described previously (205). The hexa his-tag followed (or preceded in case of the carboxy-terminal tag) by an enterokinase recognition site contains the amino acids sequence MH6E4KA. As a control plasmid, the linking sequence coding for the his-tagand enterokinase site (205) was inserted in pET324 (202) resulting in pET302. All constructs were confirmed by sequence analysis on a Vistra DNA sequencer 725 using the automated Δtaq sequencing kit (Amersham, Buckinghamshire, U.K.). All other DNA techniques followed standard procedures.

Bacterial strains and growth conditions

For all experiments, the OmpT- and OmpP-(A.K., unpublished results) protease-deficient strain SF100 (570) was used. Strains were grown aerobically at 37°C in L-broth in a shaking incubator until the end of the logarithmic phase. Ampicillin was used at 50 µg/ml. For the induction of plasmid encoded genes under control of an IPTG-inducible promoter, exponentially growing cultures were supplemented with 0.5 mM IPTG at OD660 of 0.5 and grown for another 2 hrs. Large scale production of cells was done in a 15 litre fermentor (ADI 1065 Biobench, Applikon, The Netherlands) in L-broth supplemented with 1% glycerol. At an OD660 of about 4, cells were induced for 2 hrs, collected by centrifugation, resuspended into 20% sucrose, 50 mM Tris-HCl, pH 8.0, and frozen as nuggets in liquid nitrogen.

Isolation of inner membrane vesicles (IMVs)

Cells were quickly thawed at 37°C and diluted with an equal volume of 20% glycerol, 50 mM Tris-HCl, pH 8.0 (buffer A) supplemented with 1 mg/ml of DNase and RNase, and 1 mM phenylmethylene sulfonethyl fluoride (PMSF). The suspension was subjected to French press treatment (4 times at 8,000 psi), diluted with an
equal volume of buffer A, and cleared from
debris by centrifugation (10 min at 4,000 x g).
Membranes were isolated from the supernatant
by centrifugation (90 min at 40,000 x g),
resuspended in buffer A, and applied onto a
30-60% sucrose gradient in 50 mM Tris-HCl,
pH 8.0. After 18 hrs, IMVs were collected,
diluted with 5 volumes of buffer A, and re-
collected by centrifugation (90 min at 40,000 x
g). Purified IMVs were resuspended in buffer A
at 20 mg/ml, and stored in liquid nitrogen.

**Solubilization and purification of SecYEG**

SecYnEG\(^{+}\) or SecYcEG\(^{+}\) IMVs (60 mg of protein) were solubilised on ice for 20 min at 1 mg/ml in 1.25% \(n\)-octyl-\(\beta\)-D-glucopyranoside (octylglucoside), 10 mM Tris-HCl, pH 8.0, 20% glycerol, 0.5 mg/ml *E. coli* lipids and 5 mM \(p\)-aminobenzamidine (buffer B). Non-solubilised proteins and aggregates were removed by centrifugation (30 min at 40,000 x g) and the cleared supernatant was loaded onto a DEAE column (volume 60 ml) (Whatman, DE-52) equilibrated with buffer B. The column was washed with 2 volumes of buffer B, and proteins were eluted with a linear KCl gradient (0-300 mM) in 3 volumes of buffer B. The ionic strength of the elution was monitored continuously with a conductivity monitor (Pharmacia Biotech, Uppsala, Sweden). Fractions of 6 ml were collected, immediately supplemented with 87% glycerol, 1.25% octylglucoside to yield a final glycerol concentration of 40% and stored at -18°C. Fractions were analysed on a 15% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel (571) and stained with Coomassie Brilliant Blue (CBB). Immunoblotting was performed using a semidy blotter (Transblot-SD, BioRad Laboratories, Hercules, CA) and blots were developed using several different pAbs and the \(p\)-nitroblue tetrazolium chloride/sodium 5-bromo 4-chloroindolyl-phosphate staining (Boehringer Mannheim, Mannheim, Germany) of alkaline phosphatase conjugated secondary antibody (Boehringer Mannheim, Mannheim, Germany).

**Preparation of polyclonal antibodies directed against SecY and SecE**

To obtain pAbs directed against purified SecY and SecE, membranes were isolated from *E. coli* strain SF100 harbouring pET320 (SecYhEnG\(^{+}\)) or pET349 (SecYnEG\(^{+}\)), respectively. His-tagged proteins were purified from the octyl-glucoside solubilised IMVs by Ni\(^{2+}\)-NTA affinity chromatography using a HiTrap Chelating column (Pharmacia Biotech), and a buffer containing 100 mM NaCl and 50 mM NaPi, pH 8.0, and a gradient of 0-500 mM imidazole. Fractions containing SecY and SecE were separated on a preparative 15% PAGE gel, and the individual proteins were excised from the gel and collected by electrophoresion (Electro-Eluter, BioRad Laboratories, Hercules, CA). Purified proteins (over 99% pure as checked by SDS-PAGE followed by CBB staining) were used as antigens to immunize rabbits to yield pAbs against the his-tagged SecY (\(\alpha\)-SecY IgG) and SecE (\(\alpha\)-SecE IgG).

**Reconstitution of SecYEG into liposomes**

The SecYnEG complex was reconstituted into liposomes by a modification of the detergent dilution technique (226). Solubilised SecYnEG (100 \(\mu\)l; 0.25 - 0.5 mg/ml) was mixed with 20 \(\mu\)l of *E. coli* lipids (20 mg/ml), and incubated for 5 min on ice. The sample was diluted into 4 ml of buffer containing 50 mM Tris-HCl, pH 8.0, and 50 mM KCl. After 5 min of incubation, proteoliposomes were collected by centrifugation (30 min, SW-28, 50,000 rpm, 4°C) and resuspended in 100 \(\mu\)l of 50 mM Tris-HCl, pH 8.0 and 50 mM KCl. Reconstituted SecYnEG proteoliposomes were frozen and stored in liquid nitrogen. Before use, samples
were thawed at 37°C and sonicated 3 times for 10 sec in a bath sonicator.

**Translocation and SecA translocation ATPase assays**

Translocation of \(^{35}\)S-proOmpA into proteoliposomes was assayed by its accessibility to added proteinase K (344). Reaction mixtures (50 µl) contained: buffer D [50 mM HEPES-KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml BSA, 2 mM DTT, and 2 mM Mg(OAc)\(_2\)], 1.6 µg of SecB, 0.5 µg of SecA, 2 mM ATP, 10 mM creatine phosphate, and 0.5 µg creatine kinase. Based on quantitative immunoblot analysis, reactions were supplemented with approximately equal amounts of SecY, i.e., with SechYnEG proteoliposomes (20 µg/ml) or SechYnEG\(^+\) IMVs (100 µg/ml). \(^{35}\)S-proOmpA was diluted 50-fold from a solution containing 6 M urea and 50 mM Tris-HCl, pH 7.8. Translocation reactions were performed at 37°C, and at various times samples were taken, chilled on ice, and treated with proteinase K (0.1 mg/ml) for 15 min. Samples were analysed by 12% SDS-PAGE and quantified with the β-imager 2000 (Biospace Measures, Paris, France).

Translocation ATPase activity of urea-treated inner membranes or SecYEG proteoliposomes was measured with proOmpA as described (179).

**Circular dichroism spectroscopy**

Circular dichroism (CD) spectra of octylglucoside-solubilised or reconstituted SecYnEG protein (95 µg/ml) were recorded in 10 mM NaPi, pH 7.8, 10 mM KCl and 1 mg/ml E. coli lipids. CD spectra of SecA (168 µg/ml) were determined in 10 mM NaPi, pH 7.6, 10 mM KCl and 2 mM MgCl\(_2\) in the absence and presence of 1.25% octylglucoside. Measurements were performed with an AVIV CD spectrophotometer Model 62a DS. The measuring cell was thermostated at 4°C. Spectra were corrected for the lipid and detergent contributions, and deconvoluted with the SELCON software package (572).

**Enterokinase digestion of his-tagged SecY**

The accessibility of the introduced enterokinase site at the amino-terminus of SecY was determined as follows: solubilised or reconstituted SechYnEG complex (50 µg/ml) was incubated at 20°C in a buffer (30 µl) containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 2 mM CaCl\(_2\). Varying amounts of SecA or BSA were added to the samples, and after 10 min, the mixture was supplemented with 1 unit of enterokinase (New England Biolabs, Beverly, MA) and incubated for 1 hour. Reactions were terminated by the addition of SDS sample buffer, and analysed by 15% SDS-PAGE followed by CBB staining or immunoblotting using the pAbs directed against his-tagged SecY or SecE.

**Formation of the \(^{125}\)I-labeled 30 kDa fragment of SecA.**

Purified SecA was labeled with carrier-free \(^{125}\)I as described (363,378). \(^{125}\)I-SecA (2.5 ng/ml) was incubated on ice in buffer D containing either SechYnEG\(^+\) IMVs (0.5 µg/ml), or purified SechYnEG (50 ng/ml) reconstituted into proteoliposomes or present in octylglucoside solution. Incubations were performed with or without 1.25 mM adenosine 5’-(β,γ-imidotriphosphate) (AMP-PNP), and in the case of the solubilised SechYnEG, the buffer was supplemented with 1.25% octylglucoside or 1% Triton X-100. After 30 min, samples were treated with proteinase K (0.04 to 1 mg/ml) for 15 min on ice, precipitated with 7.5% (w/v) trichloroacetic acid, washed with aceton, and solubilised in SDS sample buffer. Samples were separated by 12% PAGE.
and analysed by autoradiography and beta imaging.

\textit{125I-labeling and proteolysis of purified SechYnEG complex}

Reconstituted SechYnEG protein (10 µg) in 50 mM KCl, 50 mM Tris-HCl, pH 8.0 (100 µl) was photoaffinity labeled with 13.3 µCi of 3-(trifluoromethyl)-3-(m-[\textsuperscript{125}I]iodophenyl)-diazirine (\textsuperscript{125}I-TID) (Radiochemical centre, Amersham, U.K.) for 2 min at 0°C with a 254 nm lamp (Model UVG-54, UVP Life Sciences Inc., Cambridge, U.K.) placed at a distance of 1 cm.

For proteolysis experiments, proteoliposomes bearing nonlabeled or \textsuperscript{125}I-TID SechYnEG (0.5 µg), or solubilised SechYnEG complex, were incubated for 30 min at 4°C in buffer D (50 µl) in the absence or presence of 1.25% octylglucoside, 1.25 mM AMP-PNP and/or 20 µg/ml of SecA protein. Subsequently, samples were incubated with proteinase K (1 mg/ml) for 15 min at 4°C, solubilised in SDS-sample buffer, separated on 16% tricine gels (573) and analysed by silver and CBB staining, autoradiography, or by western blotting using α-SecY IgG.

\textbf{Other analytical techniques}

Protein concentrations were determined by the method of Lowry (564) in the presence of SDS using BSA as a standard. The SecYEG concentration was estimated from quantitative total amino acid determination performed by Eurosequence (Groningen, The Netherlands). SecA protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 90.85 mM\textsuperscript{-1} cm\textsuperscript{-1} (T. den Blaauwen, personal communication).

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