The Bacterial SecY/E Translocation Complex Forms Channel-like Structures Similar to those of the Eukaryotic Sec61p Complex

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Introduction

Most proteins transported across the cytoplasmic membrane in bacteria possess signal sequences that direct them to translocation sites in the membrane. In general, a translocation substrate first interacts with the cytoplasmic chaperone SecB and is then bound by SecA (Hartl et al., 1990), a peripheral membrane protein with ATPase activity (Cunningham et al., 1989; Lill et al., 1989). SecA associates with the signal sequence and other parts of the polypeptide chain (Lill et al., 1990). Subsequently, the substrate is transferred to the SecYEG complex (Hartl et al., 1990), a heterotrimeric membrane protein complex that is likely to constitute the central component of the translocation apparatus in the cytoplasmic membrane of bacteria. We have purified a translocationally active complex of the two subunits, SecY and SecE, from Bacillus subtilis. As demonstrated by electron microscopy, SecY/E forms ring structures in detergent solution and in intact lipid bilayers, often with a quasi-pentagonal appearance in projection. The particles represent oligomeric assemblies of the SecY/E complex and are similar to those formed by the eukaryotic Sec61p complex. We propose that these SecY/E rings represent protein-conducting channels and that the two essential membrane components SecY and SecE are sufficient for their formation.

The SecYEG complex is major component of the protein translocation apparatus in the cytoplasmic membrane of bacteria. We have purified a translocationally active complex of the two subunits, SecY and SecE, from Bacillus subtilis. As demonstrated by electron microscopy, SecY/E forms ring structures in detergent solution and in intact lipid bilayers, often with a quasi-pentagonal appearance in projection. The particles represent oligomeric assemblies of the SecY/E complex and are similar to those formed by the eukaryotic Sec61p complex. We propose that these SecY/E rings represent protein-conducting channels and that the two essential membrane components SecY and SecE are sufficient for their formation.

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central component of the protein translocation system in the endoplasmic reticulum (ER) membrane (Görlich et al., 1992; Görlich & Rapoport, 1993; Hartmann et al., 1994). SecY is similar in sequence and topology with the largest subunit of the trimeric complex (called Sec61p in *Saccharomyces cerevisiae* and Sec61β in mammals; Deshaies & Scheckman, 1987; Esnault et al., 1994; Görlich et al., 1992), and SecE is homologous to the smallest subunit (called Sss1p in yeast and Sec61γ in mammals; Hartmann et al., 1994), but the sequence homology is rather low (~20% identical amino acid residues). SecG does not share any obvious similarity with the β-subunit of the eukaryotic Sec61p complex. Certain functional aspects of the translocation systems must also be distinct, as SecB, SecA, and SecD/F/yajC are not present in eukaryotes, and components required for translocation in eukaryotes are not found in prokaryotes. In eukaryotes, many proteins are translocated while being synthesized on membrane-bound ribosomes, and the Sec61p complex may function to dock ribosomes to the ER membrane (Kalies et al., 1994). In contrast, a tight membrane interaction of ribosomes has not been observed in bacteria. Hence, it is not clear to what extent structural and functional information from the eukaryotic Sec61p complex can be applied to the bacterial SecYEG complex.

In eukaryotes, there is evidence that the Sec61p complex forms a protein-conducting channel in the plane of the ER membrane. Initial evidence came from cross-linking experiments, demonstrating that Sec61β is the only protein that lines the path of the translocating polypeptide chain continuously from one side of the membrane to the other (Mothes et al., 1994). Further support for the existence of a Sec61p channel was provided by electron microscopy (Hanein et al., 1996). Purified complexes from both mammals and yeast form ring structures in detergent, with pore sizes of ~20 Å. The volume and mass of these particles are consistent with their being comprised of three to four Sec61p heterotrimers, although in projection the structures are quasi-pentagonal. Similar structures were seen in freeze-fracture electron micrographs of the Sec61p complex reconstituted into lipid bilayers. Quasi-pentagonal rings were also seen in native ER membranes, although they had a somewhat larger diameter than those formed by the purified complex. A recent electron microscopy study demonstrated that the channel formed by the Sec61p complex is aligned with a central tunnel within the large subunit of the ribosome (Beckmann et al., 1997), and suggests a continuous translocation path for the nascent chain from the peptidyl transferase center to the lumen of the ER membrane. Studies of the passage of fluorescence quenching agents through the membrane of native microsomes suggest that the pore diameter of the channel may change from ~9-15 Å to ~40-60 Å when translocation is initiated, and that the pore is opened at its luminal end by removal of BiP (Hamman et al., 1998).

Evidence for the formation of a channel by the bacterial SecYEG complex is limited. Cross-linking experiments have shown close proximity of some positions of a translocating polypeptide chain with SecY (Joly & Wickner, 1993). In addition, the existence of a channel of limited pore size is indicated by the fact that translocation was stalled by the presence of a bulky group attached to the C terminus of a substrate (Bassilana & Wickner, 1993). However, systematic cross-linking or electron microscopy experiments in the bacterial system have not yet been performed.

Here, we demonstrate by electron microscopy that a complex consisting only of bacterial SecY and SecE forms ring structures in detergent solution and in intact lipid bilayers. We propose that these rings are formed from oligomers of the SecY/E complex and represent protein-conducting channels.

Results

**Purification of the SecY/E complex from *B. subtilis***

We reasoned that a putative bacterial protein translocation channel would be formed from the SecY and SecE proteins alone, because SecG is non-essential in *E. coli* (Nishiyama et al., 1994). We therefore purified the SecY/E complex from *B. subtilis* cells. Expression of the proteins, both with N-terminal His tags, was induced in a strain that carried a deletion of the chromosomal secY gene. As judged from immunoblots with antibodies directed against SecY, SecE, or the His tag, both proteins were expressed at a level approximately four- to fivefold higher than in wild-type *B. subtilis* cells (data not shown).

The SecY/E complex was purified from total membranes after solubilization with 1% digitonin and removal of insoluble material by centrifugation (Figure 1, lanes 1 and 2). The solubilized proteins were bound to a nickel-nitritoltriacetic acid (Ni-NTA)-agarose column and eluted with an imidazole gradient. SecY and SecE coeluted from the column (lane 3; immunoblots not shown). The complex was further purified by cation exchange chromatography using a HiTrap SP-Sepharose column. The SecY and SecE proteins coeluted as a complex and the final preparation was estimated to be more than 90% pure (lane 4; SecG runs as a sharp band with a faint smear below it). The identity of the SecY and SecE proteins was confirmed by amino acid sequencing of tryptic digestion products (data not shown). No evidence for a SecG band was found. A band of ~33 kDa was an occasional, sub-stoichiometric impurity. Its presence or absence had no effect on the results of translocation or electron microscopy experiments.
We then determined the translocation activity of the purified SecY/E complex. We employed proOmpA, which is normally transported across the cytoplasmic membrane of E. coli and inserted into the outer membrane, as the translocation substrate. The protein was synthesized in vitro in a reticulocyte lysate system in the presence of [35S]methionine. After sedimentation of the ribosomes to ensure that only post-translational translocation can occur, ATP was removed by gel filtration. Labeled proOmpA, SecA purified from B. subtilis, and ATP were added to proteoliposomes reconstituted from the purified SecY/E complex and a polar lipid extract from E. coli membranes. After incubation for 60 minutes at 37°C, translocation was assayed by the appearance of protease-protected material (Figure 2(a)). Approximately 23% of the input proOmpA was protected (lane 6), but could be degraded when the bilayer was disrupted by the addition of Triton X-100 (lane 5). Essentially no translocation was seen in the absence of SecY/E complex (lane 1), ATP (lane 2), or SecA (lane 3), or when incubation was omitted (lane 4). A time-course experiment demonstrated that translocation of proOmpA proceeded for about 15 minutes (Figure 2(b)). A transport efficiency of 8-10% was obtained when prepro-α-factor was used as a substrate (data not shown). These results indicate that the purified B. subtilis SecY/E complex can actively translocate proteins.

**Morphology of the SecY/E complex in detergent solution**

We used electron microscopy to determine the morphology of the purified SecY/E complex. Negatively stained specimens showed a large number of ring-like structures (see overview in Figure 3(a)); the rings are indicated by arrowheads). Many particles had a quasi-pentagonal shape with a small pore (Figure 3(b)). Similar ring-like morphologies were seen when closely packed SecY/E particles were analyzed in vitreous ice (see the arrowheads in Figure 3(b)).

For image processing we used negatively stained SecY/E complexes because of their higher contrast
compared with particles in vitreous ice. We scanned 11 micrographs and initially chose 2041 particles for analysis. A subset of particles \((n = 237)\) with quasi-fivefold rotational symmetry (such as those shown in Figure 3(c)) were then selected by rotational power spectra analysis (Kocsis et al., 1995; Yang et al., 1998) and used to generate an average (see the map in Figure 3(d)). After classification of these data, two major classes were found which differed significantly in their apparent outer diameters (Figure 3(e) and (f)). The average diameter of the SecY/E rings was \(81 \text{ Å}\) for class I (Figure 3(e)) and \(87 \text{ Å}\) for class II (Figure 3(f)), with the overall average being \(84 \text{ Å}\) (Figure 3(d)). In both size classes and the overall average, the pore was less pronounced than with individual particles (Figure 3(c)).

We next wished to obtain an average from the particles of the original larger dataset which had not been selected based on their quasi-fivefold symmetry. We used classification to select 1200 particles based on their size and ring-like morphology. This dataset was aligned against the overall average from the quasi-fivefold dataset (Figure 3(d)) to generate a global average (shown in Figure 3(g)). The global average is similar in size and shape with that obtained with rings selected on the basis of quasi-fivefold symmetry (compare Figure 3(d) and (g)), but is somewhat more rounded.

**Figure 3.** Structure of the SecY/E complex in detergent solution. (a) Overview of particles of the purified SecY/E complex (in 0.2% deoxyBigCHAP and 1% digitonin) analyzed by negative stain electron microscopy. The protein appears white. Examples of ring-like structures are indicated by the arrow-heads. (b) The same as (a), except that the particles were analyzed in vitreous ice. The protein appears black. The scale bar represents 500 Å. (c) From an initial dataset of 2041 negatively stained particles, 237 had a predominant quasi-fivefold rotational symmetry and 11 of these particles are shown. (d) The global average of particles with quasi-fivefold rotational symmetry \((n = 237)\) is shown. (e) and (f) Classification of the particles with quasi-fivefold rotational symmetry revealed two different sizes, designated class I \((n = 102)\) and class II \((n = 124)\), the averages of which are shown in (e) and (f), respectively. (g) A global average was obtained by alignment of a larger number of particles \((n = 1200)\) against the average shown in (d). The panel sizes in (c) through (g) each correspond to 121 Å × 121 Å.
We next calculated radial density profiles which facilitates size comparisons among different particle populations. The particles were aligned based on their centers of gravity and the cylindrically averaged density was plotted versus the radius (Figure 4). The negatively stained SecY/E particles had density in their center which was higher than at their exterior (Figure 4(a)). The low degree of stain penetration is also visible in galleries of individual particles (see Figure 3(c)). Since the stain cannot penetrate all the way through the center, the pore may not be entirely open. Alternatively, since the pore is often not exactly in the center of the particles, image processing might lead to the appearance of density within the pore of the averages. To test this, we took 30 randomly chosen images from the quasi-pentagonal dataset and realigned them manually for maximal overlap of their pores. The re-calculated radial density curve indicates a small peak shift (~1 Å) and a decrease of the normalized density within the pore from 67% to 54% (Figure 4(a)). Although the standard deviation in the pore region is relatively large, it appears that small misalignments of the particles are not primarily responsible for the density in the center of the rings.

We next performed a similar size analysis with SecY/E particles in vitreous ice (Figure 4(b)).

Figure 4. Radial density plots of images of the SecY/E and Sec61p particles. Cylindrically averaged densities were calculated from the images and plotted against the radius. Error bars represent one standard deviation (see Materials and Methods). (a) Negatively stained SecY/E particles (n = 30) were either aligned by computer (comp.) according to their center of gravity or manually (man.) for maximal overlap of the pores. (b) Plots for the global averages of frozen-hydrated (fh), freeze-fractured (ff), or negatively stained (ns) SecY/E particles. (c) Plots for the global averages of frozen-hydrated SecY/E and dog Sec61p particles. The high external background with dog Sec61p particles is probably due to neighboring particles in the close-packed arrays. (d) Plots for the two major classes obtained from frozen-hydrated SecY/E. (e) Plots for the two major classes obtained from frozen-hydrated dog Sec61p. (f) Plots for the two major classes obtained from SecY/E analyzed by freeze-fracture electron microscopy.
unstained particles showed a small radial peak shift in the wall of the rings, relative to the negatively stained ones. The outer diameter was slightly larger than for stained particles, as indicated by the small shift of the right-hand part of the curves (Figure 4(b), at a radius of 35 Å). The central pore in the unstained particles had a density that was almost identical with that of the external background (Figure 4(b) and (c)), suggesting that it extends completely or nearly all the way through the particles.

The SecY/E complex looked very similar to the eukaryotic Sec61p complex in both negative stain and in vitreous ice. To directly compare the size of the particles, we collected images of frozen-hydrated Sec61p complex from dog pancreas microsomes and analyzed them under exactly the same conditions as the bacterial complex. When the radial density profiles of frozen-hydrated complexes from the two species were compared, the SecY/E particles were smaller than the Sec61p particles (~9% radial difference calculated from Figure 4(c) at 50% density level). The difference in diameter is significant (see the error bars in Figure 4(c)). The pore size was about the same for the SecY/E and Sec61p particles.

We also computed radial density profiles for the two size classes observed with both negatively stained (profiles not shown) and frozen-hydrated SecY/E particles (Figure 4(d)). The two classes of frozen-hydrated SecY/E differ significantly with respect to both pore size and outer diameter (the error bars on each curve represent one standard deviation; see Materials and Methods). These curves suggest that a concerted expansion of the channel wall may be responsible for the two SecY/E classes. The smaller class (class I) seems to have a partially occluded pore (~22% density in the center; see Figure 4(d)). Two major size classes were also observed with the eukaryotic Sec61p complex visualized in vitreous ice (Figure 4(e)). In this case, the inner pore size is about the same for the two size classes.

Oligomers of the SecY/E complex in detergent

The size of the rings seen in electron micrographs suggested that they were too large to be formed by a single molecule of the SecY/E complex. To test for the existence of oligomers, we subjected the purified SecY/E complex to sucrose gradient centrifugation (Figure 5). In two experiments with different SecY/E preparations, the complex ran as relatively homogeneous material with a size of approximately 210 kDa. Direct observation of the peak fractions in the electron microscope revealed particles of a uniform size, many with a central pore (data not shown). Assuming that one micelle of the detergent digitonin (about 70 kDa) is bound to the SecY/E complex, and taking into account the combined mass of SecY and SecE (~50 kDa), the complex may contain three copies of each protein. No protein was found at the position in the gradient where monomeric SecY/E complexes would be expected. We conclude that most of the SecY/E complex in detergent is in an oligomeric state, consistent with the size of the rings seen in electron micrographs.

Structure of the SecY/E complex in proteoliposomes

Next we determined the morphology of the SecY/E complex in lipid bilayers. Purified SecY/E complex was reconstituted into proteoliposomes and analyzed by freeze-fracture electron microscopy. A large number of ring-like particles were seen with a density of up to ~1000-1200 rings per µm² (Figure 6(a) and (b)). Similar to the SecY/E particles in detergent, rings in lipid bilayers showed a quasi-pentagonal shape. The hole in their center was generally more pronounced than when viewed in detergent (see particles in Figure 6(c) and the global average in Figure 6(d)), perhaps due to the metal shadowing which gives a surface image of the molecule rather than a projection through the entire complex (as in frozen-hydrated or negatively stained specimens).

Single particle image analysis carried out with 190 selected particles demonstrated a ring-like structure with a quasi-pentagonal outline (Figure 6(d)). Again, two distinct size classes were found (Figure 6(e) and (f)), and this was confirmed by radial density plots (Figure 4(f)). For both size
classes, the density in the center of the particles was almost identical with that in the external background. The averaged outer diameter was almost the same as for particles in vitreous ice and larger than for negatively stained specimen (Figure 4(b)).

As observed for particles in ice, there appears to be a concerted radial shift of the channel wall, resulting in comparable changes of both pore size and outer diameter.

Discussion

We have isolated a translocationally active channel complex from *B. subtilis*, comprised of SecY and SecE. As demonstrated by electron microscopy, the SecY/E complex forms ring-like structures in detergent solution and in intact lipid bilayers. The particles have an outer diameter of ~85 \( \AA \), a pore size of ~15-20 \( \AA \), and often have a quasi-pentagonal shape. Together with the sedimentation behavior of the particles, these data indicate that the rings are formed from multiple copies of the SecY/E complex. The SecY/E particles are similar in shape and size to those formed by the eukaryotic Sec61p complex. We therefore propose that the oligomeric rings may represent protein-conducting channels, conserved in structure from bacteria to mammals.

We consider it unlikely that the ring structures are generated by non-specific aggregation of the SecY/E complex. The SecY/E particles were generally uniform in size and shape in detergent when visualized in negative stain and vitreous ice, or when analyzed in lipid bilayers by freeze-fracture electron microscopy. In addition, the SecY/E complex sedimented as a homogeneous, oligomeric species in sucrose gradients. These data, together with the demonstration that the SecY/E complex is functional, suggest that the ring structures are the active species in translocation. Our data do not directly prove this point, but the assumption that translocation of a polypeptide chain occurs
through the pore of the rings is supported by the fact that the similarly structured eukaryotic Sec61p complex binds to the ribosome such that the pore is aligned with the protein-conducting channel of the ribosome (Beckmann et al., 1997).

Our data suggest that the SecY and SecE subunits are sufficient to form the rings. This is consistent with the fact that the third subunit of the SecYEG complex, SecG, is non-essential for translocation, and that only SecY and SecE have homologs in eukaryotes. The SecY/E ring-structures have a somewhat smaller diameter than those formed by the heterotrimeric Sec61p complex, which may reflect the absence of the third subunit. Since the pore size is about the same, the third subunit may normally be located at the periphery of the ring-like channels and could play a role in recruiting other components to the translocation site, similar to the mammalian β-subunit, which is believed to recruit the signal peptidase cation site, similar to the mammalian homologs in eukaryotes. The SecY/E ring-structures have a similar ring-like morphology that share a similar ring-like morphology that was dramatically increased upon addition of the bacterial Sec61p complex alone could form ring structures in significant numbers. In addition, the removal of ribosomes from the ER membrane did not lead to the disappearance of the ring structures (Hanein et al., 1996). Thus, it is not clear whether the Sec61p complex assembles and disassembles during each round of translocation. It seems that both the SecY/E and Sec61p complexes have the inherent ability to form channels, but it is possible that the bacterial complexes are more stable. Results reported by Joly et al. (1994) indicate that exchange of differently tagged SecY or SecE subunits does not occur between SecYEG complexes, suggesting that the bacterial channel may never disassemble. However, it may be premature to exclude the possibility that the assembly of the SecYEG complex is regulated in some way.

Taken together, the data for both the bacterial and the mammalian complex suggest that the rings contain either a deep indentation or an open pore at the center. In projection, the averaged density in the central pore of frozen-hydrated particles was almost identical with that found in the surrounding background (especially for the larger class; see Figure 4), suggesting that the pore is open. A low density of the central pore was also observed by freeze-fracture electron microscopy. However, these data are difficult to interpret because the deposition of a metal coating on the particles (generally ~5 Å thick) results in a surface image rather than a projection through the entire particle; thus, it is difficult to distinguish between an open and a partially occluded pore. Evidence that the pore may be partially occluded under some conditions comes from the fact that the smaller of the two SecY/E classes of frozen-hydrated particles had some density at its center (~20% of maximum). Occlusion of the pore was much more dramatic in negatively stained SecY/E complexes (density level of ~55-65%). These data suggest a model in which the central pore may adopt a funnel-like structure, with a wider diameter on one side of the complex than on the other. Such a model is consistent with the low-resolution three-dimensional reconstruction from ribosome-Sec61p channel complexes (Beckmann et al., 1997). In addition, recent fluorescent quenching data suggest that the inactive ER channel itself may be narrowed but not closed, since it can let I− pass through when BiP is removed from its lumenal end (Hamman et al., 1998).

We have consistently seen two major size classes of the SecY/E complexes in detergent and in membranes using different preservation methods. Two classes were also observed with Sec61p complexes (Hanein et al., 1996). The difference in outer diameter between the two classes was ~7-10 Å. The distinct size classes are not likely to arise from different tilting of specimens in the electron micro-
scope, as this would be expected to result in a continuum of particles with different size and morphology, unlike the two discrete classes with a similar appearance which we observed in this study. In addition, we selected the particles in the freeze-fracture dataset from flat membrane areas. Given the similar morphology of the two size classes, the small average size change of \(\sim 10\%\), and the homogeneous size distribution of the SecY/E complex in sucrose gradients, we feel that it is unlikely that the two size classes reflect oligomers whose mass differs by one or more copies of the SecY/E complex. Rather, since in most datasets the class with a larger outer diameter also had a larger pore size, we suggest that the changes reflect a coordinated conformational transition of an oligomer with constant subunit composition. Changes of the pore size of the channel have been observed during translocation (Hamman et al., 1998); small diameters may be required to prevent the passage of ions through it, while larger ones would allow polypeptide transfer and perhaps the re-orientation of trans-membrane anchors of membrane proteins. Expansion and contraction of the ring might provide a mechanism by which these changes occur.

Our results suggest that the two subunits of the translocation apparatus which are related in sequence between prokaryotes and eukaryotes, the multi-spanning SecY/Sec61p/Sec61\(\gamma\) subunit and the single-spanning SecE/Ss1p/Sec61\(\gamma\) subunit, are sufficient to form protein-conducting channels. Only these two components are present in all organisms, from archaea to mammals, whereas all other components of the translocation machinery are found either in prokaryotes or in eukaryotes, but not in both (Pohlschroeder et al., 1997). Since the minimum channel formed by the two membrane components cannot transport proteins on its own, it may be a passive conduit for polypeptide chains which needs to associate with other translocation components that provide the driving force and determine directionality.

**Materials and Methods**

**Materials**

Digitonin (Merck) was purified as described (Görlich & Rapoport, 1993). The \(\text{S}^2\)-labeled proOmpA was synthesized (Panzner et al., 1995) and purified by gel filtration using a NAP-5 column (G25, Pharmacia). *B. subtilis* SecA was purified by anion exchange chromatography from *E. coli* containing the overexpression plasmid pMKL40 (Klose et al., 1993). After cell lysis, soluble proteins were applied onto a 6 ml ResourceQ column (using the Akta FPLC system, Pharmacia), equilibrated with 50 mM Hepes-KOH (pH 7.6), 50 mM potassium acetate, 5 mM magnesium acetate, 1 mM 1,4-dithiothreitol (DTT) and protease inhibitors (see below). After washing with three column volumes, elution was done with a gradient of 50 mM-500 mM potassium acetate.

**Construction of a *B. subtilis* SecY/E overexpression strain**

An *E. coli* - *B. subtilis* plasmid (pHYHE1) was constructed that contained the secY and secE genes of *B. subtilis*, each with an N-terminal hexahistidine tag. Tandem promoters, including the IPTG-dependent (pSPAC-derived) spac (Yansura & Henner, 1984) and the (pQE-9-derived) phage T5 N25/O (Qiagen) promoters were positioned upstream of secY, and the (pSPAC-derived) lacI repressor gene was supplied in cis on the same plasmid. pHYPE1 was transformed into *B. subtilis* DB104 cells and the chromosomal secY deletion, yielding *B. subtilis* strain DB104::LKA4 (pHYHE1), except that cell lysis was enhanced by incubation with lysozyme (1 mg/ml for 30 minutes at 37°C) prior to the French Press treatment, and the final sucrose gradient centrifugation step was omitted. Membranes were resuspended in \(\sim 1/1000\) of the culture volume in 50 mM Hepes-KOH (pH 7.6), 5 mM magnesium acetate, 2.5 mM \(\beta\)-mercaptoethanol, 10 mM potassium acetate, 0.3 % deoxyBigCHAP, 5 % glycerol, 1 mM DTT and protease inhibitors. The extract was centrifuged (30 minutes at 50,000 g, 4°C) and the supernatant incubated with 1 ml of Ni-NTA-agarose (Qiagen) equilibrated in buffer A. After washing two hours at 4°C, the resin was poured into a column, washed first with buffer A and then with buffer A containing 10 mM imidazole, and proteins were eluted with a linear gradient of 10 mM to 200 mM imidazole in buffer A (20 column volumes). SecY/E containing fractions were pooled, diluted two-fold and applied to a 1 ml HiTrap SP-Sepharose column (Pharmacia), equilibrated in buffer A. The column was washed with buffer A containing 5 % (v/v) glycerol and the proteins were eluted with a gradient of 150 mM-500 mM potassium acetate (20 column volumes). An additional SP-Sepharose chromatography step (50-100 \(\mu\)l column volume) was used to exchange digitonin for 0.3 % deoxyBigCHAP (Calbiochem) and to concentrate the SecY/E complex. Elution was done with 50 mM Hepes-KOH (pH 7.6), 600 mM potassium acetate, 5 mM magnesium acetate, 0.3 % deoxyBigCHAP, 5 % glycerol, 1 mM DTT and protease inhibitors.

**Expression and purification of SecY/E**

*B. subtilis* DB104::LKA4 (pHYHE1) was cultured at 37°C in 2xYT medium (Sambrook et al., 1989) supplemented with 0.2 % (w/v) glucose, 10 mg/ml chloramphenicol, 10 \(\mu\)g/ml kanamycin and 1 mM IPTG. At an \(A_{600}\) of \(\sim 5-6\), the cells were harvested and either used directly or frozen in liquid nitrogen. Total membranes were prepared as described (Douville et al., 1995), except that cell lysis was enhanced by incubation with lysozyme (1 mg/ml for 30 minutes at 37°C) prior to the French Press treatment, and the final sucrose gradient centrifugation step was omitted. Membranes were resuspended in \(\sim 1/1000\) of the culture volume in 50 mM Hepes-KOH (pH 7.6), 5 mM magnesium acetate, 2.5 mM \(\beta\)-mercaptoethanol, 150 mM potassium acetate, 5 mM magnesium acetate, 1 % digitonin, 2.5 mM \(\beta\)-mercaptoethanol, with 0.1 mM Pefabloc SC (Boehringer Mannheim), 2 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml chymostatin, 0.6 \(\mu\)g/ml elastinol and 0.2 \(\mu\)g/ml pepstatin as protease inhibitors). The extract was centrifuged (30 minutes at 50,000 g, 4°C) and the supernatant incubated with 1 ml of Ni-NTA-agarose (Qiagen) equilibrated in buffer A. After two hours at 4°C, the resin was poured into a column, washed first with buffer A and then with buffer A containing 10 mM imidazole, and proteins were eluted with a linear gradient of 10 mM to 200 mM imidazole in buffer A (20 column volumes). SecY/E containing fractions were pooled, diluted two-fold and applied to a 1 ml HiTrap SP-Sepharose column (Pharmacia), equilibrated in buffer A. The column was washed with buffer A containing 5 % (v/v) glycerol and the proteins were eluted with a gradient of 150 mM-500 mM potassium acetate (20 column volumes). An additional SP-Sepharose chromatography step (50-100 \(\mu\)l column volume) was used to exchange digitonin for 0.3 % deoxyBigCHAP (Calbiochem) and to concentrate the SecY/E complex. Elution was done with 50 mM Hepes-KOH (pH 7.6), 600 mM potassium acetate, 5 mM magnesium acetate, 0.3 % deoxyBigCHAP, 5 % glycerol, 1 mM DTT and protease inhibitors.
Reconstitution of SecY/E into proteoliposomes

E. coli polar lipid extract (Avanti Polar Lipids) was treated as described (Görlich & Rapoport, 1993) to prepare a 20 mg/ml lipid stock solution containing 6% deoxyBigCHAP. Reconstitution of the purified SecY/E complex into proteoliposomes as well as preparation of proteoliposomes was performed by incubation of the components overnight at 4°C with Biobeads SM2 (Bio-Rad) to remove the detergent. The liquid phase was separated from the beads, diluted fourfold with cold water, and centrifuged for 30 minutes in a TLA 100.3 rotor (75,000 rpm at 2°C; Görlich & Rapoport, 1993). The proteoliposomes were resuspended in translocation buffer (see below) without bovine serum albumin (BSA). Proteoliposomes for translocation experiments contained about 0.5 µg SecY/E (~10 pmol) per 100 µg lipids. For freeze-fracture electron microscopy the proteoliposomes contained about 7.5 µg SecY/E (~150 pmol) per 100 µg lipids.

In vitro translocation of proOmpA

Translocation of 35S-labeled proOmpA into proteoliposomes was assayed by protease accessibility of the precursor after incubation with proteoliposomes. Reactions (10 µl) containing SecY/E proteoliposomes (120 ng per 23 µg lipids), SecA (0.1 mg/ml), ATP (4 mM, Sigma), 0.1 mg/ml creatine kinase, 10 mM creatine phosphate (both Boehringer Mannheim) in translocation buffer (50 mM Tris-Cl (pH 8), 50 mM potassium chloride, 5 mM magnesium chloride, 0.5 mg/ml BSA, 1 mM DTT) were incubated with [35S]proOmpA for 60 minutes at 37°C. Reactions without ATP were preincubated (five minutes at 37°C) with 0.1 unit/µl hexokinase and 20 mM glucose. Translocation was stopped by transferring the reaction mixtures to 0°C and adding protease K (Boehringer Mannheim) to 0.5 mg/ml. After 45 minutes the samples were precipitated with trichloroacetic acid, the pellets washed with cold acetone and resuspended in sample buffer. Protease-protected 35S-labeled proOmpA was analyzed by SDS-PAGE using 13.75% gels followed by autoradiography with Kodak BioMax films and quantification using a phosphoimager.

Sucrose density gradient centrifugation

Gradient centrifugations were performed with 9%-18% sucrose gradients in polycarbonate ultracentrifugation tubes (7 mm x 20 mm, Beckman) in 30 mM Hepes-KOH (pH 7.6), 900 mM potassium acetate, 1% digitonin and 0.2% deoxyBigCHAP. Protein sample (20 µl) containing 450 ng of the purified SecY/E complex was loaded on top of the gradients. Centrifugation was for six hours in a TLS-55 rotor at 55,000 rpm and 2°C in an Optima TLX ultracentrifuge (Beckman) using slow acceleration. Fractions of 20 µl were analyzed by SDS-PAGE followed by immunoblotting with anti-His tag monoclonal antibodies (Qiagen) and 35S-labeled anti-mouse Ig (Amersham). Quantification of the bands was performed with a phosphoimager. Marker proteins (30 µg BSA (ICN), 66 KDa; aldolase (Pharmacia), 160 KDa; or catalase (Pharmacia), 230 KDa) were run in parallel gradients and analyzed using the Micro BCA protein assay (Pierce).

Electron microscopy

For negative staining, 2-3 µl of SecY/E or Sec61p complexes (~20 ng/µl) in detergent solution were applied to air glow-discharged carbon grids (400-mesh copper). After incubation at 4°C for 30 minutes in a humid chamber, the samples were fixed (approximately one minute at 20°C) with 4% formaldehyde/0.5% glutaraldehyde in 10 mM Bis-Tris (pH 6.5), 100 mM potassium acetate, 2 mM magnesium acetate. The grids were then rinsed with five drops of 2% uranyl acetate and air-dried. For cryo-electron microscopy, SecY/E or Sec61p complexes (30-60 ng/µl) were loaded onto air glow-discharged continuous thin carbon film supported by 300-mesh grids coated with holey carbon, blotted and plunged into liquid ethane (Dubochet et al., 1988). All the preparation steps were performed at 4°C in a humid environment (>85% relative humidity). A Gatan cryotransfer system and coldholder (model 626-DH) were used to transfer grids into a Philips CM12 transmission electron microscope equipped with a cryo-blade type anti-contaminator. All electron micrographs were recorded at 100 kV, under minimal dose conditions with a LaB6 filament, using a defocus of ~1.5 µm. Micrographs were taken at 60,000× magnification on Kodak SO163 film and developed for 12 minutes in full-strength D19 developer (Kodak). For freeze-fracture electron microscopy, SecY/E proteoliposomes (~1 µg/µl) in aliquots of about 1 µl were frozen on copper hat supports by hand-plunging into liquid Freon-22. Specimens were fractured at ~110°C in a Balzer BAF-400 freeze-etch device without etching. Replicas were prepared by rotary shadowing with platinum at an angle of 22° (~5 Å thick), followed by carbon shadowing at an angle of 90° (~35 Å thick) and cleaned with chromic acid and water before examination at 60,000× in a Philips CM12 electron microscope at 100 kV.

Image processing and radial density plots

A series of micrographs were chosen for processing which contained uniform close-packed fields of particles with good contrast and minimal astigmatism. Micrographs of negatively stained SecY/E were digitized on an Eikonics scanner at a step size corresponding to 4.0 Å/pixel and then interpolated to 2.3 Å/pixel. Micrographs of frozen-hydrated and freeze-fractured SecY/E were digitized with a Zeiss SCAI scanner at 7 µm and binned to a pixel size of 14 µm, corresponding to 2.3 Å/pixel. Image processing was done using the SPIDER software package (Frank et al., 1996). The procedures of cross-correlational alignment, averaging and classification have been described (Frank et al., 1988; Akey, 1995). Visual inspection of freeze-fractured, negatively stained and frozen-hydrated specimen suggested that many SecY/E particles shared the quasi-pentagonal ring-like morphology observed for Sec61p complexes (Hanein et al., 1996). We therefore analyzed our data for the presence of this quasi-fivefold symmetry using rotational power spectra analysis (Kocsis et al., 1995) and utilized this pseudo-symmetry to select well-preserved particles for references (Yang et al., 1998). Overall, ~10% of the selected particles displayed in their Fourier power spectra at least a twofold higher quasi-fivefold frequency when compared with other symmetries (three-, four-, six- and sevenfold). All datasets were adjusted such that protein density is white and cylinder axis radial density profiles were calculated to allow a quantitative size comparison. We obtained statistics for each point on
the radial curves by dividing datasets into four randomly selected subgroups, which were then averaged to generate a standard deviation for each density point.

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References


