Functional and structural characterization of the minimal Sec translocase of the hyperthermophile

*Thermotoga maritima*

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**Abstract** The genome of the hyperthermophilic bacterium *Thermotoga maritima* contains the genes that encode core subunits of the protein translocase, a complex consisting of the molecular motor SecA and the protein conducting pore SecYE. In addition, we identified an erroneous sequence in the genome encoding for a putative *secG* gene. The genes of the *T. maritima* translocase subunits were overexpressed in *Escherichia coli* and purified to homogeneity. *T. maritima* SecA showed a basal thermostable ATPase activity that was stimulated up to 4-fold by phospholipids with an optimum at 74 °C. Membrane vesicles and proteoliposomes containing SecYE or SecYEG supported 2 to 4-fold stimulation of the precursor dependent SecA ATPase activity. Imaging of small two-dimensional crystals of the SecYE complex using electron microscopy showed square-shaped particles with a side-length of about 6 nm. These results demonstrate that in *T. maritima* a highly thermostable translocase complex is operational.
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Introduction

Bacteria secrete a variety of newly synthesized proteins across the cytoplasmic membrane via the Sec system. The Sec system consists of a peripheral ATPase SecA, a protein-conducting pore formed by the SecY, SecE, and SecG membrane proteins, and the accessory integral membrane proteins SecD and SecF (Manting and Driessen, 2000; Driessen et al., 2001). In *Escherichia coli* secretory proteins are targeted to the Sec translocase by the molecular chaperone SecB. The minimal functional composition of the Sec translocase corresponds to SecA, SecY and SecE (177). SecG is not an essential subunit, but its presence stimulates the translocation reaction (74;110). SecA drives newly synthesized preproteins in a stepwise fashion through the SecYE pore by cycles of ATP binding and hydrolysis (178).

The current era of studies on protein translocation aim to relate structure to function. Atomic detail has been obtained on the structure of the soluble form of SecA, both from *Bacillus subtilis* (179;180) and of *Mycobacterium tuberculosis* (16). Recently, also the structure has been reported of the archaeon *Methanococcus jannashii* Sec61 complex (116) that bears similarity to the bacterial SecYEG complex. The structure reveals a monomeric unit that appears in closed pore conformation assuming that a central hydrophobic constriction is opened during the translocation reaction concomitantly with the removal of an extracellular re-entrance loop that closed the pore domain like a plug. However, archaea do not contain a SecA protein urging the need for structural information of a bacterial protein pore. In this respect, the *E. coli* and *B. subtilis* SecYEG complexes have been reported to assemble into oligomers (113;115;181;182). At this stage it is unclear if these oligomers represent a single or multiple pores.

Except for a report on the oligomeric state of the purified *Thermus aquaticus* SecYE protein (181), protein translocation in hyperthermophiles has so far not been studied. Its analysis, however, will not only promote further studies on the structure-function of the bacterial protein translocase but also provide insight in the mechanisms of protein folding and translocation under the extreme thermophilic conditions. Thermophilic proteins tend to fold into an extremely stable conformation, and such a state will be incompatible with protein translocation. Moreover, protein folding can be very rapid at high temperatures. To investigate the protein translocation reaction at high temperatures, we have analyzed the translocase complex of the hyperthermophilic Gram-negative eubacterium *Thermotoga maritima*. Its genomic sequence reveals homologs of the major translocase subunits, i.e., SecA, SecY, SecE, SecD and SecF. On the other hand, the molecular chaperone SecB and pore subunit SecG appear to be missing. We have overexpressed the *T. maritima* Sec-translocase in *E. coli*, and in addition identified the *T. maritima* SecG homolog. The proteins were purified and functionally reconstituted into *T. maritima* phospholipids yielding a thermostable minimal Sec translocase that was investigated by high resolution electronmicroscopy.
Results

Cloning, overexpression and characterization of the T. maritima SecA

The gene of the *T. maritima* ATPase SecA was cloned in a pBAD TOPO arabinose inducible expression vector and overexpressed in *E. coli* Bl21 containing an extra plasmid pM1240DS coding for archael tRNAs. This overcomes the codon usage difference between *E. coli* and *T. maritima*. Overexpression of SecA could only be detected with the co-induction of archael tRNAs (data not shown). SecA was purified by a heat step and following anion exchange chromatography. Since the purified enzyme showed an extremely resistance to heat denaturation, samples were boiled for more than 20 minutes to eliminate the 75 kDa aberrant size of SecA (Fig. 1, indicated by an asterisks). The unfolded protein migrated as a 98 kDa protein consistent with its predicted molecular mass of 104 kDa.

The purified *T. maritima* SecA showed a basal ATPase activity that was optimal at 74 °C (Fig. 2A). In contrast, the *E. coli* SecA exhibits an optimal activity at 37 °C. The pH optimum of the ATPase activity of *T. maritima* SecA was found to be slightly alkaline and maximal at pH 8.0 (Fig. 2B). At their optimal temperature, the activity of the *T. maritima* SecA is about twice as low as the activity of the *E. coli* enzyme. The enzyme was rather insensitive to the salt concentration within the range of 100 mM to 1.5 M NaCl (data not shown). The basal ATPase activity showed apparent $K_m$ and $V_{max}$ values of 0.26 mM and 28 nmol/mg of protein/min at 74 °C and pH 8.0. *E. coli* SecA exhibits stimulated ATP activity upon binding to lipid vesicles containing anionic phospholipids (123). However, lipid bound SecA becomes thermolabile and denaturates at temperatures above 40 °C, unless an unfolded precursor protein is present to stabilizes SecA. This stabilization reaction which preserves the ATPase activity of SecA is termed SecA lipid ATPase (123).

To determine if the *T. maritima* SecA basal ATPase activity is stimulated by phospholipids, the enzyme was incubated at 74 °C in the presence of increasing amounts of liposomes composed of...
phospholipids isolated from *T. maritima*. The presence of the liposomes stimulated the SecA ATPase activity by more than 3-fold (Fig. 2C). Taken together, these data demonstrate that the heterologously expressed and purified *T. maritima* SecA is functionally active and thermostable.

![Figure 2: ATPase activity of the *T. maritima* SecA.](image)

**(A)** Temperature-dependence of the basal ATPase activity of the *T. maritima* (black bars) and *E. coli* (open bars) SecA. **(B)** pH dependency and **(C)** lipid stimulation of the basal ATPase activity of the *T. maritima* SecA. For lipid activation, *T. maritima* phospholipids were used. The experiments were performed at 75 °C and pH 7.5, unless indicated otherwise.

**Cloning, overexpression and purification of the *T. maritima* SecYE(G) complex**

The *secY* and *secE* genes of *T. maritima* were cloned in tandem behind the inducible *trc* promoter of pET324 to yield pEK115. To facilitate purification, a C-terminal hexa-histidine tag was introduced in SecY. The proteins were expressed in *E. coli* BL21 RIL. Analysis of the polypeptide pattern of isolated inner membrane vesicles by SDS-PAGE indeed demonstrated the high level overexpression of SecY (Fig. 3A). Due to the small size, i.e., 7.3 kDa, the *T. maritima* SecE protein could not be resolved from the isolated inner membrane vesicles on SDS-PAGE. The identity of SecY was verified by western-blotting and immuno-staining using an antibody directed against the His-tag (Fig. 3B). In contrast to the *secY* and *secE* genes, there is no *secG* gene annotated in the *T. maritima* genome. By means of TBLAST using the *E. coli* SecG sequence, we identified an erroneous sequence encoding a putative *secG* gene in the *T. maritima* genome. The presence of an error caused a frame-shift in the sequence of the gene TM0479. Therefore, the gene was cloned by PCR in the pGEM T-Easy and re-sequenced. The correct amino acid sequence was aligned to *E. coli* SecG (Fig. 4) showing a high homology (26.5 % identity, 30 % similarity) over the first 76 amino acids including the two transmembrane segments that are connected by a glycine-rich loop.
The secG gene was cloned separately into an expression vector, and co-transformed with the SecYE expression vector, to obtain overexpression of the SecYEG complex in E. coli (Fig. 3A).

**Figure 3.** Overexpression and purification of the T. maritima SecYE complex. (A) Coomassie brilliant blue staining of the polypeptide pattern of membrane vesicles derived from E. coli BL21 RIL cells containing the empty vector pET324 C-His, pEK115 that expresses the T. maritima SecYE complex with a C-terminal hexa-his tag on SecY, or containing pEK115 together with pEK125 that expresses the SecYEG complex. (B) Western blot analysis of using an antibody directed against the His-tag on the C-terminus of SecY. (C) Coomassie brilliant blue-stained 15 % SDS-PAGE of proteoliposomes reconstituted with the purified T. maritima SecYE complex reconstituted into T. maritima lipids.

In E. coli, the ATPase activity of the SecYE(G)-bound SecA is stimulated by a translocation competent precursor protein. This activity is termed SecA translocation ATPase (15;183;184). The T. maritima SecYE and SecYEG complexes were overexpressed in E. coli and combined with purified T. maritima SecA for translocation ATPase assays at 55°C. Attempts to observe translocation ATPase activity with the urea-denatured precursor of the E. coli outer membrane protein A (proOmpA) were unsuccessful. This most likely related to the rapid aggregation of proOmpA as observed in a steep increase in light scattering of urea-diluted proOmpA at high temperatures (data not shown). Therefore, we cloned a gene encoding a native precursor protein,
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TM0593, which specifies a putative amino acid binding protein from the *T. maritima* genome. The full length precursor has a molecular mass of about 28 kDa. The gene was fused at its C-terminus with a hexa-his tag and cloned into pET324 which allows expression controlled by the inducible *trc* promoter. The protein was overexpressed in *E. coli* and purified by Ni-NTA affinity chromatography.

Membrane vesicles containing *T. maritima* SecYE or SecYEG were assayed for translocation ATPase activity with *T. maritima* SecA using the urea-denatured precursor protein TM0593. In the presence of control vesicles, SecA showed only a basal ATPase activity which increased with the temperature (Fig. 5, white bars). However, in the presence of precursor protein, SecYE membranes supported an up to 2-fold increase in SecA ATPase activity at 55 ºC (Fig. 5, grey bars). Furthermore, with SecYEG membranes, an up to 4-fold stimulation of the ATPase activity was observed (Fig. 5, black bars). These data demonstrate that the SecYEG complex of *T. maritima* is functionally expressed in *E. coli*.

**Figure 5.** Temperature dependence of the translocation ATPase activity of *T. maritima* SecA. The Tm0593 precursor protein stimulated ATPase activity of SecA was measured in the presence of membrane vesicles derived from control cells (empty bars), SecYE (grey bars) and SecYEG (black bars) expressing cells.

Functional reconstitution of the *T. maritima* SecYE complex

The use of *E. coli* membranes may present a limit to the temperature range that can be used to perform functional assays with the *T. maritima* translocase. For instance, the lipid composition of the *E. coli* membrane is vastly different from that of *T. maritima*. To investigate the *T. maritima* translocase in its native lipid environment, the minimal functional unit of the translocase, m i.e., the SecYE complex was purified from the *E. coli* membrane employing the C-terminal hexa-his tag on SecY and Ni-NTA affinity chromatography (data not shown). Purified *T. maritima* SecYE complex was reconstituted by means of a detergent absorption method into liposomes composed of *T. maritima* or *E. coli* phospholipid. With the reconstituted complex both the SecY and SecE polypeptide could be resolved on an SDS-PAGE (Fig. 3C). Proteoliposomes were tested for translocation ATPase activity of the *T. maritima* SecA in the presence of the urea-denatured precursor protein TM0593. In the presence of empty liposomes composed of *T. maritima* (Fig. 6A)
or *E. coli* (data not shown) phospholipids, the ATPase activity of SecA was not significantly activated. In contrast, SecYE proteoliposomes composed of *E. coli* (Fig. 6B) or *T. maritima* (Fig. 6C) phospholipids, showed a substantial level of the SecA translocation ATPase. At 75 °C, high levels of SecA translocation ATPase were observed with the SecYE complex reconstituted into *T. maritima* phospholipids. Taken together, these data demonstrate the functional reconstitution of a thermostable translocase complex of *T. maritima*.

**Figure 6.** *T. maritima* SecYE proteoliposomes support the translocation ATPase activity of SecA. The ATPase activity of SecA was measured in the absence (empty bars) and presence (black bars) of the precursor *Tm*0593 in the presence of (A) liposomes prepared from *T. maritima* phospholipids or SecYE proteoliposomes composed of (B) *E. coli* or (C) *T. maritima* phospholipids.

**Two-dimensional crystallization and electron microscopy of the purified *T. maritima* SecYE complex**

The monolayer approach was used to reconstitute *T. maritima* translocase. The His-tagged SecYE surrounded by detergents and solubilized lipids bind a Nickel-functionalized lipid layer adopting a unidirectional orientation (185). Subsequent detergent removal induces the protein reconstitution at the interface. The best crystalline patches were obtained using a DOPC/DOPA lipid mixture with a lipid-to-protein ratio (LPR) of 0.6. Fig. 7A displays reconstituted membranes where single SecYE complexes (arrows) and rectangular crystalline patches (arrow heads) are distinguishable. Diffraction patterns calculated from 6 crystalline areas yielded unit cell dimensions of $a = 6.59 \pm 0.14$ nm, $b = 13.35 \pm 0.41$ nm, $\gamma = 90.2 \pm 1.6^\circ$. Although the crystals are not well ordered, and diffracted to a resolution of only 3 nm (Fig. 7 B), some substructure within the SecYE complexes is resolved after correlation averaging (Saxton 1996). They have a square-shaped appearance and reveal two small and one elongated domains (Fig. 7C). One unit cell houses two SecYE complexes, which exhibit a side-length of about 6 nm.
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Figure 7. 2D-crystallization of \textit{T. maritima} SecYE on the planar lipid film.

(A) Electron micrograph of membrane formed at the interface after detergent removal shows crystalline patches (arrow heads) and single complexes (arrows). Scale bar, 100 nm. (B) The diffraction pattern of the area marked in panel A exhibits spots to a resolution of 3 nm. Scale bar 5 nm. (C) The projection map has been calculated by correlation averaging from the crystalline array marked in panel A. Scale bar, 10 nm.

Discussion

Here we describe the biochemical and structural characterization of the minimal protein translocase of a hyperthermophilic bacterium, \textit{Thermatoga maritima}. This bacterium grows between a temperature range of 55 and 90 °C (174). Our data show that the \textit{T. maritima} ATPase SecA is highly thermostable as compared to the \textit{E. coli} SecA. Complete denaturation in SDS required an extensive boiling step which underscores the high thermostability of this protein. The protein exhibited a low basal ATPase activity that was stimulated by the presence of phospholipids. In addition, we have identified an erroneous sequence encoding for SecG of \textit{T. maritima} and co-overexpressed this protein with SecYE in \textit{E. coli}. The SecYE complex supported translocation ATPase activity of SecA with the urea-unfolded precursor protein Tm0593. Moreover, the presence of SecG resulted in a further 2-fold higher stimulation of the translocation ATPase activity which demonstrates that the SecYEG complex of \textit{T. martima} can be functionally overexpressed in \textit{E. coli}.
The purified *T. maritima* SecYE complex was also functional reconstituted into liposomes composed of *T. maritima* phospholipids as shown by the thermostable translocation ATPase activity of SecA. Remarkably, unlike the *Thermus aquaticus* SecYE complex (181), the *T. maritima* complex was not activated by the *E. coli* precursor proOmpA. We noted, however, that proOmpA readily aggregates at high temperature and it is likely that this renders this precursor unsuitable as a substrate for a hyperthermophilic translocase. The reconstituted *T. maritima* SecYE complex was optimally active at a temperature close to the growth temperature when reconstituted into its native lipids. Because of the high growth temperature, *T. maritima* mainly contains phospholipids with saturated acyl chains. About 10% of the lipids are membrane spanning C₃₀⁻, C₃₂⁻, C₃₄⁻, ester lipids containing either one central internal methyl branch or two central vicinal methyl branches (174). The majority of the lipids, however, appear to be of uncharacterized structure. Unfortunately, it proved to be impossible to analyze the translocation of the urea-unfolded precursor protein *Tm*0593. Translocation across the membrane is routinely assayed by means of protection against externally added protease. However, even non-translocated *Tm*0593 precursor readily folds into a protease-resistant conformation resulting in a high background of protease-resistant protein. The increased ATPase activity of SecA in the presence of SecYE proteoliposomes and urea-denatured precursor suggest that the system is also capable of translocation.

Based on the high level of SecA translocation ATPase activity of SecYE reconstituted into liposomes, electron microscopy was used to characterize the SecYE after reconstitution in membrane and 2D-crystallization. The translocase was successfully incorporated in DOPA/DOPC lipids, and electron microscopy analysis revealed well defined single molecules and ordered crystalline patches of SecYE (Fig. 7A). By its shape and dimension, the projection map of the reconstituted translocase (Fig. 7A) is compatible with the SecY 3D X-ray structure of *Methanococcus jannaschii* (116). A central pore or indentation is distinguishable on single particles, but it is less pronounced in the projection map as result of limited resolution (see diffraction pattern, Fig. 7B). Unfortunately a higher resolution projection map could not be obtained as result of the limited size and order of the crystalline patches.

A question that remains is how precursor proteins in hyperthermophiles such as *T. maritima* are maintained in an unfolded state prior to their translocation. In *E. coli*, the molecular chaperone SecB fulfils this role and in addition, it targets precursor proteins to the SecA subunit of the translocase. In order for SecB to stimulate translocation, it needs to transfer the precursor protein to SecA (186). This transfer reaction involves the highly conserved C-terminus of SecA that binds to SecB where upon the transfer reaction is initiated (126). The C-terminus of the *T. maritima* SecA is not homologous to the SecB binding domain. It does not contain the highly conserved cysteine and histidine residues that constitute a zinc atom binding site. We noted that addition of SecB to the *in
vitro \( T. \) maritima system had no effect in the translocation ATPase activity (data not shown). It is therefore likely that the \( T. \) maritima SecA is unable to interact with the \( E. \) coli SecB. Moreover, \( T. \) maritima and other thermophilic bacteria lack a SecB homolog. These organisms therefore either use another molecular chaperone or proteins are translocated in a co-translational mode which would prevent premature folding of the precursor protein in a stable conformation. The in vitro assay now allows for a direct biochemical identification of such putative chaperones and will deepen our insight in the protein translocation reaction at high temperatures.

Materials and methods

Organism, growth conditions and materials

\textit{Thermotoga maritima} MSB8 DSM3109 was grown at 80 °C as described (174) and used as a source of phospholipids. \textit{Escherichia coli} DH5\( \alpha \) (Hanahan, 1983) and BI21/RIL (Stratagene, La Jolla, USA) were used for genetic manipulation and protein overexpression, and grown on Luria both supplemented with the appropriate antibiotics at 37 °C. Phospholipids including Ni\( ^{2+} \)-chelated 1,2-(Dioleyl-sn-Glycero-3-[N-(5-amino-1-carboxypentyl iminodiacetic acid) succinyl]) (Ni\(^{+}\)-NTA-DOGS) were purchased from Avanti Polar Lipids. N-dodecyl-\( \beta \)-D-maltopyranoside (DDM) was from Anatrace. Biobeads from Bio-Rad.

Isolation of phospholipids and preparation of liposomes

Lipids were isolated from freeze-dried \textit{T. maritima} cells as described (187). Lipids were dried by vacuum rotary evaporation, hydrated in buffer (50 mM HEPES, pH 7.5, 30 mM KCl) to a final concentration of 9 mg/ml. Liposomes were prepared by five consecutive freezing and thawing steps followed by extrusion through 200 nm polycarbonate filters (Avestin, Ottawa, Canada) using Lipofast\textsuperscript{TM} (Basic, Avestin) extrusion apparatus.

Cloning and plasmid construction

Chromosomal DNA from \textit{T. maritima} was prepared by CsCl-buoyant density centrifugation (188). Oligonucleotide primers for the amplification of sec genes were designed according to the genome sequence of \textit{T. maritima} available at TIGR (http://www.tigr.org/). The forward primer for \textit{secE} (5\textsuperscript{\prime} - CCCCATGGAGAACTCCGAAAGTTCTTCAGGG) and reverse primer (5\textsuperscript{\prime} - CCCGTCGACTTATCCTATCCAGCG) contained an NcoI and SalI endonuclease restriction site. The forward primer for \textit{secY} (5\textsuperscript{\prime} - CCCGTCGACCTATCCTATCCAGCG) and reverse primer (5\textsuperscript{\prime} - CCCGTCGACCTATCCTATCCAGCG) contained an NcoI and SalI endonuclease restriction site. The forward primer for \textit{secG} (5\textsuperscript{\prime} - CCCGTCGACCTATCCTATCCAGCG) and reverse primer (5\textsuperscript{\prime} - CCCGTCGACCTATCCTATCCAGCG) contained a Sall and BamHI sites, respectively. Digested PCR products were ligated into the expression vector pET324 (189) at the NcoI and BamHI sites, downstream from the inducible \textit{trc} promoter yielding pEK115. The forward primer for \textit{secG} (5\textsuperscript{\prime} - CCCGTCGACCTATCCTATCCAGCG) contained an NcoI and SalI endonuclease restriction site, and the resultant PCR fragment was ligated into the pGEM T-EASY cloning vector (Promega Benelux b.v.). The correct sequence was confirmed by oligonucleotide sequencing. The plasmid was digested with NcoI and PstI, and the resulting DNA fragment was ligated into pK184 resulting in the vector pEK125 that contains the \textit{secG} gene under control of the inducible \textit{trc} promoter and the kanamycin resistance marker.
The gene encoding for SecA was amplified by PCR using the forward (5’-CCCGGATCCGATACTCTTCGATAAGAACAAGCG) and reverse (CCCGTCGACTTACCTCTTCACTCTTATTCTACC) primers and cloned with the pBAD TOPO TA Expression Kit (Invitrogen, The Netherlands). The gene encoding the putative amino acid binding protein Tm0593 was amplified by forward (5’-GGGCCATGGTAAAAAAATACTTGCGG) and a reverse (5’-CGCGGATCCCTCTGAGAACATTTTCTATAAGCAGC) primer NcoI and BamHI digested PCR product was ligated into pET15b yielding pEK126.

Overexpression of SecYE and SecYEG complex

*E. coli* BL21 cells were co-transformed with plasmids pEK125 (Kanr) and pEK115 (Ampr) encoding for SecG and SecYE, respectively. Cells were grown on LB medium supplemented with 0.5 % glucose and ampicillin and kanamycin (both at 50 µg/l). For expression of SecYE, plasmid pEK115 was also transformed separately into *E. coli* strain BL21 RIL and cells were grown on LB medium supplemented with ampicillin (50 µg/l), chloramphenicol (20 µg/l) and 0.5 % glucose. Precultures of 30 ml were grown at 37 ºC for 10 hrs, diluted into 1 l medium and growth was continued up to an OD$_{600}$ of 1. Next, cells were induced for protein expression by the addition of 0.8 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG), and growth was continued for an additional 6 hrs. Cells were harvested by centrifugation, and resuspended in 50 mM TrisHCl pH 8.0 and 20 % glycerol, and stored at -80 ºC.

Overexpression of SecA

For the heterologous expression of the *T. maritima* SecA *E. coli* BL21 was co-transformed with plasmid pEK123 (Ampr) which encodes SecA and with pM1240DS (Spcr). pM1240DS (Spcr) is a derivative of pSJS1240 (190;191) and contains genes encoding rare *E. coli* tRNAs; i.e., *argU* which specifies an arginyl tRNA which reads AGG and AGA codons; and *ileX* which encodes an isoleucyl tRNA that reads AUA codons. Cells were grown in 1 l LB supplemented with ampicillin and spectinomycin (both at 50 µg/l) for 8 hrs at 37 ºC. At an OD$_{600}$ of 1, cells were induced with 0.2% arabinose and growth was continued for additional 6 hrs where upon the cells were harvested by centrifugation and stored at – 80 ºC as described above.

Purification of *T. maritima* SecA

Frozen cell suspensions were thawed, and supplemented with 1 mM DTT, 1 mM phenylmethylsulphonylfluoride (PMSF) and the proteinase inhibitor cocktail Complete (Roche, The Netherlands). Cells were lysed by French pressure treatment, and the cell debris was removed by low speed centrifugation. The supernatant was cleared by ultra centrifugation (100,000 x g, 60 min). The cell lysate was supplemented with 100 mM NaCl and heat treated at 70 ºC for 20 minutes. The suspension was cooled on ice and precipitated proteins were removed by ultracentrifugation (100,000 x g, 60 min). The supernatant was applied to a MonoQ column which was washed intensively with 50 mM TrisHCl, pH 8.0, 10 % glycerol and 140 mM NaCl. SecA was eluted with the same buffer with a linear NaCl gradient from 140 mM to 1M. The purified protein was dialysed against 50 mM TrisHCl, pH 8.0, 10 % glycerol and stored in aliquots at -80 ºC.

Purification and reconstitution of the *T. maritima* SecYE complex

Membranes of *E. coli* containing overexpressed *T. maritima* SecYE proteins were isolated at the concentration of 20 mg/ml as described (192). For precursor stimulated ATPase assays, membranes were subjected to a urea-extraction to remove and inactivate the endogenous bound SecA of *E. coli*. For this purpose, 200 µl membrane vesicles (4 mg of
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protein) were incubated with 800 µl 8 M urea for 30 minutes on ice where upon the membranes were collected by ultracentrifugation (100,000 x g, 30 minutes). The pellet was washed once with a buffer containing 20 % glycerol and 50 mM TrisHCl, pH 8.0, and finally resuspended in 200 µl of the same buffer.

For purification of the SecYE complex, membranes were solubilized at 4 ºC for 30 min at 1 mg of protein/ml with 2 % DDM in 50 mM HEPES, pH 8, 30 mM KCl, 20 % glycerol and 20 mM imidazole. Non-solubilized proteins and aggregates were removed by centrifugation (30 minutes at 40,000 x g) and the cleared supernatant was loaded onto a Ni-NTA column. The SecYE complex was eluted at 100 mM imidazole, pH 8.0 and immediately reconstituted into liposomes as described below.

T. maritima lipids (4 mg/ml, 250 µl) were suspended in a buffer containing 0.05% DDM and 50 mM HEPES, pH 7.5 and 30 mM KCl, and incubated with 250 µl purified SecYE complex (0.2 mg/ml) for 30 min at 4 ºC with constant mixing. The mixture was diluted with 800 µl 50 mM HEPES, pH 7.5 and 30 mM KCl and supplemented with 80 mg (wet weight) Bio-Beads SM-2 (Biorad Laboratories, Hercules CA). The suspension was gently shaken at 4 ºC, and the Bio-Beads were replaced after 2, 4 and 6 hours. After overnight incubation, the Bio-Beads were discarded and the proteoliposomes were collected by centrifugation (20 min at 200,000 g). The pellet was washed twice with 2 ml 50 mM HEPES, pH 7.5 and 30 mM KCl and finally suspended in 10 µl 50 mM HEPES, pH 7.5 and 30 mM KCl and stored at -80 ºC.

Expression and purification of the precursor protein Tm0593

E. coli BL21 RIL cells were transformed with plasmids pEK126 (Amp') encoding for Tm0593, respectively. Cells were grown on LB medium supplemented with 0.5 % glucose, ampicillin (50µg/l) and chloramphenicol (20 µg/l). Precultures of 30 ml were grown at 37 ºC for 10 hrs, diluted into 1 l medium and growth was continued up to an OD600 of 1. Next, cells were induced for protein expression by the addition of 0.8 mM IPTG, and growth was continued for an additional 6 hrs. Cells were harvested by centrifugation, and resuspended in 50 mM TrisHCl, pH 8.0, and 20 % glycerol. The cell suspension was supplemented with 1 mM DTT, 1 mM PMSF and proteinase inhibitor cocktail Complete (Roche, The Netherlands), and lysed by French pressure treatment. Cell debris was removed by low speed centrifugation, and the pellet fraction was resuspended in 6 M Urea and the nonextractable protein was removed by ultra centrifugation (100,000 x g, 60 min). The supernatant was loaded onto a Ni-NTA column and Tm0593 was eluted with 200 mM Imidazole, pH 7.0, 6 M urea, 20 % glycerol, 100 mM NaCl and stored in aliquots at -80 ºC.

ATPase measurements

The ATPase activity of SecA was measured calorimetrically according to the method described by (193). T. maritima SecA (100 µg/ml) or E. coli SecA (20 µg/ml) protein was incubated at the indicated temperature for 1 min in 50 mM TrisHCl, pH 8.0, 5 mM MgCl2, and 30 mM KCl with or without liposomes (0-500 µg/ml) or SecYE proteoliposomes (4 µg/ml). Where indicated, the purified precursor Tm0593 was added to a final concentration of 5 µg/ml. The reaction started by the addition of 2 mM ATP, and after 9 min, when the reaction was still linear in time, the reaction was stopped by freezing in liquid nitrogen. The amount of inorganic phosphate released was measured as described (193). The pH dependence of the ATPase activity was monitored by adjusting the buffer composition: pH 5.5-6, MES; pH 6.5-7.5, HEPES; and pH 8-9.5, TrisHCl.

2D crystallization and electron microscopy

His-tagged SecYE was reconstituted on Ni2+-chelating lipid film using the method described by Levy et al (185). The reconstitutions were achieved with ternary mixtures made of 0.3 mg/ml of His-tagged T. maritima SecYE translocase,
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0.5 mg/ml DDM and dioleylglycerolphosphocholine-dioleylglycerophosphatid acid lipid mixtures (DOPC/DOPA, 90/10, w/w ratio). The detergent concentration of the mixtures was adjusted in order to have a final DDM concentration of 5 mg/ml in the monolayer well after ternary mixture addition. The lipid-protein-detergent micellar solution was injected below the Nickel-containing lipid layer in a 10 mM TrisHCl pH 7.6, 50 mM NaCl buffer. To induce reconstitution and crystallization, detergent was removed by adding 10 mg of polystyrene beads (biobeads). The interfacial surfaces were transferred to carbon-coated grids and negatively stained with 2% uranyl acetate. Electron micrographs were recorded on a Hitachi 7000 electron microscope operating at 100 kV. Electron micrographs of crystalline patches were digitized using a Leafscan-45 scanner (Leaf Systems, Inc., Westborough, MA) and processed with the SEMPER image processing system (Saxton 1996; Synoptics Ltd., Cambridge, UK).

Other methods

Protein levels were determined by using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

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