doi:10.1016/j.jmb.2006.07.017 J. Mol. Biol. (2006) 361, 839–849

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Identification of Two Interaction Sites in SecY that Are Important for the Functional Interaction with SecA

Eli O. van der Sluis[1], Nico Nouwen[1], Joachim Koch[2] Jeanine de Keyzer[1], Chris van der Does[1], Robert Tampé[2] and Arnold J. M. Driessen[1]*

[1]*Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute University of Groningen 9751 NN Haren The Netherlands*

[2]*Institute of Biochemistry Biozentrum Frankfurt Johann Wolfgang Goethe University, Marie-Curie-Strasse 9, 60439 Frankfurt am Main Germany*

**Corresponding author*

The motor protein SecA drives the translocation of (pre-)proteins across the SecYEG channel in the bacterial cytoplasmic membrane by nucleotide-dependent cycles of conformational changes often referred to as membrane insertion/de-insertion. Despite structural data on SecA and an archaeal homolog of SecYEG, the identity of the sites of interaction between SecA and SecYEG are unknown. Here, we show that SecA can be cross-linked to several residues in cytoplasmic loop 5 (C5) of SecY, and that SecA directly interacts with a part of transmembrane segment 4 (TMS4) of SecY that is buried in the membrane region of SecYEG. Mutagenesis of either the conserved Arg357 in C5 or Glu176 in TMS4 interferes with the catalytic activity of SecA but not with binding of SecA to SecYEG. Our data explain how conformational changes in SecA could be directly coupled to the previously proposed opening mechanism of the SecYEG channel.

Keywords: protein translocation; SecA; SecY; cysteine crosslinking; peptide scanning

Introduction

The Sec machinery is a universally conserved multi-component enzyme complex involved in two biological key processes: the integration of membrane proteins into lipid bilayers and the translocation of pre-proteins across these bilayers.[1,2] In bacteria, a central role in both processes is fulfilled by the integral membrane complex SecYEG, that forms the protein conducting channel within the cytoplasmic membrane.[3,4] Two different cytoplasmic partners can bind to SecYEG to induce opening of the channel and to provide the driving force for the translocation process. Membrane proteins are mostly inserted co-translationally by SecYEG-bound ribosomes whereas secretory proteins and large extracellular domains of integral membrane proteins are translocated post-translationally by the motor protein SecA.[5]

The overall mechanisms of both co and post-translational translocation have been unraveled in the early nineties with purified components from *Saccharomyces cerevisiae* and *Escherichia coli*, respectively. The last five years have been characterized by a tremendous progress in our structural view on protein translocation, by the appearance of high resolution crystal structures of individual components[6–12] and medium resolution cryo-electron microscopy (cryo-EM) structures of co-translational translocation intermediates.[13,14] Despite the availability of these structures, there remains a large gap in our understanding of the interactions between the individual components, most notably on the highly dynamic interaction between SecYEG and SecA.

Binding of SecA induces oligomerization of SecYEG protomers,[15,16] and once bound to SecYEG, SecA undergoes multiple conformational changes that ultimately result in translocation of the pre-proteins. During these cycles of conformational changes, SecA is thought to insert (partially) into the oligomeric SecYEG complex. Hence, these cycles are referred to as membrane insertion/de-insertion cycles.[17,18] During the initiation phase of translo-

Present address: E.O. van der Sluis, Gene Center, University of Munich, Department of Chemistry and Biochemistry, Feodor-Lynenstrasse 25, 81377 Munich, Germany.

Abbreviations used: TMS, transmembrane segment; IMV, inner membrane vesicle; F-mal, fluorescein-maleimide; S-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; GST, glutathione *S* transferase; BSA, bovine serum albumin.

E-mail address of the corresponding author: a.j.m.driessen@rug.nl

cation, the conformational changes in SecA are transmitted to SecYEG, resulting in opening of the channel and co-insertion of the signal sequence whereas in later stages it results in a stepwise forward translocation of the polypeptide chain in the translocation channel.[19] Thus, a detailed understanding of the SecA–SecYEG interaction is of fundamental importance for understanding the mechanism of post-translational protein translocation on a molecular level. Despite the fact that the interaction has been studied extensively, relatively little is known about the regions of SecYEG that mediate it. Ligand affinity blotting experiments indicated that SecA interacts with the N-terminal 107 amino acid residues of SecY,[20] but the relatively large size of this region prevents a more detailed understanding of the interaction. Genetic studies suggest that the fifth and sixth cytoplasmic loop (C5 and C6) of SecY interact with SecA,[21,22] but such an interaction has never been demonstrated biochemically.

Here we have used a combination of cysteine-directed cross-linking and peptide scanning to identify regions in SecYEG that interact with SecA. We have identified two regions in SecY that both contain a highly conserved charged amino acid: Glu176 in the fourth transmembrane segment (TMS4) and Arg357 in the C5 loop. Both amino acids are important for membrane insertion of SecA and thus for functionality of the complex, but not for binding of SecA to SecYEG *per se*. The results will be discussed within the framework of the previously proposed opening mechanism of the SecYEG channel.

Results

Cysteine scanning of cytoplasmic loop C5 of SecY

To identify regions in SecY that interact with SecA, we continued our previously initiated cysteine-directed cross-linking approach[23] now focussing on a single cytoplasmic loop of SecY. The C5 loop that connects TMS8 with TMS9 is one of the most conserved regions of SecY (Figure 1(a)), and according to genetic studies it could be involved in SecA binding.[22] The corresponding loop of the archaeal *Methanococcus jannaschii* SecY protrudes into the cytoplasm, and thus forms a likely candidate for an interaction with SecA in bacteria.[12] Within the C5 loop, Arg357 has been shown to play a crucial role in SecY functioning,[24] and therefore we mutated 15 amino acids around this residue (Val353 to Asp367) into single cysteine residues. Cysteine-less SecYEG, which behaves identically to wild-type SecYEG,[25] was used as a control throughout this work. All 15 single cysteine SecY mutants were overexpressed to similar levels as cysteine-less SecY (data not shown), and except for the SecY(R357C) mutant, all were equally active for *in vitro* translocation of proOmpA (Figure 1(b), compare lane 6 to all other lanes).

To determine if the cysteine residues within the mutated region are accessible for cysteine modifying reagents, we incubated inner membrane vesicles (IMVs) containing the mutants with the fluorescent probe fluorescein-maleimide (F-mal). Except for the mutants with cysteine residues at the positions of Val353, Ile356, Ala363 and Ile366, SecY was efficiently labeled with F-mal (Figure 1(c)). This indicates that the majority of the mutated region is easily accessible for chemical compounds, thereby allowing a site-specific chemical cross-linking approach to identify putative interacting partners of this region.

Cytoplasmic loop C5 of SecY is in close proximity of SecE and SecA

To investigate whether the mutated region of C5 interacts with SecA and/or other components of the Sec machinery, IMVs containing the mutant SecYEG complexes were incubated with the heterobifunctional chemical cross-linker *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (S-MBS), and cross-link products were analyzed by immunoblotting. SecY mutants P354C, G355C (Figure 2(a), lanes 3 and 4),[26] Y365C (lane 14) and D367C (lane 16) yielded cross-link products of around 50 kDa. In the case of SecY(P354C) and SecY(Y365C), these products correspond to SecY–SecE cross-links (Figure 2(b), lanes 3 and 14), while the cross-link products of 48 and 52 kDa observed with the SecY (D367C) mutant remain unidentified as they did not react with SecE or SecG antibodies. However, we cannot exclude that the latter products, as well as the similarly sized weak bands in lanes 6 to 10 of Figure 2(a), do correspond to SecY–SecE cross-links. These cross-links might not react with the polyclonal SecE antiserum if a dominant epitope is involved in the cross-linking reaction.

In addition to the SecY–SecE cross-links, several SecY mutants showed a specific but faint cross-link product of around 140 kDa that reacted with both SecY and SecA antibodies (data not shown). To improve the detection by circumventing the low blotting efficiency of high mass cross-link products, we made use of iodinated SecA (Figure 2(c)). In particular SecY mutants P354C, G355C and R357C (Figure 2(c), lanes 3, 4 and 6) showed relatively strong SecY–SecA cross-links when incubated with S-MBS. The weak background cross-linking observed with cysteine-less SecY could either be mediated by the cysteine residues in SecA, or by a non-specific reaction of the maleimide group from S-MBS with residues in SecY. Taken together, these data show that the C5 loop of SecY is in close proximity of both SecA and SecE.

Arg357 in C5 of SecY is important for functionality but not for binding of SecA

From the set of SecY mutants only IMVs of SecY (R357C) showed a significant reduction in translocation activity. Since Arg357 is highly conserved and

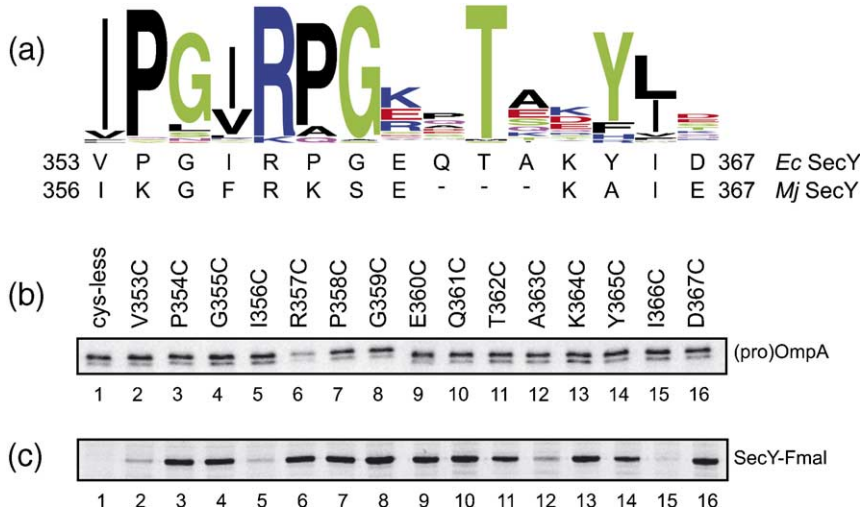

Figure 1. Cysteine scanning of cytoplasmic loop C5 of SecY. (a) Sequence logo of the mutagenized C5 region, based on an alignment of 56 SecY sequences. Indicated below are the amino acid sequences of *E. coli* (*Ec*) and *M. jannaschii* (*Mj*) SecY. (b) *In vitro* translocation of fluorescein labeled proOmpA into IMVs overexpressing the single cysteine SecY(EG) complexes. Limiting amounts of IMVs were used and to ensure that the amount of translocated proOmpA still increases linearly in time when the reaction is stopped translocation reaction were performed for 7 min at 37 °C. (c) Fluorescein-maleimide (F-mal) labeling of mutant SecYEG complexes. IMVs overexpressing the mutant SecYEG complexes were labeled with 250 μM F-mal whereafter proteins were separated by SDS–PAGE and F-mal labeled proteins were visualized by in gel UV-fluorescence.[23]

lies in the region that showed the strongest cross-links with SecA we focused on this position to study the SecY–SecA interaction in more detail. Although mutants of Arg357 have been studied before,[24] those studies did not determine if such mutants are specifically disturbed in the interaction with SecA. Since the SecY(R357C) has a residual translocation activity (Figures 1(b) and 3(a)), we also constructed a SecY(R357E) mutant that is virtually inactive in proOmpA translocation (Figure 3(a), open bars). To study the binding of SecA to the SecY(R357) mutants, we immobilized IMVs containing the mutant SecYEG complexes on a Biacore Pioneer L1 chip and determined the association and dissociation rates for SecA by surface plasmon resonance (SPR).[27] The dissociation rates of the Arg357 mutants did not differ significantly from those of cysteine-less SecYEG (Table 1). The association rate of SecY(R357E) was slightly lower than that of cysteine-less SecYEG, resulting in a lower affinity as defined by k_{off}/k_{on}. However, the differences are only small, and the K_D values for both mutants as determined by Scatchard analysis of the response levels attained at equilibrium for different SecA concentrations were within the error margins of cysteine-less SecYEG (data not shown). This shows that mutation of Arg357 does not significantly disturb the binding of SecA to SecYEG.

Since the binding of SecA to SecYEG remained unaffected in the Arg357 mutants, we reasoned that the translocation defect must be caused by a step in the translocation cycle that occurs after the initial binding of SecA. To study the functional interaction between SecA and SecYEG we assayed the mutants

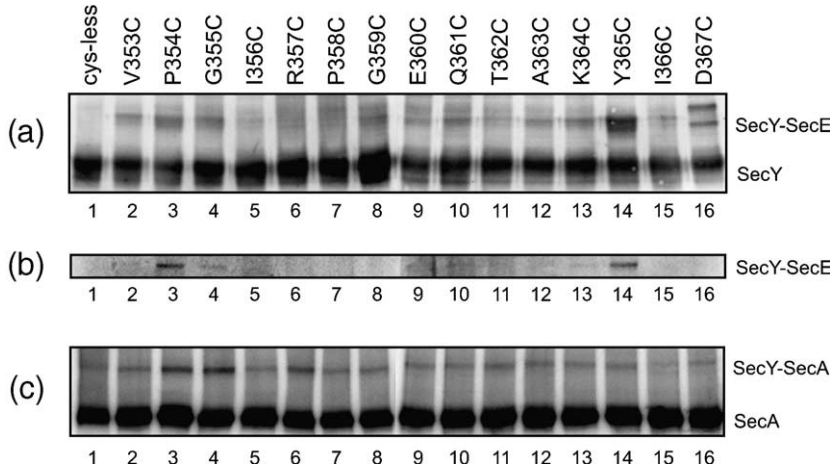

Figure 2. Cytoplasmic loop C5 of SecY is in close proximity of SecE and SecA. IMVs (10 μg) overexpressing mutant SecYEG complexes were incubated for 10 min at room temperature in translocation buffer supplemented with 1 mM S-MBS. Subsequently, proteins were separated by SDS–PAGE and blotted onto PVDF membranes. Cross-linked products were detected with antibodies raised against SecY (a) or SecE (b). (c) Cross-linking was performed as in (a) and (b) with addition of 30 nM [^{125}I]SecA. Proteins were separated by 8% SDS–PAGE (ProSieve acrylamide) and cross-linked products were detected by autoradiography. SecY, SecA, SecY–SecE and SecY–SecA cross-link products are indicated.

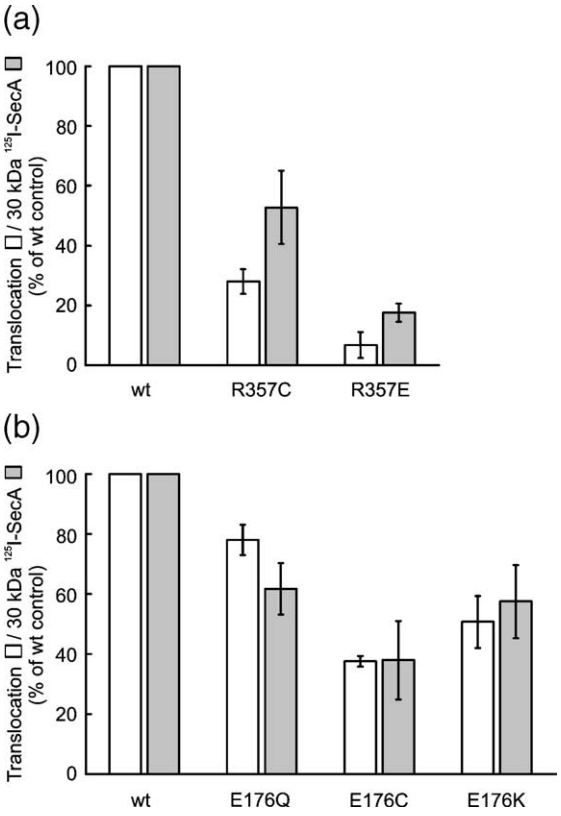

Figure 3. The effects of Arg357 and Glu176 mutagenesis on translocation activity and SecA membrane insertion. IMVs containing overexpressed SecY(EG) mutants of Arg357 (a) or Glu176 (b) were analyzed for *in vitro* translocation of proOmpA (open bars) and membrane insertion of SecA (grey bars). SecA membrane insertion was assayed by AMP-PNP induced generation of the protease-resistant 30 kDa SecA fragment[17] (for details, see Materials and Methods).

for their ability to stably generate the well-characterized 30 kDa protease resistant fragment of SecA.[17,18] The formation of this protease-resistant fragment, also referred to as the "membrane inserted state" of SecA, represents an early step in the translocation cycle. Formation of this conformational state of SecA requires either SecYEG, a preprotein and ATP or only SecYEG and the non-hydrolysable ATP analog AMP-PNP.[17] To ensure that the assay only reflects the interaction between SecA and SecYEG and is not influenced by the preprotein, we chose to generate the 30 kDa fragment under the latter conditions. The SecY(R357C) mutant was reduced to around 50% as compared to cysteine-less SecYEG in supporting the formation of the 30 kDa fragment, whereas the level of 30 kDa fragment generated with the SecY(R357E) mutant was less than 20% (Figure 3(a), grey bars). Taken together, these data demonstrate that the SecY (R357) mutants bind SecA normally, but that they are disturbed in supporting the nucleotide-dependent SecA conformational change required for translocation.

Identification of a SecA interaction site in SecY by peptide scanning (TMS4c)

Considering the size of the proteins and the dynamic nature of the SecA–SecYEG interaction, it is unlikely that the C5 loop of SecY is the only SecA interaction site at SecYEG. To identify additional interaction sites we made use of the SPOT-technology that involves miniaturized synthesis of peptides that are covalently linked to a cellulose membrane.[28] This technology is commonly used to define determinants for peptide–protein interactions, but can also be used to identify important regions of protein–protein interactions.[29] The underlying assumption is that the peptides can adopt the same structure as the corresponding region of the intact protein. For our purpose we synthesized the entire sequences of SecY (see Supplementary Data), SecE and SecG in fragments of 15 amino acid long peptides with an off-set of 1. This so-called peptide scan should allow us in principle to identify all the SecA interaction sites on SecYEG. The cellulose membrane was incubated with SecA, washed, and bound SecA was detected with antibodies. The peptides derived from SecE and SecG did not give any SecA binding signals above the background (data not shown), but a stretch of eight consecutive peptides (peptides 176 to 183) derived from SecY showed a strong signal that was strictly dependent on the addition of SecA (Figure 4(a)). The amino acid sequences of the SecA binding peptides are shown in Figure 4(b), and the minimal motif present in each of these peptides is "F^{170}LMWLGEQ^{177}" (or "WLGEQ" if the loose spot 186 is included).

To verify the detected interaction we created two GST-fusion constructs, containing SecY residues Ser163–Gly184 (all residues present in peptides 176 to 183) or Phe170–Gly184 (corresponding to peptide 183) fused to the C terminus of GST. Since the GST-Ser163–Gly184 construct was insoluble, only the glutathione *S* transferase (GST)-Phe170–Gly184 fusion could be used in a GST pull down assay. Glutathione-Sepharose beads with the immobilized

Table 1. Kinetic constants of SecA binding to SecYEG

IMVs	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	k_{off1} (s^{-1})	k_{off2} (s^{-1})	K_{D1} (M) (k_{off}/k_{on})
SecYEG	$1.84(\pm0.05)\times10^{6}$	$8.0(\pm0.1)\times10^{-3}$	$6.0(\pm0.2)\times10^{-2}$ (37%)	$5.5(\pm0.3)\times10^{-3}$ (63%)	$4.3(\pm0.3)\times10^{-9}$
SecY(R357C)EG	$1.82(\pm0.06)\times10^{6}$	$7.4(\pm0.1)\times10^{-3}$	$6.6(\pm0.7)\times10^{-2}$ (40%)	$4.8(\pm0.6)\times10^{-3}$ (60%)	$4.8(\pm0.6)\times10^{-9}$
SecY(R357E)EG	$1.42(\pm0.13)\times10^{6}$	$8.2(\pm0.4)\times10^{-3}$	$5.5(\pm0.1)\times10^{-2}$ (42%)	$5.0(\pm0.2)\times10^{-3}$ (58%)	$5.8(\pm0.6)\times10^{-9}$
SecY(E176Q)EG	$1.55(\pm0.04)\times10^{6}$	$7.3(\pm0.3)\times10^{-3}$	$3.6(\pm0.2)\times10^{-2}$ (31%)	$4.4(\pm0.2)\times10^{-3}$ (69%)	$4.7(\pm0.3)\times10^{-9}$
SecY(E176C)EG	$1.44(\pm0.04)\times10^{6}$	$8.4(\pm0.5)\times10^{-3}$	$6.2(\pm0.2)\times10^{-2}$ (45%)	$5.2(\pm0.7)\times10^{-3}$ (55%)	$5.8(\pm0.5)\times10^{-9}$
SecY(E176K)EG	$1.60(\pm0.05)\times10^{6}$	$8.0(\pm0.3)\times10^{-3}$	$5.4(\pm0.3)\times10^{-2}$ (37%)	$5.2(\pm0.1)\times10^{-3}$ (63%)	$5.0(\pm0.4)\times10^{-9}$

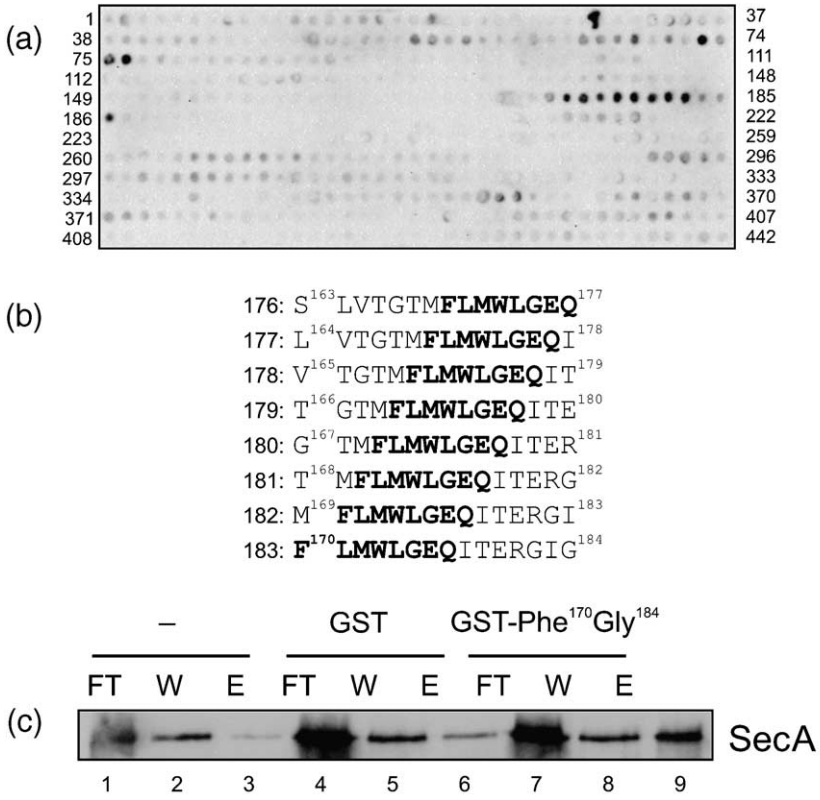

Figure 4. SecA interacts with the cytoplasmic end of TMS4 from SecY (TMS4c). (a) SecY peptide scan analysis of 15-mer peptides with an off-set of 1 between adjacent peptides. After overnight blocking in TBST with 2% skim-milk and 1% BSA, the membrane was incubated with 10 nM SecA in TBST with 1% skim-milk and 0.5% BSA (TSB buffer). Bound SecA was detected with polyclonal antibodies raised against SecA followed by anti-rabbit IgG conjugated with alkaline phosphatase, both in TSB buffer. All incubation steps were followed by three consecutive washes of 10 min with TSB buffer. (b) Amino acid sequences of the eight consecutive SecA binding peptides identified by peptide scanning, with the minimal SecA binding sequence (Phe170–Gln177:TMS4c) in bold. The sequences of all peptides employed in the SecY scan are provided as Supplementary Data. (c) SecA binding in solution to Phe170–Gly184 of SecY C-terminally fused to GST. Empty glutathione beads and immobilized GST alone were used as controls. Lanes labeled FT, W and E represent the flow-through, wash and elution steps, respectively, as described in the text.

GST fusion protein were incubated with SecA and after a washing step (Figure 4(c), lane 8), bound protein was eluted by free glutathione (lane 9). As compared to empty glutathione beads (lane 3), the GST control (lane 6), or a different GST-SecY fusion construct (GST-Tyr332–Tyr365; see Discussion) only the GST-Phe170–Gly184 fusion showed significant binding of SecA (lane 9). This shows that the Phe170–Gly184 region of SecY also binds SecA in solution, and that the interaction identified with the peptide scan is not induced by the cellulose membrane. In the structure of *M. jannaschii* SecY the residues corresponding to the minimal SecA binding motif (Phe170–Gln177) constitute the cytoplasmic end of TMS4, and therefore we will refer to this SecA interaction site as TMS4c.

Glu176 is the main determinant for SecA binding to TMS4c

To identify amino acids within TMS4c that are important for the interaction with SecA, it was subjected to an exhaustive mutagenesis study. We used the SPOT-technology to create 19 point mutations at each position of peptide Phe170–Gly184 (cysteine was omitted because it gives rise to disulfide bonding between peptides) and analyzed all 285 peptides for binding of SecA. The results are shown in Figure 5(a), with mutant peptides exhibiting a significantly decreased SecA binding in black and the remaining peptides in grey (for details, see the legend to Figure 5(a)). Especially Glu176 appeared very sensitive to mutations. Only the conserved charge substitution to aspartate did not interfere with SecA binding (Figure 5(a)). Interestingly, the negative charge at this position is absolutely conserved in the SecY/Sec61α family (Figure 5(b)). Several substitutions of the other two charged residues in the peptide (Glu180 and Arg181) also decreased SecA binding, but those residues appeared less sensitive than Glu176 and are not located in the minimal sequence required for SecA binding (TMS4c: Phe170–Gln177). These data show that the conserved Glu176 is the main determinant for SecA binding to TMS4c of SecY.

The majority of TMS4c is inaccessible from the cytoplasm

In the structure of *M. jannaschii* SecYEβ the region corresponding to TMS4c is only partially accessible from the cytoplasm (data not shown). Glu159 of *M. jannaschii* SecY corresponds to Glu176 from *E. coli* SecY, and forms a salt bridge with Lys112 in TMS3 (Arg121 in *E. coli* SecY; Figure 5(c)) that is located roughly at the membrane/water interface. The charge at both positions is conserved in all members of the SecY/Sec61α family (data not shown), suggesting that the salt bridge plays a functional role in protein translocation. Since the *E. coli* SecY structure might differ locally from *M. jannaschii* SecY, we created the SecY(G175C) and SecY(E176C) mutants to investigate the accessibility of this region with the cysteine-specific probe fluorescein

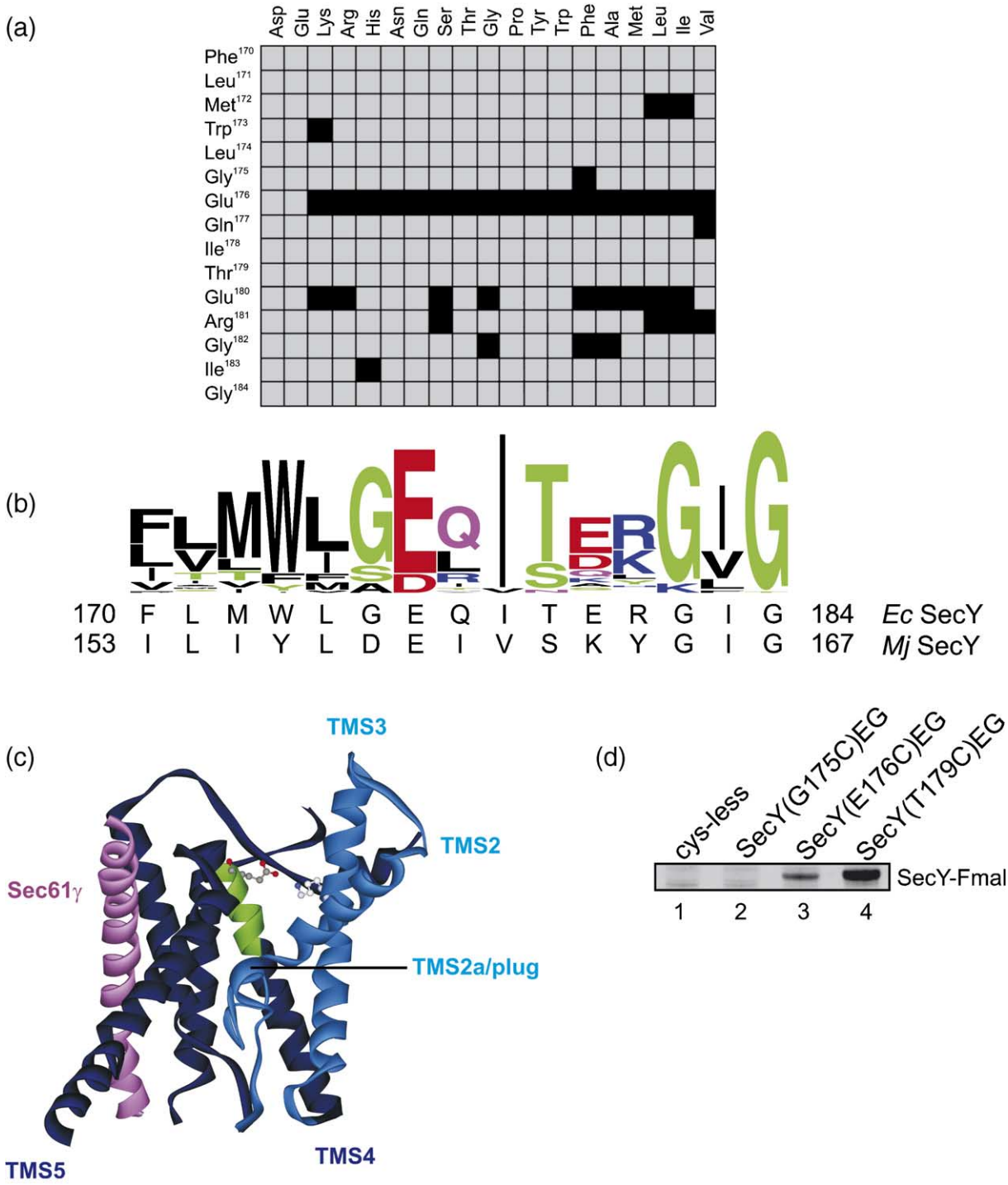

Figure 5. Glu176 plays a crucial role in SecA binding to TMS4c and is located at the membrane/water interface. (a) Each position of the SecA binding peptide Phe170-Gly184 was mutated individually to all amino acids except cysteine, and the peptides were simultaneously assayed for SecA binding. Since 15 peptides correspond to the "wild-type" sequence, statistic information could be gained from the quantified signals. The standard deviation (S.D.) of the wild-type peptides corresponded to approximately 25% of the average value, and for the mutant peptides we defined a binding decrease of ≥50% (2 S.D.) as significant. Mutants exhibiting significantly decreased SecA binding are depicted in black, the remaining mutants are in grey. (b) Sequence logo of the region of SecY corresponding to Phe170–Gly184, showing the absolute conservation of negative charge at the position of Glu176. The sequences of *E. coli* (*Ec*) and *M. jannaschii* (*Mj*) SecY are depicted underneath. (c) Side-view of the N-terminal half of *M. jannaschii* SecY, roughly parallel to the membrane from within the interior of the channel. Cytoplasmic loops are facing up, and the C-terminal half of SecY and the amphipathic helix of SecE have been removed. The tilted transmembrane segment of SecE is colored pink, the plug domain, TMS2 and TMS3 are colored light blue, the residues corresponding to TMS4c (Phe170–Gln177) are colored green, and the remaining residues dark-blue. The side-chains of the residues corresponding to *E. coli* SecY Glu176 and Arg121 (Lys112 and Glu159) involved in the conserved salt bridge are indicated. (d) Fluorescein-maleimide labeling of single cysteine SecYEG complexes as described in the legend to Figure 1(c).

maleimide. SecY(E176C) could be partially labeled by fluorescein maleimide (Figure 5(d), lane 3) as compared to SecY(T179C) in the C3 loop that shows a maximum labeling efficiency[23] (Figure 5(d), lane 4). The SecY(G175C) mutant was completely inaccessible for fluorescein maleimide (Figure 5(d), lane 2). Since the crystal structure and hydropathy profiles indicate that residues preceding Gly175 are part of the fourth TMS of SecY which is inaccessible from the cytoplasm, these data indicate that the majority of TMS4c is most likely buried within the membrane region of SecYEG.

Glu176 in TMS4c is important for functionality but not for binding of SecA

To address the importance of Glu176 in the context of the SecYEG complex in more detail, three mutations of this residue were assayed for *in vitro* translocation of proOmpA. Mutagenesis of Glu176 to glutamine, cysteine, or lysine leads to a translocation activity of 78%, 38% or 51% as compared to wild-type SecYEG, respectively (Figure 3(b), open bars). This confirms the functional importance of the TMS4c region that was identified with the SPOT-technology.

To determine if Glu176 is important for SecA binding to SecYEG, we also studied the SecA–SecYEG interaction by SPR as described.[27] The dissociation rates of the Glu176 mutants do not differ significantly from cysteine-less SecYEG (Table 1), while the association rates of the mutants are slightly lower than that of cysteine-less SecYEG. The resulting changes in K_D (k_{off}/k_{on}) are therefore small. This shows that mutation of Glu176 does not significantly disturb SecA binding to the SecYEG complex.

The SecY(E176) mutants were also tested for generation of the 30 kDa fragment of [^{125}I]SecA. As compared to wild-type SecYEG, all three mutants show a reduction in the amount of stable membrane inserted SecA that parallels the reduction in translocation activity (Figure 3(b), grey bars). Although the effects are smaller than those observed upon mutagenesis of Arg357, these data demonstrate that Glu176 of SecY is also important for supporting the nucleotide-dependent SecA conformational change required for translocation.

Discussion

In order to understand the molecular mechanism of post-translational protein translocation in bacteria, detailed knowledge of the interaction between the motor protein SecA and the protein-conducting channel SecYEG is required. Although the interaction between SecA and SecYEG has been studied extensively, no exact sites of interaction had been identified. By a combination of peptide scanning and cysteine-directed cross-linking, we have identified two functionally important regions in SecY that are involved in the SecA interaction: Phe170–Gln177 in TMS4 (TMS4c) and Pro354–Arg357 in C5. In the structure of *M. jannaschii* SecY, the C5 loop protrudes far into the cytoplasm, which makes it a likely candidate for an initial SecA interaction. TMS4c on the other hand is concealed for an initial interaction, and therefore we hypothesize that this region interacts with SecA during the translocation cycle as will be discussed below.

Unexpectedly, the two experimental approaches yielded different interaction sites. To corroborate the lack of SecA binding to C5-derived peptides in the peptide scan, we studied this SecA interaction site in more detail with an alternative approach. We reasoned that more than 15 residues might be required for SecA binding to C5, and therefore we constructed a GST-fusion protein containing nearly the entire C5 loop (Tyr332–Tyr365), and used this in a GST pull-down assay with SecA. In analogy with the peptide scan however, no SecA binding could be detected to this fusion protein (data not shown). In the context of SecYEG, the amphipathic helix of SecE is in direct contact with C5 *via* Pro354 and Tyr365 (Figure 2(b)).[12,26] The lack of SecA binding to the isolated C5 domain can be explained by the absence of SecE that could be required for the correct SecA binding conformation. Analogously, additional SecA interaction sites that are expected considering the size of SecA and SecYEG might have been missed by the peptide scanning approach, either because the peptides do not adopt the same structure as the corresponding region of the intact protein, or because the interactions are too weak. Nevertheless, peptide scanning proves to be a suitable method to systematically identify protein–protein interactions.

We have also attempted to cross-link SecA under various conditions to cysteine residues in TMS4c, but this did not result in the formation of SecY–SecA cross-links (data not shown). Most likely this is caused by the poor accessibility of TMS4c (Figure 5(d)),[12] which will not allow the chemical cross-linkers to react with the cysteine residues. Taken together, this shows that multiple strategies need to be followed to identify interacting regions within multi-component enzyme complexes such as the Sec machinery. It should be stressed that the high binding affinity of SecA for SecYEG (K_D 4–5 nM) most likely involves multiple sites of contact. Binding will thus not only be restricted to the two interaction sites described here. This would also explain why mutation of the single amino acids Glu176 or Arg357 does not significantly reduce SecA binding to SecYEG.

The SecA interaction site we identified by cysteine-directed cross-linking is located in a loop of SecY that was previously shown to be important for functionality of SecYEG.[24] However, the molecular basis of the importance for SecYEG functioning had not been established. Here, we show for the first time that several amino acids in the C5 loop are in close proximity of SecA. Moreover, we demonstrate that the most critical residue within this region (Arg357) is important

for SecA "insertion", whereas this residue is not critical for SecA binding in contrast to previous speculations.[24] The *in vitro* translocation activity of SecY(R357C)EG could not be restored by modification of the cysteine residue with the positively charged reagent (2-(trimethylammonium)ethyl)-methane-thiosulfonate (MTSET) (unpublished data), suggesting a high specificity of Arg357 for the functional SecY–SecA interaction. The central role of Arg357 in SecY functioning is further illustrated by the recent findings that mutations in that region interfere with SecYEG oligomerization and movement of the plug domain.[30]

The SecA interaction site we identified by peptide scanning (TMS4c: Phe170–Gln177) constitutes the cytoplasmic end of TMS4 from SecY. Instead of being an authentic SecA–SecY interaction site, the observed interaction could represent binding of SecA to a pre-protein. This possibility is considered unlikely for the following reasons: The TMS4c peptides have a net negative charge, whereas that of signal peptides is usually positive due to the characteristic positively charged residues in the N terminus. It has recently been shown that these positively charged residues are directly involved in signal sequence binding to SecA,[31] which is in marked contrast to the negatively charged residue (Glu176) that is the main determinant for binding of TMS4c to SecA. Furthermore, if the interaction with TMS4c would represent SecA binding to the mature region of a pre-protein, many more peptides are expected to bind SecA.

Genetic data also suggest that the TMS4c region is important for SecYEG functioning. First, SecY mutants within TMS4c (W173C, G175C and E176C) are less efficient than wild-type SecY in complementing the cold-sensitive phenotype of *secY39*.[32] Second, SecY mutants within (G175D) or just after (G184D) TMS4c cause a cold-sensitive growth phenotype.[33] Third, replacement of six residues just preceding TMS4c (Leu164–Met169) by an unrelated polypeptide stretch strongly interferes with protein translocation.[34] The region following TMS4c (Gly182–Pro200) is the most conserved region of the SecY/Sec61α family,[35] that has been proposed to form a hinge region for separation of the two domains of the channel.[12]

The data presented here provide clues on how the conformational changes of SecA could result in opening of the SecYEG channel. In the structure of *M. jannaschii* SecY, the C5 loop protrudes far into the cytoplasm[12] and the residues that yield the strongest SecA cross-links (including Arg357) are located at the tip of this loop. This location suggests that C5 could be involved in the initial contact between SecA and SecYEG. The second interaction site we identified is only half accessible from the cytoplasm, and thus SecYEG needs to undergo a substantial conformational change to allow SecA binding to the complete TMS4c region. Since the Glu176 mutants of SecY are disturbed in supporting 30 kDa formation of SecA and not in SecA binding, we hypothesize that this conformational change is induced by "membrane insertion" of SecA, and thus that SecA only interacts with the TMS4c region in the "inserted" state. Although the extent of membrane insertion of SecA as originally proposed[17] is questionable,[36] TMS4c could be part of the membrane region where SecA actually inserts.

Importantly, the location of the two interaction sites we identified may explain how conformational changes in SecA (or insertion) could be directly coupled to the proposed opening mechanism of the translocon, i.e. separation of the two SecY domains. Since C5 is located in the C-terminal domain and TMS4c in the N-terminal domain of SecY, a simultaneous interaction of inserted SecA with both interaction sites (green in Figure 6) could induce the outward directed force on each domain that is required for their separation. A similar mechanism could underlie the ribosome induced opening mechanism as well, as the ribosome also interacts with the C5 loop[37] and with the cytoplasmic domain of SecG which is bound to the N-terminal domain of SecY.[14]

Taken together, our studies provide the first demonstration of two SecY regions that interact with SecA, including a region where SecA possibly

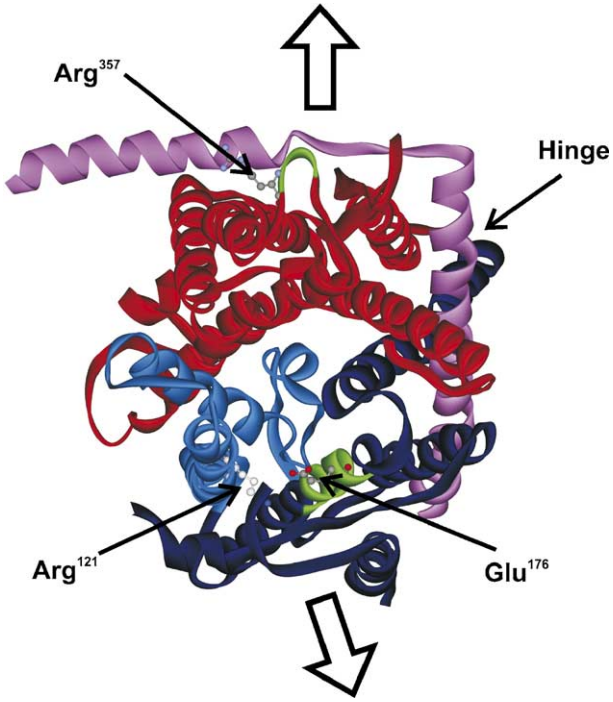

Figure 6. Overview of the identified SecA interaction sites and proposed conformational changes of SecYEG occurring upon membrane insertion of SecA. Cytoplasmic view of *M. jannaschii* SecYEβ colored as for Figure 5(c), with the C-terminal half of SecY colored red. The residues corresponding to Pro354–Arg357 and TMS4c (Phe170–Gln177) of *E. coli* SecY are colored green, and the side-chains of the residues corresponding to Arg121, Glu176 and Arg357 are indicated. Arrows indicate the global conformational change separating the two halves of the channel that has been proposed as the opening mechanism for SecYEG.

inserts into the SecYEG channel. In addition, our data provide a framework that couples conformational changes in SecA to the previously proposed opening mechanism of the SecYEG channel. It will be important to establish which region of SecA associates and inserts into SecYEG, and how these processes relate to the oligomeric state of SecYEG. The interaction sites identified here could facilitate these studies.

Materials and Methods

Chemicals and biochemicals

m-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (S-MBS) was purchased from Pierce (Rockford, IL, USA), dithiothreitol (DTT) from Roche (Basel, Switzerland), fluorescein-5-maleimide (F-Mal) from Molecular Probes (Eugene, OR, USA), glutathione-Sepharose 4B and pGEX-4T3 from Amersham Biosciences (Piscataway, NJ, USA). Fmoc protected and OPfp activated amino acids were purchased from Bachem (Switzerland). All other chemicals were purchased from Sigma (St Louis, MO, USA). SecA,[38] SecB[39] and proOmpA[40] were purified essentially as described. GST, GST-SecY(Tyr332–Tyr365) and GST-SecY (Phe170–Gly184) were purified with glutathione-Sepharose 4B according to the manufacturer's instructions. Translocation buffer consists of 50 mM Hepes/NaOH (pH 7.0), 20 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (BSA), 5 mM DTT. SecA was iodinated[41] and IMVs were isolated[23] as described.

Bacterial strains and plasmids

E. coli strain DH5α (*sup*E44, Δ*lac*U169 (ϕ80 *lac*Z ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1 *rel*A1) was used for the expression of GST constructs and standard DNA manipulations. Expression of SecYEG was performed in *E. coli* SF100 (F^-, Δ*lac*X74, *gal*K, *thi*, *rps*L, *str*A Δ*pho*A(pvuII), Δ*omp*T). Plasmids encoding *secY* mutants and wild-type *secE/secG* (see Table 2) were created with the Stratagene QuikChange™ mutagenesis kit using pEK20[23] as template. Plasmid pEK63 and pEK65 were created by ligation of SalI/NotI or SmaI/XhoI digested PCR products into pGEX-4T3, respectively. The PCR products were generated with standard oligonucleotides pairs containing the appropriate restriction sites, using pEK20 as template. All constructs were confirmed by sequence analysis.

SPOT-methodology

Peptide arrays were synthesized by Fmoc chemistry at activated PEG spacers on cellulose membranes using a semi-automated spot robot (ASP222; Intavis, Germany) as described.[42] SecY (including the N-terminal hexahistidine-tag and enterokinase cleavage-site; see Supplementary Data), SecE and SecG were synthesized as overlapping peptides (15-mers off-set by one amino acid) covering the entire sequence of the proteins. After blocking with 2% (w/v) skim-milk and 1% BSA in TBST (25 mM Tris-HCI pH 7.4, 150 mM NaCl, 0.05% Tween 20), the membranes were incubated to for 1 h with 10 nM SecA. Bound SecA was detected with a SecA-specific polyclonal antibody and visualized using a secondary antibody conjugated to alkaline phosphatase. Binding signals were detected by a Roche Lumi-Imager F1, and quantified using OptiQuant software.

Table 2. Plasmids used in this study with their relevant characteristics

Plasmid	Characteristics	Mutation
pEK20	Cysteine-less SecYEG	–
pEK44	SecY(V353C)EG	GTA→TGC
pEK45	SecY(P354C)EG	CCA→TGC
pEK46	SecY(G355C)EG	GGA→TGC
pEK47	SecY(I356C)EG	ATT→TGT
pEK48	SecY(R357C)EG	CGT→TGT
pNN260	SecY(R357E)EG	CCG→GAG
pEK49	SecY(P358C)EG	CCG→TGC
pEK50	SecY(G359C)EG	GGA→TGC
pEK51	SecY(E360C)EG	GAG→TGC
pEK52	SecY(Q361C)EG	CAA→TGC
pEK53	SecY(T362C)EG	ACG→TGC
pEK54	SecY(A363C)EG	GCG→TGC
pEK55	SecY(K364C)EG	AAG→TGC
pEK56	SecY(Y365C)EG	TAT→TGT
pEK57	SecY(I366C)EG	ATC→TGC
pEK58	SecY(D367C)EG	GAC→TGC
pEK69	SecY(G175C)EG	GGC→TGC
pEK70	SecY(E176Q)EG	GAA→CAA
pEK71	SecY(E176C)EG	GAA→TGC
pEK72	SecY(E176K)EG	GAA→AAA
pEK23	SecY(T179C)EG	ACT→TGT
pGEX-4T3	*Schistosoma japonica* GST	–
pEK63	GST-SecY(Tyr332–Tyr365)	–
pEK65	GST-SecY(Phe170–Gly184)	–

Surface plasmon resonance(SPR)

SPR measurements were performed on a Biacore 2000 essentially as described[27] but Tris–HCl (pH 8.0) was replaced by Hepes/NaOH (pH 7.0) in all steps. Approximately 3200 response units of IMVs were loaded on a Biacore Pioneer L1 chip, and SecA was injected at a (dimeric) concentration of 50 nM with a flow speed of 20 μl/min. Fitting of the association and dissociation rates from the corrected response curves was performed as described, and gave nearly identical values to those described for the previous setup.[27]

Miscellaneous

Labeling of cysteine residues, chemical cross-linking, and *in vitro* translocation assays were performed as described.[23] Membrane insertion of SecA was assayed by AMP-PNP induced generation of the 30 kDa protease resistant fragment of SecA, essentially as described.[17] Briefly, urea stripped IMVs (20 μg) were mixed with 30 nM [^{125}I]SecA and 0.5 mM AMP-PNP in translocation buffer and incubated for 5 min at 37 °C. After chilling on ice, the samples were digested for 30 min with 0.1 mg/ml of TPCK-treated trypsin (4 °C). After inactivation of the protease, proteins were separated by 10% (w/v) SDS-PAGE and the 30 kDa fragment was visualized by autoradiography. Bands corresponding to the 30 kDa fragment (and translocated (pro)OmpA) were quantified with OptiQuant software, and values of wild-type SecYEG were set to 100%. The averages and error bars in Figure 3 are derived from at least two independent experiments.

For the GST pull-down assay, GST(-fusion protein) was immobilized on 25 μl glutathione-Sepharose and incubated for 60 min at 4 °C with 200 nM SecA in 50 mM Hepes/NaOH (pH 7.0), 50 mM KCl. The beads were washed three times with 50 mM Hepes/NaOH (pH 7.0), 150 mM KCl, and proteins were eluted by 15 mM reduced

glutathione in the same buffer. SecA was detected by Western blotting. Sequence logos were created on line†.

Acknowledgements

The authors thank Juke Lolkema and Eric Geertsma for valuable discussions, and Greetje Berrelkamp for technical assistance. This work was supported by the Council for Chemical Sciences (CW) that is subsidized by the Dutch Organization for the Advancement of Scientific Research (NWO) and by a fellowship to N.N. by the Royal Netherlands Academy of Arts and Sciences.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.07.017

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Edited by I. B. Holland

(Received 3 May 2006; received in revised form 3 July 2006; accepted 12 July 2006)
Available online 15 July 2006