SecA mediated protein translocation in Escherichia coli

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Summary and concluding remarks

The movement of proteins across or integration of proteins into the cytoplasmic membrane of *Escherichia coli* is mediated by the multimeric membrane protein complex translocase. The core of the translocase consists of a motor protein, the ATPase SecA, and a protein-conducting channel, formed by the integral membrane proteins SecY and SecE. The SecYE complex is highly conserved, with homologs in the cytoplasmic membrane of Archaea, the chloroplast thylakoid membrane, and the eukaryotic endoplasmic reticulum (ER). SecA is unique for the bacterial post-translational translocation pathway. It is absent both in Archaea and in the eukaryotic ER, but a homolog exists in plant chloroplasts. SecA is a soluble protein that distributes between cytoplasmic and membrane associated states. The interaction with the cytoplasmic membrane occurs via low affinity interactions with anionic phospholipids and by a high affinity interaction with the SecYEG complex. At the membrane SecA forms a receptor for preproteins and drives their stepwise movement through the SecYEG channel [93, 173]. The ATPase activity of SecA can be stimulated by the association of the SecYE complex with the integral membrane protein SecG [7, 8]. In this study, both novel and classical methods were exploited to increasing our insight in the mechanism and kinetics of the protein translocation reaction.

Kinetics of protein translocation

Although the majority of secretory proteins that are translocated by the Sec-translocase pass the cytoplasmic membrane in an unfolded state, the SecYEG-pore is sufficiently wide or flexible to permit the passage of preproteins conjugated with small molecules. When the model preprotein proOmpA was labelled site specifically with fluorescent probes of a size up to 13-16 Å, it was readily translocated into inner membrane vesicles or proteoliposomes containing the purified SecYEG complex in the absence of the proton motive force (PMF) (Chapter 2). The translocation efficiency of labelled proOmpA was largely independent on the size and position of the fluorophore, provided that the label was not too close to the signal sequence. A fluorophore at the +4 position from the signal sequence cleavage site caused a severe protein translocation defect, but this defect was not observed when the probe was moved to the +33 position. The probe on the +4 position possibly interferes with the initiation of translocation as it is located in the region of the mature polypeptide domain that was proposed to form a looped structure with the signal sequence at the onset of translocation [283].

The translocation compatibility of fluorescent proOmpA was used to renew and extend the application of the protein translocation assay. In the classical version of this assay, movement of a translocation competent precursor protein into the lumen of the isolated inner membrane vesicles or proteoliposomes is monitored by their protection against treatment with an externally added protease. Protease protected polypeptides are subsequently visualized by western blotting or autoradiography. The fluorescent label on proOmpA allowed *in gel* fluorescence imaging of the protease protected fragments, which significantly increased the ease and speed of the detection.

When monitored spectroscopically, translocation of fluorescently labelled proOmpA resulted in a progressive decrease in fluorescence. The mechanism behind this fluorescence-quenching phenomenon is unclear as it was observed with a wide range of different probes with various spectroscopic properties. However, as the decrease was

directly related to the amount of proOmpA molecules translocated, the method could be used for real-time monitoring of the kinetics of translocation. Inner membrane vesicles containing wild-type SecYEG were found to translocate proOmpA with a turnover of 4.5 molecules proOmpA/ SecYEG complex/ minute and an apparent K_m of 180 nM. Although *in vitro* translocation can be driven by the hydrolysis of ATP alone, the efficiency is enhanced by the presence of a PMF [93, 177]. This effect of the PMF was also detected by the spectroscopic translocation assay. The turnover number was reduced more than 3-fold upon the dissipation of the PMF while the apparent K_m of the translocase for the precursor was barely changed.

The real-time spectroscopic translocation assay can be a useful tool for future research on the translocase. The assay gives accurate information on the initial rate of translocation, which is difficult to estimate in the classical protease protection assay. In addition, the method can be easily adapted to other preprotein substrates and might be a first step towards high throughput automation of the translocation reaction.

The PrlA4 mutation increases the activity of the translocase

Secretory proteins are synthesized as preproteins with an N-terminal signal sequence. The signal sequence is important for the targeting of the preprotein to the translocation site and, as a consequence, defective signal sequences can lead to severe protein translocation defects. In the past this property has been used to identify components that are involved in bacterial protein secretion. Screens for mutants that allowed translocation of preproteins with a defective signal sequence resulted in the identification of the genes for SecY (*prlA*), SecE (*prlG*) and SecA (*prlD*). Later, such signal sequence suppressor mutations were also identified in SecG (*prlH*). PrlA4 [250] is one of the strongest *prlA* mutants. As has been observed for other *prl* mutants [185], PrlA4 does not only increase the translocation of preproteins with aberrant signal sequences, but also of preproteins with functional signal sequences [184]. When saturated with proOmpA, the turnover rate of PrlA4 was almost 10-fold increased as compared to wild-type SecYEG and barely affected by dissipation of the PMF (Chapter 2)[181]. The *prlA4* mutation also relieves defects caused by aberrant structural elements that are not part of the signal sequence, like the fluorophore at the +4 position of proOmpA (chapter 2).

PrlA4 is a double mutant, containing a F286Y substitution in TMS 7 and an I408N substitution in TMS 10. The ability to suppress the negative effects of defective signal sequences results from the I408N substitution [267]. On its own, I408N dramatically increases the activity of the translocase (Chapter 3), but this mutation cannot be stably maintained without second site mutations as F286Y or S188L in TMS 5 [266]. Separate overexpression of SecY(I408N)EG severely reduced the expression level of the SecYEG complex and caused a slower migration of SecY on SDS-PAGE (Chapter 3). Since the net charge is not affected by the mutation, the aberrant migration points at an altered conformation of SecY. Reintroduction of the F286Y mutation restored the expression level to the level of wild-type SecYEG, but did not reverse the altered mobility on SDS-PAGE. *Prl* mutants are more thermolabile in detergent solution than the wild-type SecYEG complex, and readily dissociate upon prolonged incubation at 37°C [183]. The low expression of SecY(I408N)EG compared to PrlA4 suggests a reduced stability of the mutant SecYEG complex, but the SecY(I408N)EG and PrlA4 complex do not differ significantly in thermolability in detergent solution ([183], Chapter 3). It is also important to note that the thermolability is even not manifested when the complexes are present in the lipid membrane (Chapter 3). This suggests that these mutant SecY and SecE proteins normally interact, provided that they are retained in their native lipid environment. We therefore conclude that

the F286Y mutation restores the expression of PrlA4 by another mechanism than just increasing the (thermo-)stability of the PrlA4 complex.

Although it is generally assumed that signal sequence suppression does not result from a direct restoration of signal sequence recognition, the mechanism of signal sequence suppression is still not fully understood. It has been proposed that *prl* mutations alter the gating properties of the protein-conducting channel via relaxation of the SecY-SecE interaction [183], but with the current insight in the structure of the SecYEG complex it is unclear how this could be achieved. The crystal structure of the homologous *M. jannaschii* Sec61 complex shows multiple sites of interaction between the Sec61α (SecY) and Sec61γ (SecE) subunits [50]. Many of the *prlA* mutations are located in the internal site of a funnel like cavity that was proposed to form the protein conducting channel of the translocase [50]. In a model of the *E. coli* complex the *prlA* mutations in SecY point away from the sites of contact with TMS 3 of SecE [132]. This suggests that the *prlA* mutations alter the overall SecYEG conformation instead of directly affecting the SecY-SecE interaction.

According to the proofreading model, *prl* mutations affect a mechanism that prevents preproteins with aberrant signal sequences to enter the translocation pore [266]. In agreement with this model, van der Wolk *et al.* attributed the *prl* phenotype of PrlA4 to its increased SecA binding affinity, which stabilizes the SecA-preprotein complex at the membrane resulting in an increased efficiency of the initiation of translocation [184]. As expected, the I408N mutation was found to be responsible for the increased SecA binding affinity of PrlA4. It causes a four times higher affinity for SecA (K_d 0.8-1 nM) as compared to the wild-type SecYEG complex (K_d 4 nM) (Chapter 3). The SecY(F286Y) mutant appeared to be a very poor binding partner for SecA (K_d 8 nM) and its low affinity for SecA was accompanied with a severely reduced translocation and SecA-ATPase activity. In the complete PrlA4 mutant, the F286Y substitution partially reversed the effects of the I408N mutation. The second site mutation reduces the strength of the I408N mutation of PrlA4 and lowers the SecA binding affinity. Biochemical analysis indicates that the interaction of SecA with the SecYEG complex occurs at least via SecY. The first TMSs [98, 100] and the 5th and 6th cytoplasmic loops of SecY [101, 102] seem to be important for the interaction, but the exact identity of the SecA interacting domain(s) is still elusive. As the F286Y and I408N mutation are not located in the regions implicated in SecA binding, they might alter the conformation of the SecYEG complex into a state that is more or less favourable for the interaction with SecA.

Interestingly, the I278C *prl* mutation in TMS 7 has also been reported to increase the SecA binding affinity [185]. In the crystal structure of the *M. jannaschii* Sec61 complex, the residues corresponding to *E. coli* I278 and I408 are located in a ring-like structure that is lined with six hydrophobic residues [50]. The isoleucine at position 278 is a hot-spot for *prlA* mutations and all substitutions identified so far involve a change into a polar residue (S, N, C or T) [266]. The I191S [266] and the I187 *prl* mutations are located in the same ring, but besides their capability to suppress signal sequence defects little is known about their phenotype. The ring forms a constriction in the middle of the putative protein conducting channel (Fig. 1) and was proposed to form a seal around passing polypeptide chains [50]. Van den Berg *et al.* suggested that *PrlA* mutations in ring residues stabilize the "open state" or facilitate the widening of the channel [50]. Opening or widening of the channel could also alter the accessibility of the SecA binding domain(s) and thereby increase the affinity for SecA (see below).

Of the "pore ring" mutations only the I408 mutation is known to be stabilized by "second site mutations" (S188L or F286Y) [266]. Comparison of these mutations with the corresponding residues of *M. jannaschii* Sec61α provides insight in their position in respect to the *prlA* mutation (Fig. 2).

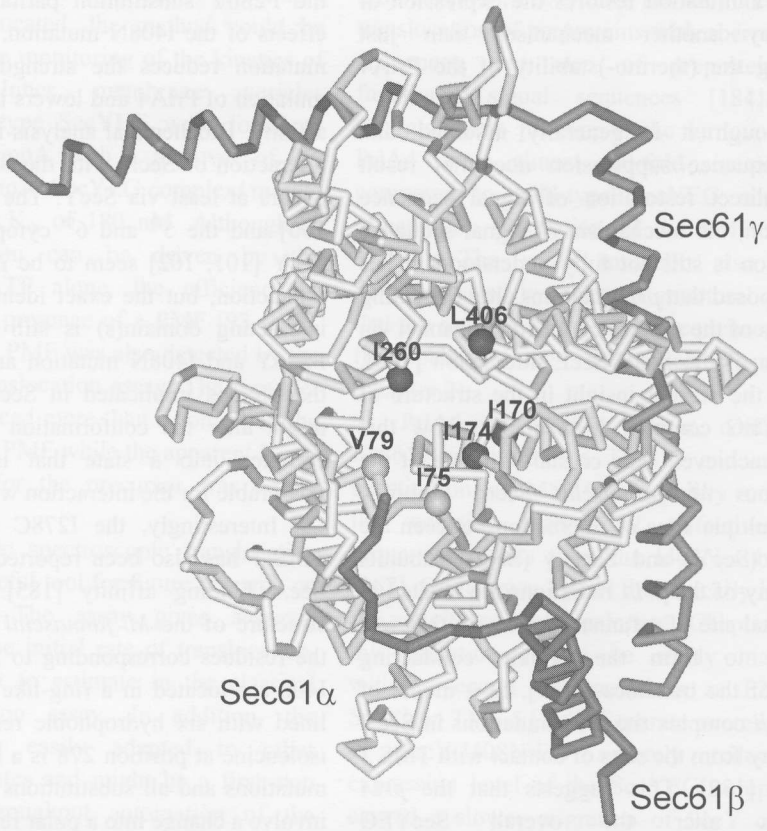

Fig. 1. Position of *prlA* mutations in the pore ring of the *M. jannaschii* Sec61 complex. View from the cytosolic side of the complex. Sec61α (homologous to *E. coli* SecY) is shown in light grey, Sec61γ (homologous to *E. coli* SecE) and Secβ are shown in dark grey. The Cα atoms of the pore ring residues corresponding to *E. coli prlA* mutations (I187, I191, I278 and I408) are indicated by black spheres, the Cα atoms of the pore ring residues for which no *prlA* mutant have been described are indicated by grey spheres. Pdb entry 1RHZ [50].

The residue S188 (corresponding to *M. jannaschii* G171) is located close to I408 (*M. jannaschii* L406) and points towards the inside of the putative channel. The substitution to leucine might compensate for the reduced hydrophobicity of the pore ring as a result of the I408N mutation. The residue corresponding to F286 (*M. jannaschii* N268) is located more distally at the periplasmic site of the pore ring. Although this residue is located in a helix that lines the putative channel (TMS 7), it does not point towards the inside of the cavity. Second site mutations are also found in combination with another strong *prlA* mutant in TMS 10 (L407R, *M. jannaschii* L405). This *prl* mutation is accompanied by either A277E (*M. jannaschii* V259) in TMS 7 or V411G (*M. jannaschii* S409) in TMS 10 [266]. In the *M. jannaschii* Sec61α structure, these residues are located at the inside of the putative channel at the same height and one helical turn above the *prl* mutation, respectively. The position and nature of the second site mutations do not provide clear insight in how these mutations compensate the effects of the *prlA* mutation. Small differences between the *E. coli* and *M. jannaschii* complex or a different conformation due to the (*prl*) mutations could, however, alter the position of the residues. It should also be kept in mind that the position of the residues

might change upon the interaction with SecA or during protein translocation. Therefore, it is not possible at this stage to explain the *prl* phenotype on the basis of the location of the mutations in the structure of Sec61α protein. We also observed that the SecY(I408N) mutation changes the protease accessibility of a synthetic translocation intermediate, which would be in line with the suggestion that the conformation of the active translocase complex is altered by the *prl* mutation (appendix of Chapter 3).

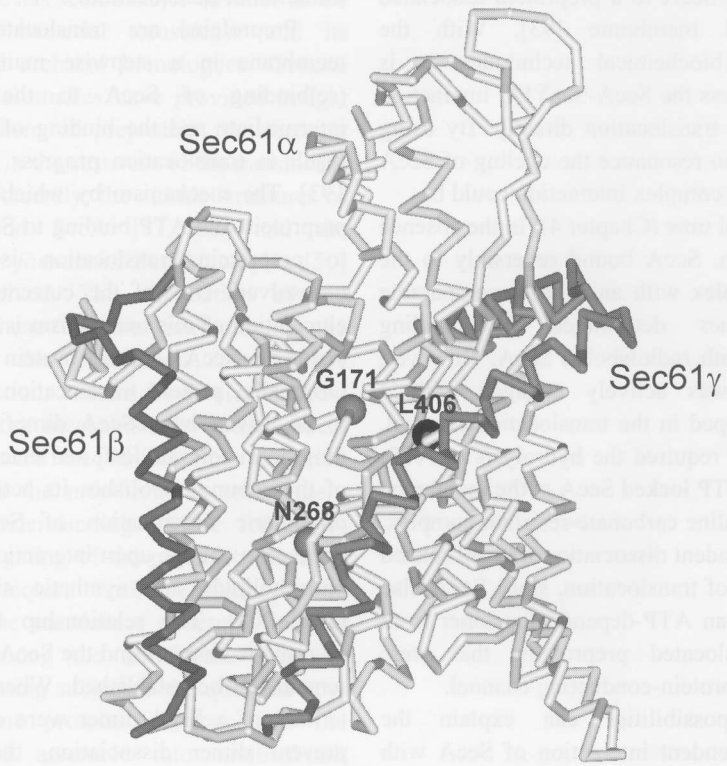

Fig. 2. Position of the second site mutations of *E. coli* SecY(I408N) in the *M. jannaschii* Sec61 complex. Sec61α (homologous to *E. coli* SecY) is shown in light grey, Sec61γ (homologous to *E. coli* SecE) and Secβ in dark grey. The Cα atoms of the residues corresponding to the *prlA* mutation (*E. coli* I408N in TM10) and second site mutations (*E. coli* S188L and F286Y in TM5 and TM7, respectively) indicated by black and grey spheres respectively. Pdb entry 1RHZ [50].

The dynamics of the SecA reaction cycle

At the onset of translocation, SecA is associated with the SecYEG complex, but during its reaction cycle SecA is thought to distribute between cytosolic and membrane-associated states. This "SecA cycling" model is based on the appearance of protease protected domains of SecA [188, 193] and analysis of the binding and dissociation of SecA to a preprotein associated with a lipid membrane [93]. With the conventional biochemical techniques it is difficult to assess the SecA-SecYEG interaction during protein translocation directly. By using surface plasmon resonance the cycling of SecA at the SecYEG complex interaction could be followed in real time (Chapter 4). In the absence of a preprotein, SecA bound reversibly to the SecYEG complex with an affinity comparable to the values determined by binding experiments with radiolabeled SecA. However, when SecA was actively engaged with a preprotein trapped in the translocation channel, release strictly required the hydrolysis of ATP. Depletion of ATP locked SecA at the membrane in a stable alkaline carbonate-resistant complex. The ATP dependent dissociation was not related to completion of translocation, since SecA also dissociated in an ATP-dependent manner from partially translocated preproteins that were trapped in the protein-conducting channel.

Several possibilities can explain the nucleotide-dependent interaction of SecA with the preprotein:SecYEG complex. SecA may interact with high affinity with the exposed non-translocated polypeptide domains of the preprotein. In this way, SecA may be anchored more stably to the SecYEG pore complex such that ATP hydrolysis is needed to dissociate the SecA-preprotein interaction [93]. Alternatively, the activated preprotein containing SecYEG complex might exist in a different conformational state that permits a tight interaction with SecA. Interestingly, *prlA* mutations located in the "pore-ring" of the SecYEG complex also confer an increased affinity for SecA [185] (Chapter 3). It is possible that these mutations alter the conformation of the SecYEG complex into a state that resembles the preprotein containing SecYEG complex. This scenario would be consistent with the hypothesis that *prl* mutations stabilize the open state of the translocation channel [50]. The activated conformation of the SecYEG complex could stabilize SecA at the membrane, resulting in a reduced the rejection rate of preproteins upon initiation of translocation.

Preproteins are translocated across the membrane in a stepwise manner. Both the (re)binding of SecA to the translocation intermediate and the binding of ATP to SecA result in translocation progress [93, 173, 188, 193]. The mechanism by which the energy of preprotein and ATP binding to SecA is coupled to preprotein translocation is still largely unresolved. One of the current issues in the elucidation of this mechanism is the oligomeric state of SecA during protein translocation. Originally, protein translocation was proposed to be driven by a SecA dimer [81]. SecA is dimeric in solution [80] and inactivation of one of the subunits abolishes its activity [81]. The oligomeric organization of SecA, however, appears to change upon interaction with anionic phospholipids and synthetic signal peptides [200, 201]. The relationship between these oligomeric changes and the SecA reaction cycle remains to be established. When the carboxy-termini of a SecA-dimer were cross-linked to prevent dimer dissociation, the cross-linked form of SecA was as active in protein translocation as wild-type SecA (Chapter 5). This observation demonstrates that dimer dissociation is not a critical element of the catalytic cycle of SecA.

The availability of both carboxy termini of the SecA dimer appeared, however, to be critical for the translocation of preproteins associated with the molecular chaperone SecB. Crystallographic and biochemical studies have shown that a SecA dimer binds to a negatively charged surface on the SecB tetramer via its extreme carboxy-termini [44, 47, 48]. The fold of the carboxy-termini is stabilized by a zinc ion that is coordinated by three cysteine residues

and a histidine residue [47, 48]. As the oxidized SecA dimer was unable to support the translocation of preproteins associated with SecB, disulfide bridge formation between the cysteines probably abolishes the surface that interacts with SecB.

Concluding remarks

The last decade has seen a major advance in the study of the bacterial translocase and its eukaryotic and archaeal homologues. Genetic and biochemical studies have provided insight in the mechanism of preprotein translocation and the insight in the structure of the translocase is increasing. Recently, the atomic structures of SecB [28] and SecA [87, 88] and the *Methanococcus jannaschii* Sec61 complex [50] have been solved. The elucidation of the structure of these subunits attributes to the understanding the translocase, but many intriguing questions remain for future research. For instance, the mechanism by which SecA generates a macro mechanical force to drive proteins across the membrane is still largely unresolved. Understanding of the formation, activation and gating of the protein-conducting channel will require further studies on the dynamics of the translocase structure, and the interactions among the translocase subunits. Another interesting subject is the question how inner membrane proteins integrate into the membrane and assemble into multisubunit complexes, and how this process is linked to the incorporation of non-protein cofactors.

The recently solved crystal structures are a step towards the answering of these questions. However, the conformation, stochiometry and subunit assembly of the translocase may change in response to different functions and substrates. Detailed information of the various states of the translocase will be essential to obtain insight in the dynamics of the translocase. As elucidation of the structure of the translocase 'caught' in the various stages of its reaction cycle will be difficult, biochemical and biophysical studies will remain important to relate structure to function. Together these techniques will make possible a detailed knowledge about the way in which proteins cross and integrate into the cytoplasmic membrane.