Chapter 6

Summary and discussion
Introduction – role of the SecA motor protein in translocation

The preprotein translocase is a multi-subunit membrane complex that facilitates the movement of proteins across or into the membrane. In the bacterial kingdom, the main translocation pathway is provided by the Sec-translocase (for review see Osborne et al., 2005), although in some organisms the twin arginine translocase (TAT) pathway for folded proteins seems to be prevalent (Widdick et al., 2006). In Escherichia coli the translocase consists of a protein conducting channel (PCC) formed by the SecY, SecE and SecG proteins, and several associated proteins, among which the motor protein SecA, an ATPase (reviewed in de Keyzer et al., 2003) (Figure 1). In post-translational protein translocation, the preprotein associates with the cytosolic chaperone SecB, during or just after its synthesis. SecB is a secretion-dedicated chaperone that fulfills two functions: 1) to maintain preproteins in a translocation competent state (Randall et al., 1986; Collier et al., 1988) and 2) to target preproteins to the SecYEG bound SecA at the cis-side of the membrane (Hartl et al., 1990; Fekkes et al., 1999). Upon a successful interaction between the preprotein and SecA, multiple cycles of ATP binding and hydrolysis by SecA result in the progressive movement of preprotein segments through the PCC (Schiebel et al., 1991; van der Wolk et al., 1997). The proton motive force (PMF) provides an additional energy source for translocation (Driessen, 1992).

How does post-translational translocation occur? In bacteria, SecA functions as a cis-acting motor, but for protein translocation into the ER and mitochondria, luminal and matrix Hsp70 proteins act as trans-acting motor proteins. Two main mechanisms have been proposed: the power-stroke and the Brownian ratchet (for review see Alder et al., 2003). In the Brownian ratchet model the polypeptide is directed through the translocation pore towards the trans-side by spontaneous movement, i.e., diffusion. At the trans-side, Hsp70 proteins specifically trap the polypeptide to provide unidirectionality to the process. The power stroke model implies that Hsp70 exerts an active pulling force on the protein which in addition to a possible trapping, facilitates the translocation of the protein (Chapter 1).

How do these models relate to translocation mechanism facilitated by cis-acting motors like SecA? In the power-stroke model, the binding and/or hydrolysis of ATP result in conformational changes in SecA that cause the mechanical movement of the preprotein binding domain in the direction of the PCC. This would push the preprotein through the PCC in distinct steps. Intuitively, a Brownian ratchet mechanism for cis-acting motor proteins seems less likely as trapping of the diffusing polypeptide at the cis-side appears less effective as compared to trans-trapping. Since preproteins are transferred from SecB to SecA in a completely unfolded state (Chapter 4), it seems possible that the spontaneous (Brownian) movement of a polypeptide in the PCC could be driven by a folding event at the trans-side of the membrane. In such a model, the trapping of the preprotein by SecA at the cis-side would allow more time for refolding of the polypeptide at the trans-side, and this would result in an acceleration of directional translocation. Indeed, in the absence of SecA a spontaneous movement of the polypeptide (protein intermediates) in the PCC was observed (Schiebel et al., 1991; Arkowitz et al., 1993), but without the input of energy (PMF) or SecA and ATP, this spontaneous movement does not result in the complete translocation of the
preprotein. However, several preproteins translocate in a SecB-independent manner, and their folding state prior to translocation has not been determined. Possibly, the initial insertion of the signal sequence, as a loop-like structure with the N-terminal mature domain into the PCC, suffices to partially unfold the preprotein.

The power stroke model suggests that the protein is pushed through the PCC in discreet steps. The step size must be mechanistically coupled to a conformational change in SecA. On the other hand, with a Brownian ratchet mechanism, no defined step size is expected and translocation progress will depend mostly on the folding characteristics of translocating polypeptide segments. Since discrete translocation intermediates have been observed during the in vitro translocation of proOmpA, translocation has been proposed to be the result of an active pushing by SecA. Herein, a catalytic cycle of the SecA ATPase causes the translocation progress of about 40 amino acids in two distinct sub-steps that result from the (re-)binding of SecA to the translocating polypeptide chain and the binding of ATP to SecA (Schiebel et al., 1991; van der Wolk et al., 1997). The power-stroke mechanism appears consistent with the structure of SecA that reveals the presence of a DEAD motor domain (Vrontou et al., 2004). In DNA helicases as PcrA, the DEAD motor domain is involved in the ATP-dependent mechanically unwinding of the dsDNA (Ye et al., 2004). The solution structures of SecA, however, do not yet reveal large conformational changes of SecA domains induced by nucleotide binding. Various speculations exist on possible movements of the preprotein binding domain (PBD) by comparison of different crystal structures of SecA (Osborne et al., 2004). However those “movements” appear to be too small to account for a power-stroke that drives the translocation of polypeptide segments of about 20 amino acids in length.

Unequivocal experimental discrimination between the power stroke and Brownian ratchet models seems difficult, as the observed step size could be the result of both pushing and trapping events. Also other subunits than SecA, may participate in translocation and affect the step size. A more detailed analysis, especially on how the physicochemical properties of polypeptide segments influence the translocation behavior, will shed more light on the molecular mechanism of preprotein translocation.
Summary and discussion

Kinetics of translocation

How are the kinetics and energetics of translocation affected by preprotein length?

An unresolved question concerns how is the kinetics of translocation affected by the length of a preprotein? Since translocation of proOmpA seems to occur in consecutive steps of 40 amino acids (Schiebel et al., 1991; van der Wolk et al., 1997), shorter polypeptides will require less time to be translocated as compared to longer polypeptides. This is correct only when the actual translocation reaction is rate determining for the overall process, while other factors such as preprotein targeting, polypeptide amino acid composition and folding of the preprotein at the trans-side only marginal affect the translocation kinetics. To study the effect of preprotein length on the translocation rate, we constructed a series of proOmpA derivatives up to 4-fold its original length (Chapter 2). ProOmpA consists of two very distinct domains: an N-terminal hydrophobic β-barrel domain and a C-terminal hydrophilic periplasmic domain (Ried et al., 1994; Pautsch et al., 1998). To increase the length of proOmpA, multiple copies of the C-terminal periplasmic domain were added at the C-terminus of proOmpA. This resulted in a protein with one (wild type, P1), two, four, six and eight repeats (P2, P4, P6 and P8, respectively). To assay SecA-dependent translocation only, the PMF was dissipated by means of ionophores. The study demonstrated that the translocation rate increased with decreasing preprotein length, but more importantly, remained essentially constant when expressed as number of amino acids translocated per time unit. Since the translocation ATPase activity of SecA was similar for all preprotein constructs, our data imply that the ATP consumption per translocated preprotein increases linearly with the length of the preprotein. These observations deviate from an earlier study in which the translocation of proOmpA was compared with a C-terminally truncated proOmpA-derivative (Bassilana et al., 1992). Herein, the translocation rate was identical for the wild-type and the truncated version, whereas the SecA ATPase activity was found to be higher for the wild-type proOmpA. The latter was attributed to the presence of a disulfide bond in the C-terminus of proOmpA, which obstructs translocation and causes an uncoupled SecA translocation ATPase activity. In our studies, a single cysteine proOmpA variant was used that was labeled with a fluorescent maleimide derivative. Therefore, uncoupling because of a disulfide bond can be excluded. Moreover, in the studies of Bassilana and coworkers (Bassilana et al., 1992), translocation rates were determined in the presence of PMF, while the ATPase activity was measured in the absence of PMF. Also, it is not known if these proOmpA derivatives differ in PMF-dependence of translocation. Therefore, no general conclusion about the kinetics and energy requirements of translocation can be derived for the aforementioned study. Our studies show that there is a constant rate of translocation, implying that, at least in vitro, protein targeting and translocation initiation are not rate limiting for the overall reaction, at least for the proOmpA variants studied. This is in accordance with the notion that for SecA-dependent translocation, the actual translocation event itself is rate-determining.

1 Obviously, this assumption does not hold for polypeptide segments that contain long stretches of hydrophobic amino acids that may function as a transmembrane domain. In that case, the protein will insert into the membrane instead of being translocated vectorially across the membrane.
How do these data fit into the proposed models of SecA functioning? A constant translocation rate and ATP consumption per amino acid suggest that a fixed length of protein is translocated during a single catalytic cycle of SecA. This is consistent with a stepping model of translocation favoring the power stroke mechanism. However, since the preproteins are relatively similar in amino acid composition, at least as far as the repetitive C-terminal regions is concerned, a trapping mechanism cannot be ruled out. Therefore, it would be of interest to systematically analyze how a different amino acid composition affects the translocation.

**How does the protein folding state affect the translocation kinetics and energetics?**

Another question which was addressed in this work is how the folding status of the preprotein mature domain affects translocation? A generally accepted view is that preproteins are translocated in an unfolded conformation (Randall *et al.*, 1986; Matouschek, 2003). Stable folding of the mature preprotein domain severely compromises translocation, and folded subdomains of the preprotein can lead to the generation of translocation intermediates trapped in the PCC (Arkowitz *et al.*, 1993). Therefore during post-translational translocation, protein folding prior to translocation is a potential problem the cell needs to deal with. To prevent, or slow down folding of preproteins, two main strategies are used. The signal sequence has been shown to slow down the folding of the preprotein mature domain (Park *et al.*, 1988; Liu *et al.*, 1989; Beena *et al.*, 2004). Moreover, the secretion-dedicated chaperone SecB also affects the folding of preprotein (Liu *et al.*, 1989; Lecker *et al.*, 1990). SecB binds early preprotein folding intermediates in a stochiometric complex and thereby stabilizes their unfolded state (Lecker *et al.*, 1990). Previously, it has been suggested that the degree of preprotein folding affects the SecA dependence of translocation (de Cock *et al.*, 1998). In these experiments, preMBP (precursor of maltose binding protein) derivatives were employed containing point mutations in the mature region that were shown to either stabilize or destabilize the folding state of the mature domain (Chun *et al.*, 1993). The SecA requirement was found to increase with the stability of the folded state, indicating that part of the energy requirement for translocation is utilized for active unfolding. This was a rather surprising observation, as preMBP translocation is known to be strictly dependent on SecB (Collier *et al.*, 1988; Weiss *et al.*, 1988), and SecB is expected to be a major determinant of the preMBP folding. We have studied the folding and translocation of the preMBP variants using a well defined in vitro translocation system and saturating amounts of purified mutant forms of preMBP (Chapter 3). The effect of mutations in the mature domain of preMBP appeared rather marginal as compared to the impact of the signal sequence and SecB on preprotein folding. Consequently, the SecA-dependence and ATP-requirement for the translocation of the preMBP mutants was indistinguishable as compare to the wild type. How can this be reconciled with the results of de Cock and Randall (de Cock *et al.*, 1998)? It is important to stress that those experiments were performed with a crude in vitro system using a cellular lysate and radiochemical amounts of preprotein. Lysate components, such as other chaperones, may affect the translocation of the preMBP variant differently. Because of the use of non-saturating concentrations of preprotein it is also difficult to discriminate between mechanistic differences in the energy requirement and kinetic effects caused by differences in affinity. Also, it is uncertain if with the used in vitro translation and translocation approach, all preMBP variants were translocated post-translationally. Our data suggest that for post-translational translocation of a strictly SecB-dependent preprotein (like MBP), unfolding of the
mature domain prior to or during translocation through the PCC is not a rate determining step. As will be discussed below, our observations are consistent with findings that SecB stabilizes MBP in a completely unfolded state (Chapter 4). These data suggest that SecB-dependent proteins require only a limited energy input for protein unfolding prior to translocation. However, many preproteins are translocated in a SecB-independent manner. Possibly, the SecA-mediated unfolding is a more significant issue with such proteins. In this respect, recent evidence shows that the Sec-translocase is able to translocate the tightly folded human cardiac Ig-like domain I27 fused to the C-terminus of proOmpA (N. Nouwen, unpublished data). Moreover, stably unfolded titin I27 domain was shown to translocate with faster kinetics and with less ATP consumption than the native prefolded protein. This implies that the ATP requirement for translocation is dependent on the preprotein folding state.

Are there any other features of a preprotein that may affect the translocation kinetics and energetics? For protein translocation into mitochondria it was shown that the length of the signal sequence determines mechanism as well as the rate of initiation of translocation (Matouschek et al., 1997; Huang et al., 1999). Also, the structure of the N-terminus of the mature protein seems to affect the translocation rate presumably because of different requirements of protein unfolding before translocation (Huang et al., 1999; Wilcox et al., 2005). In particular, β-sheet structures that form intimate contacts are unfavorable. The mitochondrial system can actively unfold proteins (Huang et al., 1999). As mentioned before, a recent study on the translocation of the tightly folded titin domain (N. Nouwen, unpublished data) suggests that the bacterial translocase is also equipped with unfolding activity. Thus it would be of interest to determine how the secondary structure of the mature N-terminus affects bacterial preprotein transport. Since SecB stabilizes preproteins in a completely unfolded state (Chapter 4; see below) again a distinction should be made between SecB-dependent and independent proteins. For the latter group, alkaline phosphatase or ribose binding protein might be interesting candidates, and the question arises if these proteins require more ATP for translocation than the SecB-dependent proteins.

**Optical tweezers measurements**

Biochemical studies have resulted in a major insight into the mechanism of bacterial protein translocation. However, in the biochemical analysis, the values obtained from bulk assays reflect averages of all active translocases and are therefore not informative for movements, forces and energy requirements for single translocation reactions. Single molecule studies can reveal explicit information on, for instance, the step size of translocation. To analyze the molecular mechanism of translocation at the single molecule level, we have applied the optical tweezers technique (Appendix). This method uses the ability to trap small particles, like beads, by a laser beam (Ashkin, 1998). The optical tweezers setup comprises two beads, one of which is in a fixed position and second one is trapped in the laser beam. The ends of a protein or other macromolecule under study are attached to these two beads. During the measurement, changes in the distance between beads as well as force applied can be measured with nm and pN precision. Optical tweezers were successfully employed to study piconewton forces associated with molecular motors like myosin.
and kinesin and the elasticity of biopolymers like protein or DNA (for review see Mehta et al., 1999). For the optical tweezers measurements described in this thesis, we used a micropipette and DNA linker to attach the measured molecule to a bead in the laser trap (Figure 2). The advantage of using a DNA linker and micropipette is that it prevents effects of the laser beam and the surface on the studied molecule. However, since optical tweezers had not been used before to study the translocation reaction, the entire setup had to be designed and validated.

The effect of SecB on protein folding

The optical tweezers setup turned out to be an excellent platform to analyze the interaction between the SecB chaperone and secretory proteins. SecB maintains preproteins in a translocation competent state, which is a non-aggregated loosely folded conformation with possibly native like secondary structure and lack of stable tertiary structure (Lecker et al., 1990; Breukink et al., 1992). The exact preprotein conformation is, however, not known. SecB is a homotetramer with four putative polypeptide binding sites (Zhou et al., 2005) that bind only one preprotein. The question arises how the four seemingly independent peptide binding domains are used to bind single preproteins. Are preproteins wrapped around the SecB-tetramer as a continuous binding frame (Lecker et al., 1989), or are separate segments of the preprotein bound to the four putative peptide binding sites? To study the interaction between SecB and preproteins in more detail, optical tweezers were applied (Chapter 4). Because of its strict SecB-dependence for translocation, maltose binding protein (MBP) was used as a model protein (chapter 3, Randall et al., 1986; Collier et al., 1988; Kumamoto et al., 1988).

For the optical tweezers experiments, a single MBP molecule was stretched between two beads. One of the beads was immobilized on a pipette, while the other bead was placed in the optical trap. Force-extension curves obtained by moving the pipette bound bead away from the optical trap, showed the unfolding of MBP as a sudden change in the length of the protein at a specific force, i.e. ~25 pN. Relaxation of the protein by the reverse movement in the optical trap resulted in the refolding of MBP. The MBP unfolding force is in line with the values reported for tenascin,
spectrumin and ribonuclease (Oberhauser et al., 1998; Rief et al., 1999; Cecconi et al., 2005). Incidentally, stable unfolding intermediates were found. Interestingly, by the use of a construct with four covalently linked MBP molecules (MBP4), the presence of such intermediates during unfolding could be more accurately detected. This partially unfolding at around 10 pN, results in a distinct change in the contour length of the protein. Simulation of the MBP unfolding during mechanical pulling by steered molecular dynamics (SMD) suggests that the early unfolding event is due to unwrapping of the C-terminal α-helices from the MBP surface. The predicted length of the remained 280 amino acid core, 90 nm (assuming 0.32 nm per amino acid) is in good agreement with the length of the stable intermediate as determined by the optical tweezers measurements (92 nm). This intermediate likely corresponds to the β-strand rich core domain that requires higher forces for unfolding. Interestingly, mutations which disturb the MBP folding also localize to the same core domain of MBP (Chun et al., 1993).

Since MBP was shown to undergo reversible cycles of unfolding/refolding, the effect of the SecB chaperone on the folding could be studied. Interestingly, when SecB was added to a refolded MBP, no difference in force characteristics was observed during the unfolding transition as compared to MBP only. This implies that SecB does not promote unfolding. However, once the protein was unfolded, no refolding occurred in the presence of SecB. This suggests that SecB prevents protein refolding only when it can bind to an unfolded protein. Interestingly, the lack of changes in force or extension in the presence of SecB suggests that SecB may interact with the protein at forces that are below the detection limit of the optical tweezers setup. This interaction may be very transient.

Our observations with the tweezers may explain the biological function of SecB. In the cell the folding of newly synthesized protein is delayed by the presence of the signal sequence allowing SecB to bind (Park et al., 1988). This interaction involves fast binding and unbinding event as suggested previously in kinetic studies (Fekkes et al., 1995). This process is termed kinetic partitioning where the rate of SecB-preprotein interaction is determined by a kinetic partitioning between the rate of protein folding and the rate of association with SecB (Khisty et al., 1995; Topping et al., 1997). The observed strong, all-or-nothing, effect of SecB on protein folding suggests that the protein is targeted and transferred to SecA in an unfolded state. This implies that for SecB-dependent proteins, there is little need for protein unfolding in order to effect translocation. In line with this conclusion is the observation that mutations that stabilize or destabilize the fold of the mature MBP domain have little effect on the energy requirement for translocation (Chapter 3). Importantly, the MBP mutants remain strongly dependent on SecB for translocation, and it would be of interest to analyze similar types of mutations in preproteins that do not require SecB for translocation.

The optical tweezers setup now provides an excellent platform for protein folding studies. Therefore, it would be of concern to study the interaction between MBP and other chaperones, such as DnaK/DnaJ or GroEL/ES. These chaperones use the energy from ATP for protein release and protein folding, and it will be of interest to determine how nucleotides affect the folding in the optical tweezers setup.
Protein translocation on the single molecule level

Optical tweezers have been employed to monitor active processes such as the moving of kinesin on microtubule (Svoboda et al., 1993) or the transcription of DNA by RNA polymerase (Yin et al., 1995; Wang et al., 1997). This suggests that low forces used in the optical tweezers measurements are compatible with these active biological entities. Indeed, it was shown that this method could be used with live bacteria for the observations of DNA-uptake in Bacillus subtilis (Maier et al., 2004) as well as the pilus retraction and elongation by Neisseria gonorrhoeae (Maier et al., 2002).

The optical tweezers setup would be excellently suited to measure the step size of protein translocation. In previous studies (Schiebel et al., 1991; van der Wolk et al., 1997), the step size has been estimated from the translocation progress of translocation intermediates using SDS-PAGE for visualization. The accuracy of such a measurement is not high, and the question remains as to whether translocation occurs with steps of a fixed or variable size. Single molecule studies could be applied to address this and the following questions: Is this a real step size of a single translocation reaction? Does translocation occur indeed in the step-wise manner? What is the true mechanism of translocation? Is it the result of active pushing by the motor protein SecA or is the passage through the channel solely driven by the Brownian motion, while SecA prevents the retrograde movement of protein (Mitra et al., 2006)? In an attempt to analyze protein translocation by optical tweezers technique, we have utilized the in vitro translocation system consisting of inner membrane vesicles (IMVs) with embedded SecYEG translocases (Chapter 5). In the presence of SecA, SecB and ATP, a preprotein can be translocated into the vesicle. In the optical tweezers setup, IMVs charged with a translocation intermediate were bound to the fixed bead, while the laser-trapped bead contained a DNA-linker that at its free end was biotinylated in order to create a tether with the C-terminal part of translocation intermediate. The C-terminus of the protein is exposed to the outside of the vesicles and contained a biotin label. By means of streptavidin, a tether can be generated between the DNA and the protein. For these measurements a variant of proOmpA was used with eight repeats of the periplasmic domain (proOmpA-P8) (Chapter 2). The increase in length of the protein substrate should extend the measuring time and because these domains are repetitive units, a reproducible pattern is expected in a single molecule translocation measurement. Unfortunately, major difficulties were encountered in discriminating between specific and non-specific interactions, and it has not been possible to detect a specific signature of the protein translocation reaction. Several experimental modifications may be introduced to overcome the problem of non-specificity. It is possible that the N-terminal hydrophobic β-barrel domain of proOmpA-P8 is responsible for observed non-specific interactions with IMVs. Thus, employing a more hydrophilic protein such as preMBP might decrease the non-specific interactions with IMVs. Additionally, with a soluble protein it would be possible to covalently attach a DNA linker to the C-terminus of the preprotein, resulting in a setup where the translocation could be started during the measurement itself. Apart from the difficulty to ascertain a specific interaction, the lack of a signature could be caused by many different features of the system, like folding of the protein in solution before the translocation reaction.
Moreover, folding of the protein prior to translocation may hinder the detection of translocation. Under those conditions, the unfolding concomitantly with translocation may not result in a distinct change of the inter-bead distance, and thus translocation may remain undetected. For this reason, preMBP may again provide the solution to this problem as the SecB bound MBP is entirely unfolded, which at least defines the initial state of the preprotein prior to targeting. Although we used 100 nm IMVs that are more rigid than standard isolated IMVs (150-400 nm), the deformation of the IMVs during translocation needs to be considered as technical obstacle in the measurements. Because of the complexity of the optical tweezers system other methods should be considered as well. In particular single molecule spectroscopy may provide a good alternative.

This thesis presents different approaches to reveal details of the preprotein translocation. The question whether SecA acts by active pushing or by trapping a Brownian movement of the preprotein remains unanswered, although the constant rate of translocation as observed in Chapter 2, favors a mechanistic coupling between the ATPase cycle and translocation progress. It is, however, conceivable that the two proposed mechanisms coexist, as recently hypothesized by Mitra and coworkers (Mitra et al., 2006). In their molecular peristalsis model, the translocating polypeptide is first trapped in a large cavity formed at the interface between SecA and the PCC. It is assumed that SecA essentially acts as a power stroke motor, wherein the binding of ATP to SecA is used on one hand to decreases the cavity size and volume and on the other hand, to open the PCC. Since these events would take place concomitantly, the actual polypeptide movement through the pore is mainly driven by a directed Brownian motion of the polypeptide, in effect by a volume exclusion of the protein initially trapped in the cavity. The full potential of single molecule measurements has not yet been realized, but the development and refinement of these sophisticated measurements, in combination with a further characterization of the translocase in bulk experiments, will likely yield further detailed insight in the molecular mechanisms of this biological nanomachine.