Chapter 7

Summary and concluding remarks

Introduction

SecA is the ATP-driven force generator for the Sec-dependent translocation of proteins across the inner membrane of *Escherichia coli*. Together with an integral membrane protein complex, consisting of the SecY, SecE and SecG proteins, SecA constitutes the translocase (reviewed in Wickner *et al.*, 1991; Driessen *et al.*, 1998). SecA is the precursor protein binding subunit, and the only ATPase involved in the translocation process. Translocase suffices for the reconstitution of the ATP- and proton motive force (Δp)-dependent translocation of precursor proteins *in vitro*. An additional complex of the SecD, SecF and YajC proteins is not essential for, but enhances the efficiency of, translocation (Duong and Wickner, 1997a). Precursor proteins are directed to the inner membrane by chaperones such as SecB and signal recognition particle (SRP).

ATP and the Δp are the energy sources that are required for protein translocation. ATP is essential for the initiation of translocation and is indispensable for protein export. *In vivo*, there is an absolute requirement for Δp as an energy source. *In vitro*, the Δp stimulates the rate of translocation (Driessen, 1992b) while it can drive the completion of protein translocation provided SecA is not associated with the non-translocated part of the precursor polypeptide chain (Schiebel *et al.*, 1991).

SecA-driven preprotein translocation

The high-affinity ATP-binding site on SecA

All bacterial SecA proteins identified to date are (Walker *et al.*, 1982). These ATP-binding sites are composed of the so-called Walker A and B sequence motifs. The Walker A motif is characterized by the presence of a hydrophobic β-strand, directly followed by a glycine rich loop (GxTGxG), the sequence GKT/S (the catalytic triad) and is ended by an α-helix (Walker *et al.*, 1982). The SecA sequence contains two of these putative NTP-binding motifs, one in the N-terminal part and one in the C-terminal half of the protein. For a number of NTP-binding proteins, the high resolution atomic structure has been determined (reviewed by Bourne *et al.*, 1991) and a molecular model has been proposed for ATP-binding via the Walker A motif (Story and Steitz, 1992; Yoshida and Amano, 1995; Chapters 1, 2 and 3). By site-directed mutagenesis of the conserved catalytic lysine, Lys^{106}, of the putative A motif of the high affinity ($K_{d,ADP}$ ~0.15 μM) NBS-I, we have shown that this sequence is indeed functionally needed for the translocation ATPase activity of the *Bacillus subtilis* SecA (Chapter 2). These findings were confirmed by Mitchell and Oliver (1993) for the *E. coli* SecA, who in addition demonstrated the presence of the C-terminally located NBS-II that is of low affinity ($K_{d,ADP}$ ~340 μM). The *B. subtilis* SecA is highly homologous to the *E. coli* enzyme, but ATPase mutants of the *B. subtilis* SecA protein are less toxic for the cell than the *E. coli* equivalent when expressed to high levels. This permits the isolation of the protein without having to resort to the formation of inclusion bodies and reactivation by renaturation. K106N SecA retained the ability to bind ATP, but could no longer hydrolyse the

manner. As a result, this SecA mutant is unable to support ATP-driven preprotein translocation *in vitro*. K106N SecA even prevents the ATP- and Δp-dependent chase of a partially translocated precursor (translocation intermediate) by wild-type SecA. These data suggest that the mutant SecA still forms a complex with the membrane translocation sites and with precursor protein intermediates but is unable to dissociate from these sites in an ATP-dependent manner. In *E. coli* SecA, the corresponding domain has been shown to fulfil an identical function (Economou *et al.*, 1995).

The catalytic residue in the Walker B motif (an aspartate that is located at the end of a hydrophobic β-strand) is mainly involved in the stabilization of the Mg^{2+} ion needed for ATP hydrolysis (Walker *et al.*, 1982). In NBS-I of SecA, this B motif is present as a tandem repeat. In Chapter 3 it is shown that both aspartate residues of the respective occurrences of the B motif of NBS-I are indispensable for SecA function. These data confirm the observations that the first aspartate in the B motif of *E. coli* SecA (Asp^{209}) is involved in the catalytic function of SecA (Mitchell and Oliver, 1993), but Chapter 3 furthermore shows that the spatial arrangement of this domain is more complex than previously proposed. Substitution of either Asp^{207} (corresponding to Asp^{209} in *E. coli*) or Asp^{215} of *B. subtilis* SecA results in an inactive protein that is still able to interfere with protein translocation, both *in vivo* and *in vitro*. Asp^{215} seems to be most intimately involved with Mg^{2+}-binding as the D215N SecA mutant is no longer capable of Mg^{2+}-ion and ATP-binding. Asp^{207} appears to fulfil a more subtle role. The D207N SecA mutant still binds Mg^{2+} and ATP, but is unable to hydrolyse the nucleotide. This indicates that both aspartate residues are involved in binding and hydrolysis of ATP by SecA.

Role of ATP binding to NBS-I of SecA

The research presented in this thesis and work done by other groups has shown that ATP binding and hydrolysis at NBS-I is required for SecA membrane and precursor association and dissociation. During this process, SecA undergoes major conformational changes as revealed the thermal unfolding characteristics (Den Blaauwen *et al.*, 1996). Thermodynamically, SecA consists of at least two independent folding domains of approximately equal size (Den Blaauwen *et al.*, 1996), *i.e.* the N- and C-domain, respectively (Den Blaauwen, manuscript in preparation). Binding of ADP to NBS-I leads to the formation of a more compact state of the SecA protein, and promotes the interaction between the N and C-domains. Remarkably, binding of ATP to the NBS-I results in an elongated conformation of the protein with little interaction between the two domains (Den Blaauwen *et al.*, 1996). ATP hydrolysis at the high-affinity NBS-I appears to be used to drive a thermodynamically unfavourable conformational change yielding a more compact protein, while ATP binding reverses this conformational change (Den Blaauwen, manuscript in preparation). In this manner, the energy of ATP binding is converted into a mechanical force, *i.e.* a physical movement of the two SecA domains relative to each other.

Translocation has been proposed to involve the nucleotide-dependent cycles of membrane-insertion and de-insertion of the SecYEG-bound SecA. According to this model, translocation is the result of the co-insertion of precursor protein segments. The model of SecA membrane-insertion and de-insertion is mainly based on protease-resistance experiments. Under translocating conditions, *i.e.* in the presence of a preprotein and ATP, a stable C-terminal 30 kDa SecA fragment can be formed after proteolysis of the SecYEG-bound SecA (Economou and Wickner, 1994; Economou *et al.*, 1995; Price *et al.*, 1996). Overproduction of the SecD and SecF proteins stabilizes this proteolytic fragment, which is thought to insert into the membrane based on the finding that disruption of the membrane relieves the protease resistance (Economou and Wickner, 1994). The same sets of conditions also lead to the formation of a proteolytic 65 kDa fragment that originates from the N-terminus (Eichler and Wickner, 1997). Together with the 30 kDa fragment this would imply that SecA completely emerges into the membrane during translocation. The 30 and 65 kDa fragments (Eichler *et al.*, 1997) and the intact SecA (Van Voorst *et al.*, 1998) are largely

presence of detergents that maintain the SecA-SecYEG interaction, efficient formation of the 30 kDa SecA fragment is also possible in the absence of membranes (Van der Does *et al.*, 1998). The SecYEG is proteolysed to small fragments under these conditions, which argues against a model in which the membrane-inserted SecA domains are protected against proteolysis by a belt of SecYEG α-helices. The 30 and 65 kDa fragments of SecA can also be formed with low efficiency with the soluble enzyme (Price *et al.*, 1996), indicating that they must originate from pre-existing domains that become more protease-resistant upon the interaction with SecYEG. Other data demonstrate that domains of SecA bound to SecYEG are accessible to proteases and chemical reagents from the periplasmic face of the membrane (Van der Does *et al.*, 1996; Ramamurthy and Oliver, 1997). Taken together, it is not certain that SecA penetrates the membrane in a nucleotide-dependent manner. An alternative explanation seems more feasible. Electron microscopical investigation of the ultrastructure of the active translocase complex indicates that SecYEG units oligomerize into a pore-like structure (Manting, Van der Does, Engel and Driessen, manuscript in preparation). The cytosolic SecA may function as a plug for this pore that may be open from the periplasmic face of the membrane thereby rendering domains of cytosolic SecA accessible from the outside. Translocation may be driven by the nucleotide-dependent disposition of the SecA domains thereby converting the chemical energy into a mechanical force. Such a model does not require the actual membrane penetration of SecA domains.

Role of NBS-II in the SecA protomer

The role of the low-affinity NBS-II in protein translocation is less well resolved. Mutants of NBS-II are defective in the release of SecA from the membrane, but still undergo the conformational changes that are induced by the binding of nucleotides at NBS-I (Economou *et al.*, 1995). In Chapter 4 it is shown that NBS-II is localized at the subunit interface of the SecA dimer. The photo-activatable bifunctional cross-linking agent diN_3ATP was used to show that the UV-induced cross-linking of diN_3ATP-bound *E.* dimeric species of SecA. Using mutants that were altered in both NBS-I and NBS-II, it is demonstrated that NBS-II is the site of cross-linking and that NBS-II binds nucleotides at or near the subunit interface of the SecA dimer. The interfacial localisation of NBS-II may provide a mechanistic explanation for the action of the dimer as the functional unit. Binding of ADP at NBS-II causes further conformational changes in the SecA protein, and a major reduction in the amount of solvent accessible protein surface (Den Blaauwen *et al.*, 1996). Hydrolysis of ATP at NBS-II could promote the formation of this highly compact conformation by increasing the interfacial contact between the subunits of the SecA protomer, while it may displace the two subunits when it binds ATP. The highly compact state appears to be essential for membrane release. In this respect, lipid monolayer experiments have shown that NBS-I and NBS-II saturating concentrations of ADP favour the surface-bound conformation of SecA, rather than the lipid-inserted state.

ATP-driven translocation is a stepwise process.

Translocation is thought to be driven by cycles of ATP binding and hydrolysis by SecA (Schiebel *et al.*, 1991). According to this hypothesis, translocation would be a stepwise process. This hypothesis is supported by the finding that non-hydrolysable nucleotide analogues, such as ATPγS (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991) cause the translocation progress of translocation intermediates by approximately 2-2.5 kDa. Experiments in which translocation is synthetically arrested by the introduction of a stable disulfide-bond in the mature domain also indicate that translocation occurs in discrete steps (Uchida *et al.*, 1995). Chapter 5 shows that ATP-dependent translocation is indeed a stepwise process. A complete catalytic cycle of SecA, which involves turnover of the ATP, permits ~5 kDa translocation progress. This is twice the size of the previously suggested step size of ~2.5 kDa which does not involve a complete turnover of SecA. On the other hand, a retardation of the catalytic cycle of the SecA ATPase by azide results in the accumulation of intermediates that are spaced in intervals of about ~2.5 kDa. These data now lead to a new model for the

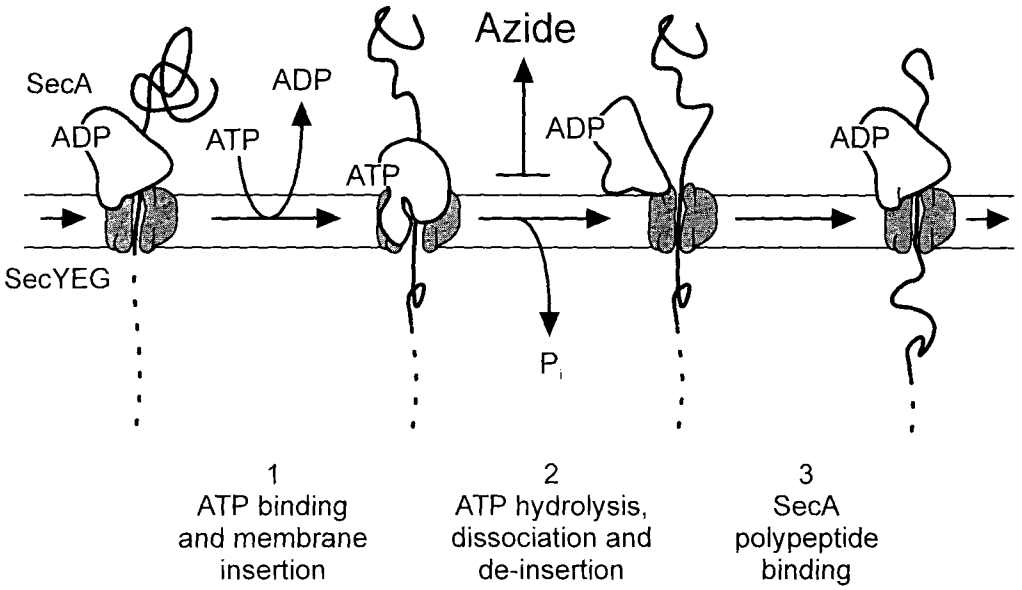

Figure 1. Model for the catalytic cycle of the SecA translocation ATPase during intermediate stages of precursor protein translocation. See text for details.

catalytic cycle of SecA (Figure 1). Binding of ATP to SecA, complexed with a translocation intermediate, results in the conformational change specified by the protease-resistant 30-kDa fragment (Economou and Wickner, 1994) concomitant with the translocation of a ~2.5 kDa polypeptide segment (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991). Hydrolysis of the bound ATP-hydrolysis reverses this conformational change and elicits the release of the precursor protein from its association with SecA. Subsequently, SecA can rebind to the precursor protein in transit, and this reaction results in the translocation of another 2.5 kDa segment of the preprotein (Schiebel *et al.*, 1991). This binding reaction activates SecA for ADP-ATP exchange, and the catalytic cycle is repeated in order to translocate another ~5 kDa of polypeptide mass.

The role of ATP during initiation of translocation

ATP is essential for the initiation of translocation (Schiebel *et al.*, 1991). During these first stages of the translocation process, binding of

domain of the preprotein to the extent that the signal sequence can be processed by leader peptidase. It has been proposed that at this stage "proof-reading" of the signal sequence occurs to select for proper secretory proteins (Osborne and Silhavy, 1993). Aberrant signal sequences normally do not support the translocation of secretory proteins, even though they are recognized with low affinity by SecA (Chapter 6). The so-called **p**rotein **l**ocalisation (*prl*) mutations have been identified in all essential components of the translocase, and restore the translocation of such defective precursor proteins. (Emr *et al.*, 1981; Bieker *et al.*, 1990; Dermann *et al.*, 1993; Bost and Belin, 1997). This implies that all essential subunits of the translocase are involved in the process of proof-reading. A direct interaction between the signal sequence and one of the components of the translocation machinery has only been demonstrated for SecA (Cunningham and Wickner, 1989; Lill *et al.*, 1990; Kimura *et al.*, 1990). Binding of SecA to a signal sequence induces a conformational change in SecA (Shinkai *et al.*, 1991). This may be sensed by the other

these proteins. In this way, a functional complex may be formed to allow the initiation of translocation. Mutations in the components of the translocase that do not directly interact with the signal sequence can in this way still lead to a *prl* phenotype. One of these mutants of SecY, *prlA4*, was analysed in greater detail in Chapter 6. PrlA4 is one of the most potent mutants in the translocation machinery that allows the translocation of defective, or even completely missing, signal sequences (Emr *et al.*, 1981; Dermann *et al.*, 1993). The data in Chapter 6 demonstrate that the *prlA4* mutation does not restore the recognition of the defective signal sequence by SecA, but rather gives rise to a stabilization of the SecA-SecYEG interaction during the initiation of translocation. With the wild-type SecY, the SecA-preprotein-SecY complex is very unstable in the presence of ATP and this results in a significant level of premature release of the precursor from the translocation site. With the *prlA4* mutant, the affinity of SecY for SecA is dramatically enhanced and little premature release of preprotein occurs, even in the presence of ATP. As a consequence, the initiation of translocation of wild-type precursor proteins is much more efficient in the PrlA4 strain, leading to more efficient translocation. The increased affinity also results in a disruption of the proof-reading mechanism as preproteins with a defective signal sequence can now enter the translocation channel. This phenomenon is not simply due to the increased activity of the translocase since high concentrations of SecA do not suffice for the translocation of defective preproteins with appreciable rates (Huie and Silhavy, 1995). The PrlA4 strain also shows a very low Δp dependency for translocation (Nouwen *et al.*, 1996b). When this is considered in the context of the increased SecA binding-affinity, which leads to a longer retention of SecA at the site of translocation, this can be regarded as another line of evidence that indicates that the Δp only acts as a direct driving force for translocation when the preprotein is not bound to SecA (Schiebel *et al.*, 1991; Driessen, 1992; Chapter 2).

Concluding remarks

SecA performs a crucial role during preprotein translocation. Not only is SecA the converging point where all components of the translocase interact, but it functions as the actual motor of the translocation reaction by converting the chemical energy provided by ATP into mechanical force. In this thesis not only the molecular details of the ATP-SecA interactions have been investigated, but also more insight has been generated in the mechanistic implications of these interactions for the ATP-driven protein translocation across the cytoplasmic membrane of *Escherichia coli*.

SecA is one of the largest enzymes of *E. coli*, with a functional size of 204 kDa. For function, SecA interacts in a complicated manner with the membrane domain of the translocase. As shown in Chapter 6, these interacts have a major impact on the specificity and activity of the enzyme. Therefore, the static high-resolution atomic structure of the protein in solution by X-ray crystallography will provide only a limited view on its mechanism of action. It will be important to visualize the dynamics of this protein translocation reaction at the atomic level. The recent visualization of an active preprotein-SecA-SecYEG complex by high resolution electromicroscopy is a first step towards this goal (Manting *et al.*, manuscript in preparation). Elucidation of the low resolution structure during all stages of translocation will already provide us with answers on the actual physical distance that is covered by the SecA domains during turnover (Den Blaauwen *et al.*, 1996; Chapter 4), and whether this involves the actual membrane insertion of protein domains. Also, many questions remain on how the various membrane components of the translocase accommodate SecA in an active conformation. The two transmembrane segments of SecG, for instance, appear to spectacularly inverse their topology upon interacting with SecA (Nishiyama *et al.*, 1996). Another pertinent challenge for future research concerns the role of the Δp in the translocation process. Δp can drive the

the absence of SecA association (Schiebel *et al.*, 1991), suggesting that in the presence of a Δp, translocation would have a discontinuous step size. This finding, however, is based on an artificial situation in which SecA is depleted from the inner membrane using an polyclonal antibody. In the cell SecA is always present, even in a 5-fold excess relative to the SecY monomer. *In vitro* translocation simultaneously driven by ATP/SecA and the Δp, however, retains its stepwise nature, although the amounts of detectable intermediates are significantly lower than in the absence of a Δp (J.P.W. van der Wolk, unpublished results). The Δp has been shown to affect many different stages of the translocation reaction, *i.e.* initiation, intermediate stage, and the final release. It is not clear if Δp acts on these stage according a common mechanism, nor is it known how the Δp would mechanistically act as a direct driving force in the absence of SecA.