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Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme

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Summary

Preprotein translocase, the membrane transporter for secretory proteins, is a processive enzyme. It comprises the membrane proteins SecYEG(DFYajC) and the peripheral ATPase SecA, which acts as a motor subunit. Translocase subunits form dynamic complexes in the lipid bilayer and build an aqueous conduit through which preprotein substrates are transported at the expense of energy. Preproteins bind to translocase and trigger cycles of ATP binding and hydrolysis that drive a transition of SecA between two distinct conformational states. These changes are transmitted to SecG and lead to inversion of its membrane topology. SecA conformational changes promote directed migration of the polymeric substrate through the translocase, in steps of 20–30 aminoacyl residues. Translocase dissociates from the substrate only after the whole preprotein chain length has been transported to the *trans* side of the membrane, where it is fully released.

Introduction

Bacteria devote more than 30% of their genome-coding capacity to genes for exported, periplasmic, outer and inner membrane proteins. Polypeptides that reside in specialized cellular compartments or that are fully secreted have to cross hydrophobic lipid bilayers to reach their final destinations. During protein export, cytosolically synthesized polypeptides are targeted to the *cis* side of a membrane and then translocated through the membrane plane for release on the *trans* side. The unique features of the translocated substrate (a polymer that is several times as long as the membrane is wide, with charged and apolar regions and a tendency to fold rapidly) required the invention of a dedicated membrane transporter, termed preprotein translocase or translocon.

Our current understanding of the bacterial (read *Escherichia coli*) preprotein translocase and, particularly, of the mechanism of export of hydrophilic proteins is advanced. This development has largely been due to the unrivalled efficiency of bacterial genetics (which identified most of the responsible secretion genes) and to the post-translational nature of bacterial translocation (which allowed biochemical dissection). Efficient *in vitro* reconstitution using purified components allowed the initial characterization of energetic and kinetic parameters and has defined the order of some of the catalytic steps. Recent exciting developments have revealed that translocase promotes translocation of substrates through it by using metabolic energy to drive cyclic alterations in its conformation and in the dynamic associations between its subunits. In reviewing this work, I shall focus mainly on the better understood ATP-driven component of the reaction.

How substrates find the translocase at the membrane

Secretory preproteins carry signal or leader peptides at their N-termini. Signal peptides mediate initial recognition of secretory nascent polypeptides by the cell and are proteolytically removed on the *trans* side of the membrane. Targeting of preproteins to the membrane is facilitated by chaperones: either general purpose ones (GroEL, DnaK, etc.) or secretion-specific ones, such as SecB and the signal recognition particle (SRP) (reviewed by Kumamoto, 1991; Arkowitz and Bassilana, 1994; Randall and Hardy, 1995). Chaperones bind to signal (SRP) or mature (SecB) regions of preproteins and prevent native folding and aggregation. These associations are of particular importance, as bacterial protein secretion is largely uncoupled from ongoing translation (Randall and Hardy, 1995). In a well-characterized reaction, SecB/preprotein complexes are targeted to the membrane (Randall and Hardy, 1995; Wickner and Leonard, 1996) by virtue of their high affinity for the membrane-embedded translocase and, in particular, the SecA subunit, which contains a C-terminal SecB binding site (Breukink *et al*., 1995; Fekkes *et al*., 1997).

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An inventory of translocase parts: integral membrane subunits

Translocase comprises the integral membrane polypeptides SecY, SecE and SecG and the peripheral protein SecA and requires acidic phospholipids (reviewed by Oliver, 1993; Arkowitz and Bassilana, 1994; Wickner and Leonard, 1996). SecYEGA are necessary and sufficient in reconstituted proteoliposomes to allow preprotein translocation at high efficiency (approximately 25–50% of the yield and rates obtained with intact inner membranes). SecY, SecE and SecG associate with each other, as they can be isolated (chromatographically and by immunoprecipitation) as a stable trimer, and stable overexpression of SecY requires concomitant overexpression of SecE. Individual subcomplexes of SecY/SecE (Joly *et al*., 1994; Duong and Wickner, 1997a; Homma *et al*., 1997) and SecY/SecG (Homma *et al*., 1997) may also exist in the membrane.

SecY, a 10-transmembrane helix protein, has been conserved in all organisms and small membrane proteins, similar to SecE and SecG found in archaeal and eukaryotic translocases. SecY, SecA and SecE are essential *E. coli* proteins, while SecG is only required for viability at low temperatures (Hanada *et al*., 1996; Nishiyama *et al*., 1996). SecYA are the only subunits that have been demonstrated to lie in the vicinity of translocating chains (Joly and Wickner, 1993; Sato *et al*., 1997). Membranes significantly depleted of SecE are still proficient in the *in vitro* translocation of some polypeptide substrates (Yang *et al*., 1997). These observations indicate that the minimal catalytic core of a bacterial preprotein translocase is SecYEA and, perhaps, even SecY and SecA alone, as is the case for the spartan bacterial genome of *Mycoplasma genitalium*. Biochemical and genetic assays have begun to map interacting regions between the translocase subunits (Breukink *et al*., 1995; Flower *et al*., 1995; Pohlschroder *et al*., 1996; Homma *et al*., 1997; Snyders *et al*., 1997).

The translocase core can associate with additional membrane polypeptides. After solubilization with the non-ionic detergent digitonin, SecYEG can be co-immunoprecipitated together with the integral membrane proteins, SecD and SecF, and a small, uncharacterized membrane polypeptide, YajC. This association was seen most clearly after overexpression of the non-essential *secDFyajC* genes (Duong and Wickner, 1997a). Deletion of these genes results in slight (*secD*, *secF)* or no (*yajC*) reduction in optimal translocation (Pogliano and Beckwith, 1994a; Economou *et al*., 1995; Duong and Wickner, 1997a). These data, taken together with genetic analyses and the finding that SecF overexpression stabilizes overexpressed SecY (Sagara *et al*., 1994), have suggested that SecDF and YajC may associate with the translocase core (Duong and Wickner, 1997a). SecDF(YajC) may also exist in the membrane as a distinct complex, because they can be immunoprecipitated together independently of SecYEG (Duong and Wickner, 1997a). In the absence of any detailed subunit stoichiometry, it is not clear how many of the translocase core units in the cell contain SecDF(YajC), particularly as SecYEG were estimated to exist in a 10-fold excess over SecDF (Pogliano and Beckwith, 1994b). Bacterial translocase could exist in both a tetrameric (SecYEGA) and a heptameric (SecYEGADFYajC) form, an organization reminiscent of that of eukaryotic translocase (Duong and Wickner, 1997a). SecDF can stabilize a membrane-inserted form of SecA at SecYEG (Economou *et al*., 1995; Duong and Wickner, 1997a), and SecDF overexpression causes an accumulation of the total amount of membrane-associated SecA (Kim *et al*., 1994). Neither SecDF nor SecG is essential for SecA binding to SecYE or for catalysis; however, the *in vivo* and *in vitro* translocation proceeds more efficiently in their presence (Pogliano and Beckwith, 1994a; Economou *et al*., 1995; Hanada *et al*., 1996; Duong and Wickner, 1997a,b). Even when both SecG and SecDF are absent simultaneously, overexpression of the catalytic core is sufficient to overcome the defect in translocation efficiency (Duong and Wickner, 1997a). Using combinations of these proteins co-reconstituted with SecYEA, Duong and Wickner (1997b) have demonstrated that the accumulation of translocation intermediates within translocase is prevented in the presence of SecDF. SecDF(YajC) and SecG can, therefore, be considered as regulatory subunits of the translocase (Economou *et al*., 1995; Duong and Wickner, 1997a,b).

SecY has been cross-linked to translocating preproteins from within the translocation channel (Joly and Wickner, 1993). This and the vast majority of available data, including evolutionary considerations, support the proposal that the translocation of preproteins is catalysed by SecYA(EG) complexes. Nevertheless, some *in vitro* experiments have indicated that SecA may mediate translocation even in vast super-stoichiometric excess to SecYE (Watanabe and Blobel, 1993). It is not known if additional proteinaceous factors would be required for the observed translocation, as SecA cannot translocate hydrophilic proteins into liposomes in the absence of SecYEG (Wickner and Leonard, 1996). The inherent ability of SecA to bind and insert in lipid bilayers (Ulbrandt *et al*., 1992; Breukink *et al*., 1995) may be important in these reactions.

An inventory of translocase parts: the peripheral SecA subunit

In contrast to the universality of SecY, SecA is a translocase accessory that is unique to bacteria. Despite its late arrival on the translocation scene, SecA has become the central component of the bacterial secretion pathway, presumably by offering efficient translation-independent,

ATP-driven translocation of large hydrophilic proteins. SecA performs five essential tasks: (i) it is the physical link between all of the components of the reaction: preprotein, SecB chaperone, SecYEG, lipids, SecA mRNA and ATP (Ulbrandt *et al*., 1992; Oliver, 1993; Arkowitz and Bassilana, 1994; Breukink *et al*., 1995; Fekkes *et al*., 1997); (ii) it acts as the primary translocase receptor for secreted substrates and their chaperones (Wickner and Leonard, 1996) and probably directs the substrate to the export channel; (iii) it is a building block for the export conduit (Joly and Wickner, 1993; van der Does *et al*., 1996; Chen *et al*., 1996; Snyders *et al*., 1997); SecA, together with SecY(EG), may shield polar segments of substrates from phospholipids (Joly and Wickner, 1993); (iv) it generates the mechanical work necessary for substrate translocation by converting ATP free energy (Economou and Wickner, 1994); and (v) it senses and regulates the secretion status of the cell by tightly controlling its own translation through mRNA binding (Oliver, 1993) and RNA helicase (Park *et al*., 1997) activities.

SecA, a 101 kDa hydrophilic homodimer (Oliver, 1993), has been dissected biochemically in two distinct primary domains: the N-terminal 70 kDa domain and the C-terminal 30 kDa domain (Economou and Wickner, 1994; den Blaauwen *et al*., 1996; Price *et al*., 1996). The 70 kDa domain shares homology with RNA helicases and contains two nucleotide binding domains (NBDs). NBD1 is located approximately between residues 100 and 220 of the protein, contains characteristic ATP binding motifs (Walker boxes A and B) and has been demonstrated to be a high-affinity site in several experiments (Mitchell and Oliver, 1993; Oliver, 1993). NBD2 has been poorly characterized and was proposed to contain a non-canonical Walker box A nucleotide binding site around residues 500–520 and an undefined Walker box B motif further downstream (Mitchell and Oliver, 1993). SecA was invented early on in the evolution of the bacterial domain. It was probably created after an ancestral RNA helicase-like enzyme fused N-terminally to a polypeptide, which allowed the protein to acquire novel specializations: to dimerize (Hirano *et al*., 1996); to acquire the ability to interact with acidic phospholipids (Breukink *et al*., 1995) and with SecY (Snyders *et al*., 1997); to insert in the membrane at SecYEG (Economou and Wickner, 1994; Economou *et al*., 1995); to interact with SecB (Breukink *et al*., 1995; Fekkes *et al*., 1997); and, importantly, to interact directly not only with RNA but also with aminoacyl polymers.

Energizing the translocase machine: ATP and the proton motive force

One of the salient features of the bacterial preprotein translocase is that it is driven by (at least) two different energy sources, ATP and the proton motive force (PMF) (reviewed by Driessen, 1992; Arkowitz and Bassilana, 1994). *In vitro* studies have revealed that the two energetic components act at distinct stages of the translocation reaction, with ATP being required first (Schiebel *et al*., 1991) and used via SecA, the only ATPase of the system (Oliver, 1993). The PMF is of course omnipresent even at the early stages of translocation. However, its contribution can be seen more clearly biochemically after preproteins have translocated about halfway through the membrane. At that stage, the PMF can drive completion of translocation in the absence of ATP, but only if SecA is inactivated beforehand by antibodies (Schiebel *et al*., 1991). It is possible that several membrane components of the reaction are simultaneously affected by the PMF. It may affect translocase directly by optimizing the rate of ATP hydrolysis by SecA by lowering the K_m for ATP by 100-fold (Shiozuka *et al*., 1990) and by imposing directionality to the vectorial transfer (Driessen, 1993; Arkowitz and Bassilana, 1994). A direct involvement of SecY in PMF-driven translocation was suggested by an important recent observation: a single aminoacyl residue substitution in this protein renders the *in vivo* and *in vitro* translocation PMF independent (Nouwen *et al*., 1996). While the ATP-driven subreactions are essential for translocation, those of the PMF are not (Schiebel *et al*., 1991; Driessen, 1993), but the imposition of a PMF leads to a two- to 10-fold increase in translocation rates (Shiozuka *et al*., 1990; Schiebel *et al*., 1991; Driessen, 1993). The SecA/ATP and the PMF components of the reaction are somehow co-ordinately linked, as excess of SecA allows translocation of a precursor that is otherwise absolutely PMF dependent for its translocation (Yamada *et al*., 1989). Furthermore, PMF-driven translocation (after SecA inactivation) is slowed down when SecA is added back to the system in the absence of ATP (Schiebel *et al*., 1991). The following section describes how energy intake changes the conformation of translocase as the translocation of substrates proceeds.

The translocase machine at work: an ATP-driven SecA membrane insertion

Preprotein–SecB complexes associate with SecYEGA in the apparent absence of any exogenous energy and trigger translocase mechanical movement. Binding of ATP or of non-hydrolysable ATP analogues at the NBD1 site stabilizes a SecA conformation, which is more integral or buried into the membrane (Economou *et al*., 1995). This conformational change has been detected not only at initiation of translocation but also during translocation of advanced intermediates (A. Economou, unpublished results), suggesting that it is a fundamental characteristic

of the ATP-driven component of translocation. This subreaction, termed 'SecA membrane insertion', takes place specifically at SecYEG and is detected as protease inaccessibility of the otherwise protease-susceptible C-terminal 30 kDa domain of SecA (Economou and Wickner, 1994). Regions of membrane-inserted SecA may even span the membrane to become accessible to reagents on the periplasmic side of the membrane (Economou and Wickner, 1994; Kim *et al*., 1994; van der Does *et al*., 1996; Ramamurthy and Oliver, 1997). Recently, using single cysteinyl derivatives or ^{35}S-labelled SecA, significant amounts of not only the 30 kDa but also the 70 kDa domain were found to become inaccessible to protease in the membrane (Ramamurthy and Oliver, 1997), and specifically at SecYEG (Eichler and Wickner, 1997).

Characterization of its ATP-bound conformation suggested deep membrane penetration of SecA in the inserted state. However, in the absence of nucleotide (and preprotein ligand), the precise topology and conformation of SecA bound at SecYEG is completely unknown. Low levels of membrane-inserted 30 kDa domain can be detected even in the absence of ligands (Economou and Wickner, 1994), and SecA stretches exposed to the periplasm can be detected in the absence of ongoing translocation (Kim *et al*., 1994; van der Does *et al*., 1996; Ramamurthy and Oliver, 1997). Therefore, it cannot be excluded that, as SecA binds to SecYEG and acidic phospholipids in the absence of ATP, it spontaneously 'inserts' in the membrane, in such a way that it is accessible to protease. In this topology, SecA could be in closer association with lipid (Ulbrandt *et al*., 1992; Breukink *et al*., 1995; Chen *et al*., 1996). ATP-driven conformational changes between the two states of SecA (allowing for movement of small 20–30 aminoacyl residue segments) may be localized and need not represent dramatic alterations in the overall shape of the molecule (S. Karamanou, B. Shilton and A. Economou, unpublished results). To characterize fully the extent of ATP-driven SecA movement relative to the membrane and the precise changes in the environment that SecA encounters, more sensitive, real-time and non-destructive biophysical approaches need to be used.

What is the membrane environment into which SecA inserts? How does a protein devoid of continuous apolar stretches insert in a hydrophobic milieu? Attempts to label either the C-terminal 30 kDa or the N-terminal 70 kDa domain of membrane-inserted SecA using a carbene-generating cross-linker embedded in the phospholipid bilayer have not been successful (Eichler *et al*., 1997). This negative result was taken as a preliminary indication that both the 70 kDa and the 30 kDa domains of SecA may not be neighbouring phospholipids in the inserted or deinserted states. SecA membrane insertion may take place at hydrophilic surfaces provided by the other translocase subunits, such as SecY, which was shown to interact with the negatively charged 30 kDa domain (Snyders *et al*., 1997). If, as anticipated, bacterial translocase is organized in a way analogous to that of eukaryotic translocase, which oligomerizes around a 20–60 Å channel (Crowley *et al*., 1994; Hanein *et al*., 1996), then an internal SecYEG environment could accommodate hydrophilic parts of membrane-inserted SecA, while other SecA surfaces may insert into the lipid bilayer (Ulbrandt *et al*., 1992; Breukink *et al*., 1995).

A change in SecA conformation might be expected to influence the subunits of translocase that are adjacent to it. Indeed, concomitant with ATP-driven SecA insertion, SecG undergoes a remarkable inversion of its membrane topology (Nishiyama *et al*., 1996). The hydrophilic C-terminal region of SecG, which is normally residing in the periplasm, becomes exposed to the cytoplasmic side of the membrane, as judged by acquisition of accessibility to proteases and to specific antibodies added to either the *cis* or the *trans* side of the membrane. Moreover, the central region of SecG shifts from a cytoplasmic location to one where it is exposed to the periplasm (Nishiyama *et al*., 1996). These changes could affect the conformation of SecYE and might explain the dynamic state of association and dissociation of SecG with SecYE (Joly *et al*., 1994). It has also been proposed that SecG inversion may optimize the interaction of SecA with the membrane by direct SecG/SecA interaction or indirectly via SecY (Nishiyama *et al*., 1996).

The translocase machine at work: ATP binding drives SecA membrane insertion drives limited preprotein translocation

As the translocase components alter their conformation during ATP-driven SecA membrane insertion, simultaneous co-insertion of limited segments of the preprotein substrate is observed (Schiebel *et al*., 1991). During the initiation of translocation, this involves insertion of an N-terminal sequence containing the signal peptide (20–30 amino acids) forming a loop with the succeeding 20–30 aminoacyl residues of preprotein (Schiebel *et al*., 1991). Although SecA must be the crucial component of the reaction, interaction of the signal peptide with hydrophobic surfaces of the translocase and with the lipid bilayer may also be important (Arkowitz and Bassilana, 1994). At more advanced stages of protein translocation (i.e. after the signal peptide has been cleaved off), the same ATP-driven SecA forward stroke leads to membrane insertion of 20–30 amino acids of the preprotein (Schiebel *et al*., 1991; Joly and Wickner, 1993; Uchida *et al*., 1995; Sato *et al*., 1997). Owing to their limited length, these translocated preprotein segments presumably remain in the membrane plane. As complete and efficient SecA membrane insertion at SecYEG can be stabilized by non-hydrolysable

ATP analogues alone (Economou *et al*., 1995), the presence of the preprotein substrate itself is not mechanistically or energetically essential for the SecA insertion reaction *per se*.

Use of SecA mutants and of non-hydrolysable ATP analogues and the determination of biochemical requirements and kinetics have led to the proposal that the two subreactions, i.e. SecA forward stroke and limited preprotein forward movement, are physically linked (Economou and Wickner, 1994; Economou *et al*., 1995). This model considers preprotein segments as SecA cargo and, for such a mechanism to be in place, it can be hypothesized that SecA possesses one or more substrate binding sites with particular characteristics: (i) a defined capacity for chain length (approximately 20–30 aminoacyl residues); (ii) no strict substrate sequence specificity, as SecA can translocate a huge variety of polypeptides, including enzymes normally residing in the cytosol. Backbone α-carbons could be important in this recognition; (iii) variable affinity for the substrate (or for individual stretches of the substrate), such that the energetic barrier between the bound and released state is low and yet sufficiently high to achieve SecA-mediated transfer.

SecA–substrate interaction is unlikely to involve a simple mechanism of tight lock and key type recognition by SecA. This may explain the lack of allelic specificity of SecA mutations, which can suppress different signal peptide defects. Hydrophobic patches in the SecA substrate binding pocket may facilitate the docking of N-terminal signal peptides and of internal hydrophobic stretches (Sato *et al*., 1997) found in the mature sequence of preproteins. Such substrate binding sites may exist on the C-terminal 30 kDa domain of SecA, although to date putative binding sites have only been tentatively assigned by cross-linking, genetic and biochemical studies to the 70 kDa domain (Oliver, 1993).

Approximately 20 mol of ATP are required by SecA for the secretion of 1 mol of a short, 74-residue-long substrate *in vitro* (Bassilana *et al*., 1992), indicative of a tightly coupled reaction. However, ATP hydrolysis of longer substrates becomes very inefficient, possibly as a result of back-slippage of the substrate in the *in vitro* system (Schiebel *et al*., 1991; Bassilana *et al*., 1992) or of an ATP requirement for unfolding reactions. In the absence of direct quantitation of the coupling stoichiometry of ATP expenditure for a single SecA insertion cycle/preprotein forward movement, it cannot yet be excluded that SecA membrane insertion contributes indirectly to the forward movement of the preprotein. One possibility is that membrane-inserted SecA 'primes' the translocase to acquire a translocation-competent conformation and, subsequently, short secretory polypeptide chains move through the channel by thermal motion. Limited forward movement or back-slippage of translocation intermediates have been observed (Schiebel *et al*., 1991; Arkowitz *et al*., 1993) and occur in the absence of ATP hydrolysis.

The translocase machine at work: ATP hydrolysis allows release of preprotein and recycling of SecA

If the ATP that binds at NBD1 and drives SecA insertion is not hydrolysed, the preprotein translocation reaction stalls after a few aminoacyl residues have entered the membrane. Clearly, several cycles of ATP hydrolysis are essential for the completion of transfer (Schiebel *et al*., 1991; Bassilana *et al*., 1992; Economou and Wickner, 1994; Uchida *et al*., 1995; Sato *et al*., 1997). One role of ATP hydrolysis by SecYEG-bound SecA is to mediate substrate release. Using an *in vitro* assay with preprotein bound to liposome-associated SecA (in the absence of SecYEG), it has been demonstrated that ATP hydrolysis led to an apparent change in proteolytic accessibility of the preprotein, interpreted as the release of substrate from SecA (Schiebel *et al*., 1991). Release may be further facilitated by SecG membrane topology changes (Nishiyama *et al*., 1996) and by SecD (Matsuyama *et al*., 1993). Perhaps most important of all are interactions with SecY: when advanced translocation intermediates are trapped *in statu translocanti*, preprotein chains remain stably threaded within SecYEG even after inactivation of the SecA subunit (Schiebel *et al*., 1991). This suggests that substrates can be maintained within the SecY translocation channel in a quasi-extended state, shielded from phospholipids throughout their membrane transit (Joly and Wickner, 1993; Sato *et al*., 1997).

Another role for ATP hydrolysis is to allow the translocase to be recycled and primed for the next cycle of ATP interaction. ATP hydrolysis at NBD1 allows SecA inserted at SecYEG to revert to a membrane-bound conformation, in which it is more accessible to protease (Economou and Wickner, 1994). Chase experiments have shown that a population of iodine- or sulphur-labelled SecA molecules in the 'deinserted' state can exchange with exogenously added unlabelled SecA (Economou and Wickner, 1994; Eichler and Wickner, 1997). SecA can, therefore, physically dissociate from the rest of translocase in an ATP hydrolysis-dependent manner at some point in the catalytic cycle (Economou and Wickner, 1994). At the level of a single translocase complex, the precise mechanism and stage of the SecA deinsertion reaction remains poorly understood. Several SecA insertion and deinsertion events by the same or by several SecA molecules could take place during the translocation of a single preprotein chain. Alternatively, SecA may dissociate from SecYEG only after one polypeptide chain has translocated completely. Deinserted SecA could exchange with the cytosolic pool or it could remain bound to the membrane but diffuse laterally into the lipid phase. Under different

experimental conditions, Chen *et al*. (1996) found that a large fraction of membrane-bound SecA, which could represent non-SecYEG-bound protein, does not exchange with the cytosolic pool.

How are the products of ATP hydrolysis released from SecA in the deinserted state? The PMF has been proposed to be one factor facilitating release (Shiozuka *et al*., 1990), perhaps by affecting SecY conformation (Nouwen *et al*., 1996). Furthermore, preprotein binding to soluble SecA has been shown to cause the release of ADP (Shinkai *et al*., 1991). Whenever preprotein that has been released into the translocation channel reassociates with SecA, ADP + Pi bound to NBD1 of SecA could also be released, thereby allowing a repetition of the cycle: ATP binding at NBD1, SecA/substrate co-insertion in the membrane, ATP hydrolysis, substrate release and translocase recycling. In this model, the role of the preprotein substrate is to act as a nucleotide exchange factor, thereby coupling SecA cycling to its own translocation. For as long as the preprotein is physically trapped within translocase, thermal fluctuations could lead to subsequent collisions with SecA. It has been proposed that several repeats of this cycle of events will drive the complete translocation of an average-sized 200–300 aminoacyl residue polypeptide to the *trans* side of the membrane, where it can acquire its native state (Schiebel *et al*., 1991; Economou and Wickner, 1994; den Blaauwen *et al*., 1996; Uchida *et al*., 1995). To test this proposal further, detailed kinetic measurements are necessary.

ATP interactions at the low-affinity NBD2 site are not required for SecA insertion or deinsertion (Economou *et al*., 1995). However, as a mutation in NBD2 does not prevent SecA cycling but prevents substrate translocation, it is possible that NBD2 is important in coupling SecA cycling to substrate forward movement (Economou *et al*., 1995; A. Economou, unpublished results). NBD2 may also have a regulatory role as, when mutated, ATP hydrolysis at NBD1 is reduced significantly (Mitchell and Oliver, 1993).

Concluding remarks and future questions

Bacterial preprotein translocase is a processive ATPase enzyme: (i) Like its counterparts (nucleic acid polymerases, topoisomerases, helicases, etc.), preprotein translocase recognizes and 'translocates' along polymeric substrates of random sequence, just like helicases translocate along nucleic acids. As preprotein translocase is 'fixed' in the lipid bilayer of the membrane, it is obvious that this processive movement results in a net vectorial transfer of the polymer relative to the membrane plane. (ii) To facilitate threading through the membrane, translocase maintains its substrate in non-native, energetically unfavourable states (e.g. extended) or even actively unfolds it (Arkowitz *et al*., 1993). (iii) Directed motion of preprotein translocase stems from ATP-driven conformational changes of a motor-like subunit. SecA is an RNA helicase (Park *et al*., 1997), which has learned to 'unwind' and to migrate along two chemically unrelated polymers! (iv) Translocase movement along the substrate (or substrate movement through translocase) is discontinuous and occurs in steps of 20–30 residues (Schiebel *et al*., 1991; Uchida *et al*., 1995). This could be a result of the substrate recognition mechanism (Sato *et al*., 1997) and of the physical distance covered by SecA domains during their conformational rearrangement. Movement is regulated by the kind of nucleotide bound to SecA at each state (Economou *et al*., 1995; den Blaauwen *et al*., 1996) and involves repeated cycles of substrate binding (ADP–SecA), forward translocation (ATP–SecA) and substrate release (ATP hydrolysis by SecA). (v) Substrate (or some segments of it) that is temporarily released from SecA does not dissociate from the translocase holoenzyme on account of spatial restrictions within the translocation channel and because of direct physical interaction with other Sec proteins, particularly SecY. Gain in forward translocation is maintained. Complete release from translocase occurs only after the end of the transport reaction. (vi) Directionality, prevention of back-slippage and additional driving energy for preprotein segments that are not bound to SecA is provided by the proton motive force (Schiebel *et al*., 1991; Driessen, 1993).

Pertinent questions lead to an exciting future ahead. How does SecAY recognize substrates and how is their forward movement coupled to SecA conformational changes? How do hydrophilic SecA domains enter the membrane environment and how do the other subunits of translocase alter their conformation to accommodate for such a change? How is SecA cycling/ATP hydrolysis coupled to preprotein translocation? How does the PMF affect translocase and optimize the rate of translocation? Combining biochemical, genetic and biophysical tools with three-dimensional structures at atomic resolution promises to yield deeper understanding of this fascinating molecular machine.

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