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The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events

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SecA is the ATP-dependent force generator in the *Escherichia coli* precursor protein translocation cascade, and is bound at the membrane surface to the integral membrane domain of the preprotein translocase. Preproteins are thought to be translocated in a stepwise manner by nucleotide-dependent cycles of SecA membrane insertion and de-insertion, or as large polypeptide segments by the protonmotive force (Δp) in the absence of SecA. To determine the step size of a complete ATP- and SecA-dependent catalytic cycle, translocation intermediates of the preprotein proOmpA were generated at limiting SecA translocation ATPase activity. Distinct intermediates were formed, spaced by intervals of ~5 kDa. Inhibition of the SecA ATPase by azide trapped SecA in a membrane-inserted state and shifted the step size to 2–2.5 kDa. The latter corresponds to the translocation elicited by binding of non-hydrolysable ATP analogues to SecA, or by the re-binding of partially translocated polypeptide chains by SecA. Therefore, a complete catalytic cycle of the preprotein translocase permits the stepwise translocation of 5 kDa polypeptide segments by two consecutive events, i.e. ~2.5 kDa upon binding of the polypeptide by SecA, and another 2.5 kDa upon binding of ATP to SecA.

**Keywords:** energetics/protonmotive force/SecA/secretion

**Introduction**

Preprotein translocation across the cytoplasmic membrane of *Escherichia coli* is mediated by a multisubunit enzyme termed translocase (Wickner et al., 1991; Driessen, 1994). It consists of the cognate heterotrimeric integral membrane domain with SecY, SecE and SecG as subunits, the peripheral ATPase SecA, and SecD and SecF as associated integral membrane proteins (Duong and Wickner, 1997a). The translocation of preproteins requires two forms of energy, ATP and the protonmotive force (Δp) (Geller et al., 1986; Geller and Green, 1989; Yamada et al., 1989a). Although both forms of energy are essential in *vivo*, *in vitro* translocation can be driven by ATP hydrolysis alone provided that SecA is present in excess (Yamada et al., 1989b). SecA is a large homodimeric protein (M, 102 kDa) (Schmidt et al., 1988) with two distinct folding domains, i.e. an N- and C-terminal domain (Den Blaauwen et al., 1996). SecA has two essential nucleotide-binding sites (NBSS; Mitchell and Oliver, 1993) that function in a cooperative manner. The high-affinity NBSS-I (KΔADP ~50 nM) resides in the N-terminal domain, whereas the low-affinity NBSS-II (KΔADP ~300 µM) is localized in the C-terminal domain. SecA is the only ATPase involved in translocation, and its activity is stimulated by high-affinity interactions with preproteins and the SecYEG complex (Lill et al., 1990). SecA interacts in a nucleotide-dependent manner with the membrane surface (Breukink et al., 1992). The SecYEG-bound SecA is thought to insert into the membrane with a 30 kDa C-terminal domain (Price et al., 1996; van der Does et al., 1996) upon binding of ATP at both of its NBSSs (Economou and Wickner, 1994; Economou et al., 1995). This process also allows the limited translocation of a loop of the signal sequence and N-terminal mature region of the preprotein to the extent that the signal sequence can be processed by leader peptidase (Schielb et al., 1991). After insertion, release of the bound preprotein (Schielb et al., 1991) and de-insertion of the SecA domain from the membrane requires at least the hydrolysis of ATP at the high-affinity NBSS-I (Van der Wolk et al., 1993; Economou et al., 1995), while hydrolysis of a second ATP molecule at NBSS-II is needed to release SecA from the membrane (Economou et al., 1995). Re-association of SecA with the partially translocated preprotein permits a new cycle of ATP-dependent membrane insertion and de-insertion of SecA which drives the limited translocation of another preprotein domain. The reaction can be completed by consecutive cycles of ATP-driven translocation (Schielb et al., 1991).

The mechanism by which the Δp accelerates the translocation reaction is only poorly understood. The Δp can act as the sole driving force for the completion of translocation when a partially translocated preprotein is not associated with SecA (Driessen and Wickner, 1991; Schielb et al., 1991; Driessen, 1992; Van der Wolk et al., 1993; Duong and Wickner, 1997b). Other studies indicate that the Δp acts on the SecA translocation ATPase by promoting the release of ADP from SecA (Shiozuka et al., 1990). The Δp has also been suggested to modulate the width of the translocation channel as, in its presence (plus ATP), short segments of proOmpA with a stable tertiary fold stabilized by a disulfide bridge can be translocated (Tani et al., 1990). Strains carrying a signal sequence suppressor mutation in SecY (prlA) translocate such stabilized structures even without a Δp (Nouwen et al., 1996b). With some precursor proteins, the Δp is required for the initiation of translocation (Nouwen et al., 1996a). Mutations in the signal sequence or early mature region of the preprotein can either relieve or elevate the Δp requirement (Lu et al., 1991; Geller et al., 1993; Nouwen et al., 1996a).

Based on the use of non-hydrolyzable nucleotide ana-
logues (Tani et al., 1989, 1990; Schiebel et al., 1991), and the formation of translocation intermediates that have been arrested by the introduction of a stable tertiary structure in the molecule (Uchida et al., 1995), it has been suggested that translocase mediates preprotein translocation with a step size (quantum) of ~2–2.5 kDa. Both methods, however, rely on the imposition of a synthetic translocation arrest. Here we report on the kinetic step size of an ongoing translocation reaction. A complete catalytic cycle of the SecA ATPase, which involves both binding of the preprotein and binding and hydrolysis of ATP, promotes the translocation of a polypeptide domain that is twice the size of a quantum.

Results

Formation of early intermediates in proOmpA translocation

Translocation intermediates allow a systematic analysis of the energetics of intermediate steps in protein translocation. ProOmpA, the precursor of the outer membrane protein OmpA, is a 336 amino acid polypeptide with an Mr of 38 kDa. Previously, late intermediates of proOmpA translocation have been described that were translocated for ~16, 26 and 29 kDa of the molecular mass (Tani et al., 1989, 1990; Schiebel et al., 1991). These intermediates expose their C-terminus to the outer surface when translocated into inverted inner membrane vesicles (IMVs) of E.coli, and transiently accumulate under conditions where translocation is slow (i.e. low ATP concentration and absence of a Δp). To facilitate the identification of early intermediates, a mutant proOmpA was constructed that contains methionine residues in the N-terminal region only. The three C-terminal Met residues at positions 121, 182 and 300 (Figure 1) were replaced by alanine residues, and an extra Met was introduced in the signal sequence by replacing the Ala at position 13. This mutant proOmpA was termed proOmpAM3 (Figure 1). It translocates as efficiently into E.coli IMVs as does the wild-type protein (data not shown), and was used throughout the subsequent experimental work.

In the absence of a Δp, and at a low ATP concentration, translocation intermediates can be readily detected (Schiebel et al., 1991). [35S]proOmpA, SecA, SecB and urea-extracted IMVs of E.coli, and transiently accumulate under conditions where translocation is slow (i.e. low ATP concentration and absence of a Δp). To facilitate the identification of early intermediates, a mutant proOmpA was constructed that contains methionine residues in the N-terminal region only. The three C-terminal Met residues at positions 121, 182 and 300 (Figure 1) were replaced by alanine residues, and an extra Met was introduced in the signal sequence by replacing the Ala at position 13. This mutant proOmpA was termed proOmpAM3 (Figure 1). It translocates as efficiently into E.coli IMVs as does the wild-type protein (data not shown), and was used throughout the subsequent experimental work.

In the absence of a Δp, and at a low ATP concentration, translocation intermediates can be readily detected (Schiebel et al., 1991). [35S]proOmpA, SecA, SecB and urea-extracted IMVs of E.coli D10 were pre-incubated at 37°C to allow binding of proOmpA to the translocase (Hartl et al., 1990). Next, 2 μM ATP were added to initiate slow translocation. In the presence of an excess of creatine kinase and phosphocreatine, the steady-state ADP concentration was found to be <0.1 μM. The calculated nucleotide occupancy of the SecA NBS-I and -II under these conditions is 92 and 0.5%, respectively (Den Blaauwen et al., 1996). At various times, samples of the reaction were taken and assayed for translocation intermediates by incubation on ice with proteinase K followed by SDS–PAGE using Tricine gels which are optimized for the separation of polypeptides with a molecular mass in the range of 3–30 kDa (Schägger and Von Jagow, 1987). After autoradiography, the successive appearance of proteinase K-protected proOmpA domains of increasing size was evident (Figure 2A). The species with Mr of 16 (doublet of 15 and 17 kDa) and 26 kDa correspond to the intermediates I16 and I26, respectively.
Early stage

and 12 kDa and I26. None of the other protected species are

observed, indicating that they are true kinetic intermediates

(Figure 3B). These data suggest that in analogy with the

late stage of translocation, early stages also proceed

through an ordered series of intermediates, tentatively

termed I2 (doublet of 5 and 7 kDa) and I12 (doublet of 10

and 12 kDa). In addition to the previously characterized

I16 and I26, another, less abundant intermediate can be
detected, termed I17. In the presence of 2 mM ATP and a

Δp, using native D10 IMVs, translocation is extremely

rapid without the apparent appearance of intermediates

(Figure 2C).

**Dissipation of the Δp impedes translocation at an early stage**

The ability to dissipate the Δp selectively with uncoup­lers

was used to determine the role of Δp in the kinetics of

the early stages of translocation. Translocation reactions

were performed with native _E. coli_ D10 IMVs bearing

endogenous SecA. These IMVs generate a Δp in the

presence of ATP through the activity of the _H^+_-translocat­
ing F1F0-ATPase, and exhibit extremely rapid proOmpA

translocation without detectable accumulation of early

intermediates (Figure 3B). When the Δp is dissipated by

the addition of the uncoupler CCCP (Figure 3A)
or a combination of the ionophores nigericin and valinomycin

(data not shown), translocation is slowed down severely,
and protected fragments of 5 and 7 kDa, and at later
stages of 10 and 12 kDa, appear as major intermediate

species. Unlike the urea-extracted _E. coli_ D10 IMVs,

translocation of proOmpA into the native IMVs in the

presence of CCCP or valinomycin/nigericin could not be

restored by the addition of an excess of purified SecA.

Inactivation of the _H^+_-translocating F1F0-ATPase by

DCCD also causes retardation of the rate of translocation,
yielding the same early intermediates (data not shown).

Since I7 is the major species accumulating in the absence

of a Δp, it appears that the initiation of translocation is

only marginally Δp dependent while the following stages

are strongly promoted by the Δp. This is consistent with

the notion that the the Δp is not obligatorily required for

initiation of proOmpA translocation (Geller and Green,


**Intermediates accumulate as doublets at the translocation sites**

Translocation intermediates never appear as a unique

species, but typically as a doublet. These doublets either

represent the processed and non-processed form of a

unique intermediate, or correspond to distinct inter­

mediates that have been translocated to different extents.

To discriminate between these two possibilities, IMVs

were isolated from _E. coli_ D10 harbouring plasmid pTD101
(Date and Wickner, 1981) that allows for a 3-fold over­

production of leader peptidase, LepA (Figure 4C). In urea-

extracted LepA+ IMVs, processing of fully translocated

proOmpA to OmpA is greatly enhanced, but still not

complete (Figure 4A, compare lanes 1 and 2). The

intermediates formed during translocation in the presence

of a low amount (2 µM) of ATP are similar in wild-type

and LepA+ vesicles, with the difference that the intensity

of the upper band in the doublet is diminished in LepA+

vesicles, most strikingly in the case of I16. These data

indicate that the formation of doublets is most likely due
to incomplete processing by leader peptidase.

To assay the involvement of the integral membrane
domain of the translocase, translocation reactions were performed with urea-treated IMVs derived from *E. coli* D10 harbouring plasmid pET324, which allows for the high level overproduction of the SecYEG complex (van der Does *et al.*, 1996; Figure 4D). The rate of proOmpA translocation was enhanced in SecYEG+ IMVs (Figure 4A, compare lanes 1 and 3; Douville *et al.*, 1995; Van der Does *et al.*, 1996), but the major intermediates, i.e. I12, I16 and I21, were formed with a much higher yield as compared with IMVs of the parental strain (Figure 4B, compare lanes 1 and 3). These data support the notion that intermediates accumulate at the translocation sites.

### Azide traps SecA in a membrane-inserted state and induces the accumulation of novel intermediates

Sodium azide (NaN₃) is a potent inhibitor of protein translocation, although *in vitro* the inhibition is not complete (Oliver *et al.*, 1990). NaN₃ selectively blocks the preprotein-stimulated ATPase activity of SecA (translocation ATPase), and thus provides a convenient method to reduce the activity of SecA. Translocation reactions were performed with IMVs derived from strain KM9 that lacks the entire *unc* operon. These vesicles do not generate a Δp in the presence of ATP but can be energized with an oxidizable substrate such as NADH. In the presence of excess SecA and high ATP (2 mM), translocation of proOmpA into *E. coli* KM9 IMVs appeared to be less dependent on the presence of a Δp (i.e. addition of NADH) as compared with *E. coli* D10 IMVs. Under these conditions, only a few intermediates are detectable after 10 min of translocation (Figure 5, lanes 5 and 7). Addition of 20 mM NaN₃ severely retards but does not completely block translocation (lanes 6 and 8), giving rise to intermediates. Measurements of the magnitude of the transmembrane electrical potential (ΔΨ), inside positive, and pH gradient (ΔpH), inside acid, as measured with the fluorescent dyes oxonol VI and ACMA, respectively (not shown), show that the NaN₃ does not interfere with the Δp generation. Remarkably, when NaN₃ is present, the pattern of intermediates deviates from that typically observed. In addition to the previously described I12, I16, I18 and I21, two extra abundant intermediates are observed, with estimated sizes of 13–15 and 23 kDa (indicated by asterisks, lanes 6 and 8). NaN₃ blocks proOmpA translocation completely at a low ATP concentration, i.e. 2 µM. In the absence of a Δp, the typical intermediates are formed after 10 min of translocation (Figure 5, lane 1), while the presence of a Δp dramatically stimulates translocation (lane 3). Under both conditions, NaN₃ prevents the formation of full-length proOmpA (lane 2 and 4), and, although much weaker, new intermediates were formed. These results indicate that NaN₃ interferes with the cycle of ATP- and Δp-driven translocation, possibly by trapping the translocase in a transition stage yielding novel intermediates.

To determine how NaN₃ affects the catalytic cycle of SecA membrane insertion and de-insertion, its effect on the formation of the protease-resistant and membrane-protected 30 kDa SecA fragment was investigated under translocating conditions, i.e. with urea-treated SecYEG+ IMVs, proOmpA, 2 mM ATP and 125I-labelled SecA (2.5 µg/ml) (Economou and Wickner, 1994). NaN₃ does not inhibit, but rather stimulates the formation of 30 kDa SecA fragment (Figure 6, compare lanes 2 and 5), suggesting that NaN₃ stabilizes the membrane-inserted state of SecA. SecA membrane de-insertion can be measured in a chase experiment in which a 20-fold excess of non-labelled SecA is added to membranes bearing inserted [125I]SecA. Under translocating conditions, the inserted [125I]SecA is displaced rapidly by non-labelled SecA, which is evident from the disappearance of the 30 kDa SecA fragment after protease digestion (Figure 6, lane 3). NaN₃ efficiently prevents this chase (lanes 4 and 6), and this effect is even more pronounced when excess NaN₃-treated SecA is added (lane 7). These data suggest that NaN₃ inhibits preprotein translocation by trapping the SecA in a membrane-inserted state.

### Discussion

Preprotein translocation across the inner membrane of *E. coli* requires two energy sources, ATP and the Δp...
outside. Our specifically \(\text{[35S]}\)methionine-labelled pro-
non-translocated C-terminus to protease added from the
I7, I12, I16, I21 and I26. The late intermediates I16 and I26
intervals of ~5 kDa. Depending on the translocated poly-
SDS–PAGE analysis and comparison with molecular mass
series of discrete intermediates accumulate. Based on the
preprotein proOmpA is slow. Under those conditions, a
SecA is dramatically retarded and the translocation of the
subsaturating concentrations of ATP, the catalytic cycle of
preprotein translocation is fast and intermediates in trans-
with high and low affinity, respectively (Mitchell and
binding sites, i.e. NBS-I and NBS-II, that bind nucleotides
mediated and ATP-driven translocation.
The SecYEG-bound form of SecA performs a key
role in the preprotein translocation process. It binds the
preprotein by direct recognition of the signal sequence and mature domain (Cunningham and Wickner, 1989; Lill
et al., 1990), and uses the energy of ATP binding and
hydrolysis to drive the preprotein translocation reaction (Schiebel et al., 1991). SecA has two essential ATP-
binding sites, i.e. NBS-I and NBS-II, that bind nucleotides with high and low affinity, respectively (Mitchell and
Oliver, 1993). When both NBSs are saturated with ATP, preprotein translocation is fast and intermediates in trans-
location accumulate only at a late stage. At NBS-II subsaturating concentrations of ATP, the catalytic cycle of
SecA is dramatically retarded and the translocation of the preprotein proOmpA is slow. Under those conditions, a
series of discrete intermediates accumulate. Based on the
SDS–PAGE analysis and comparison with molecular mass
standards, translocation intermediates seem to appear in intervals of ~5 kDa. Depending on the translocated poly-
peptide mass, these intermediates are tentatively named:
I\(_5\), I\(_{12}\), I\(_{16}\), I\(_{21}\) and I\(_{26}\). The late intermediates I\(_{16}\) and I\(_{26}\) have been described before (Tani et al., 1989, 1990; Schiebel et al., 1991), and in inverted IMVs expose their
non-translocated C-terminus to protease added from the
outside. Our specifically \([35S]\)methionine-labelled pro-
OmpA molecule, in combination with a high resolution
Tricine gel system, allowed the detection of the early intermediates I\(_3\) and I\(_{12}\) that after protease digestion occur as short polypeptides. In addition, a weak late intermediate I\(_{21}\) could be detected. The systematic occurrence of intermediates in intervals of ~5 kDa suggests that the functional step size of translocation may be larger than the 2–2.5 kDa translocation elicited by the binding of a
non-hydrolysable ATP analogue to SecA (Schiebel et al.,
1991). However, the latter step size is determined under
conditions where the catalytic cycle of the translocase is
blocked as hydrolysis of the SecA-bound ATP is not
possible. Hydrolysis of ATP is needed to release the
preprotein from SecA, whereupon SecA can re-enter the
translocation reaction by binding the non-translocated portion of the preprotein. Schiebel et al. (1991) have
shown that this re-binding reaction already permits the
translocation of a 2–2.5 kDa polypeptide segment. Sub-
sequent binding of a non-hydrolysable ATP analogue to
SecA effects the translocation of another 2–2.5 kDa of the
polypeptide mass (Schiebel et al., 1991). Uchida et al.
(1995) have shown that the translocation of proOmpA can be arrested at intervals of ~2.5 kDa by the introduction of
disulfide bridges in the molecule. Upon oxidation, a
stable tertiary structure is formed in the molecule that
cannot be translocated. This observation has led to
the suggestion that the catalytic cycle of the translocase allows the translocation of ~2.5 kDa, superficially termed a

\[\text{Fig. 7. Model for the stepwise mode of action of precursor protein translocation. See Discussion for details.}\]

quantum. Since this estimate is based on a synthetically
imposed translocation arrest, it cannot be ascertained that
the estimated step size indeed reflects an entire catalytic
cycle of the translocase. Rather, it agrees with the step
size defined for the partial reactions as described by

Based on the above considerations, we propose that a
complete catalytic cycle of SecA, i.e. binding and hydro-
lysis of ATP, involves two distinct translocation steps
that together permit ~5 kDa of translocation progress (Figure 7). Upon ATP binding, the SecA bound to the partially
translocated polypeptide chain will translocate ~2.5 kDa
of the precursor protein concomitant with the insertion
of a domain of SecA into the membrane (Figure 7, step 1).
This insertion mechanism for SecA was proposed based
on the finding that a 30 kDa C-terminal domain of SecA
becomes protease-resistant when SecA binds ATP and
preprotein (Economou and Wickner, 1994; Economou et al., 1995). Hydrolysis of ATP subsequently relieves the
protease resistance of the SecA domain, and this process
has been attributed to the de-insertion of the SecA domain
(Economou and Wickner, 1994) (Figure 7, step 2) while
it releases the bound precursor protein. At that stage,
SecA can re-bind to the exposed portion of the partially
translocated preprotein, and this step allows the transloca-
tion of another 2.5 kDa polypeptide segment (Figure 7,
step 3). Therefore, translocation will only be partly driven
by the SecA co-insertion reaction while the remainder
results from the preprotein binding reaction and an associ-
ated conformational change of SecA.

Particularly interesting is the impact of azide on the
catalytic cycle of the translocase. Azide inhibits transloca-
tion by blocking the translocation ATPase activity of SecA
(Oliver et al., 1990). It does not interfere with binding of
nucleotides at NBS-I, as it did not affect the photocross-
linking of \([\alpha-32P]\)ATP to NBS-I and the formation of the
staphylococcal protease V8-resistant conformation of
SecA in the presence of a NBS-I saturating concentration
of ATP or ATPyS (J.van der Wolk, unpublished data).
NBS-I has been implicated in the ATP-dependent mem-
brane insertion/de-insertion of SecA (Economou et al.,
1995). Consistent with these findings is the observation
that azide does not diminish the ATP- and preprotein-
dependent formation of the proteinase-resistant 30 kDa
SecA fragment. Rather, it promotes the formation of the
30 kDa SecA fragment, indicating that azide stabilizes the
membrane-inserted state of SecA. On the other hand,
azide blocks the de-insertion reaction as monitored by the ATP-dependent chase of membrane-inserted $^{125}$I-labelled SecA by an excess of unlabelled SecA. This step requires hydrolysis of ATP at NBS-I (Economou et al., 1995) and, therefore, it seems that azide inhibits this reaction specifically. Strikingly, in the presence of azide, a number of new intermediates are observed that are uniquely positioned in between the stable intermediates. In the presence of azide, intermediates appear to be spaced at intervals of ~2.5 kDa instead of 5 kDa. Since azide traps the SecA in a transitional state during the translocation reaction, these new intermediates may resemble such transitional states as well. This is expected, for instance, when the ATP-dependent membrane insertion of SecA is a rate-determining step in translocation. In the absence of azide, intermediates will accumulate at the step that precedes the SecA membrane insertion, and thus mainly result from the translocation progress effected by SecA preprotein binding. However, in the presence of azide, ATP hydrolysis is blocked and SecA is retained in the membrane-inserted state for longer times, yielding intermediates associated with this state. The distribution, i.e. the intensity of the various intermediates, is determined by the relative rates of SecA membrane insertion, de-insertion and preprotein release, and the preprotein (re-)binding reaction. The observation that azide reduced the ‘apparent’ step size 2-fold is in agreement with a two-step translocation reaction as discussed above.

Recently, it was suggested that the stepwise translocation of proOmpA is caused solely by the short hydrophobic segments present in proOmpA (Sato et al., 1997). Due to the association with the translocase, such hydrophobic segments would retard translocation and give rise to temporarily arrested or delayed states. However, stepwise translocation of proOmpA derivatives devoid of hydrophobic segments also occurred and, albeit with lower efficiency, the intermediates $I_{160}$, $I_{210}$, $I_{260}$, and the artificially constructed $I_{320}$ were prominently present. The phenomenological step size of translocation is probably not fixed but irregular, due, for example, to hysteresis movements of the partially translocated polypeptide chain (Schiebel et al., 1991; Driessen, 1992). Such movements may be brought about by differences in the hydrophobicity of the translocating segments (Sato et al., 1997) and/or the folding of translocated domains (Arkowitz et al., 1993). The mechanistic step size, however, must be a fixed value as this is determined by the cognitive characteristics of the ATP-dependent molecular motor that drives translocation. In this respect, SDS–PAGE analysis only provides an estimate of the step size.

The mechanism by which $\Delta p$ stimulates translocation has remained obscure as it appears to affect multiple stages of the translocation reaction (Driessen, 1994). $\Delta p$ can drive the efficient translocation of large polypeptide domains in the absence of SecA association (Schiebel et al., 1991), suggesting that the step size may be discontinuous when both energy sources are present. However, this efficient $\Delta p$-driven translocation reaction has been observed under artificial conditions, i.e. when SecA is removed from the translocation sites. In the presence of SecA, ATP and a $\Delta p$, translocation is extremely fast and seems to occur without the appearance of intermediates. However, it remains to be established if the ATP-driven translocation of proOmpA in the presence of a $\Delta p$ is indeed a continuous process, rather than being stepwise as observed for SecA-mediated translocation alone. In contrast to native E. coli D10 IMVs, the stimulatory effect of a $\Delta p$ with KM9 IMVs is only pronounced when either SecA or ATP is present at limiting concentrations. Dissipation of the $\Delta p$ with CCCP or the ionophores valinomycin and nigericin blocks translocation at a very early stage in D10 IMVs, supporting the notion that the late stages of proOmpA translocation are indeed $\Delta p$-dependent (Schiebel et al., 1991). Again, this phenomenon is less evident with KM9 IMVs. The exact reason for this discrepancy is not clear. In this respect, the cold-sensitive phenotype of $\Delta secG$ null mutants has been reported to be strain dependent (Bost and Belin, 1995) and, in some genetic backgrounds, the growth defect of this mutation is only manifested when the unc genes coding for the F$_{1}$F$_{0}$-ATPase are also deleted (Duong and Wickner, 1997a). Moreover, SecG appears to be critical for translocation in the absence of a $\Delta p$ (Nishiyama et al., 1994, 1996). Our observations of the difference in $\Delta p$ dependency of translocation into KM9 and D10 IMVs may be related to the function of SecG.

In conclusion, SecA-driven preprotein translocation is a stepwise process. Each turnover of the translocase, which involves binding and hydrolysis of ATP, results in the translocation of ~40–50 amino acid residues of the preprotein across the membrane. This process is driven by two distinct and consecutive enzymatic steps utilizing respectively the energies of preprotein and ATP binding to SecA.

### Materials and methods

#### Bacterial strains and growth conditions

Unless indicated otherwise, E. coli strains D10 (nuA10, relA1, spoT1, metE1B) and KM9 (unc::Tn10, relA1, spoT1, metE1B) (Klionsky et al., 1984) were grown at 37°C in Luria Bertani (LB) broth supplemented with 100 µg/ml of ampicillin, 0.5% (w/v) glucose, or 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), as required. Cloning and plasmid constructions were done in E. coli JM101 or JM110 (Yanisch-Perron et al., 1985). Uracil-containing single-stranded template DNA for oligo-directed mutagenesis was obtained by growing the corresponding M13KO7 helper phage on E. coli C1236 (Kalmegh et al., 1988). Overexpression of the SecYEG complex and leader peptidase (LepA) was in E. coli strain D10 harbouring plasmid pET324 (Van der Does et al., 1996) or pTD101 (Date and Wickner, 1981), respectively.

#### Biochemicals

SecA (Cabelli et al., 1988), SecB (Weiss et al., 1988), proOmpA (Crooke et al., 1988) and SecYEG (Brandage et al., 1990) were purified as described. [$^{35}$S]proOmpA was synthesized from plasmid pET033 using an in vitro transcription–translation reaction (De Vrije et al., 1987), and affinity-purified as described (Crooke and Wickner, 1987). SecA was iodinated with Na$^{125}$I as described (Economou and Wickner, 1994; Den Blaauwen et al., 1997). Inverted IMVs were prepared from E. coli strain D10 and KM9 by the procedure of Chang et al. (1978) and, when indicated, treated with 6 M urea (Cunningham et al., 1989).

#### DNA manipulation and oligonucleotide-directed mutagenesis

The procedure used for site-directed mutagenesis of the ompl4 gene was essentially as described by Kunkel et al. (1987). Mismatch oligonucleotides, resulting amino acid substitutions and the introduction of restriction endonuclease digestion sites are shown in Table I. The EcoRI-PstI restriction fragment of pRD87 harbouring the ompl4 gene was cloned in pUC18 to yield pET149. This plasmid was used to express wild-type proOmpA. Mutagenesis was done on single-stranded DNA containing the HinCl-BamHI and BamHI–PstI restriction fragments of pET149,
Table I. Mismatch primers used for oligonucleotide-directed mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amino acid substitution</th>
</tr>
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<tbody>
<tr>
<td>OMW13</td>
<td>5′-AGTGCACTTATGCTGGCTGCTAACACG-3′</td>
<td>Ala121Met</td>
</tr>
<tr>
<td>OMW121</td>
<td>5′-TGACCCCTGATGCGCAGCAAGCG-3′</td>
<td>Met121Ala</td>
</tr>
<tr>
<td>OMW182</td>
<td>5′-ACCCGTCAGTCGCCTGGCG-3′</td>
<td>Met300Ala</td>
</tr>
<tr>
<td>OMW300</td>
<td>5′-GTGTGATTGCGGCCCTGCTCCTGCGAGATCTTG-3′</td>
<td>Met300Ala</td>
</tr>
</tbody>
</table>

Nucleotides in bold and underlined mark the mismatches in the DNA sequence of the oligonucleotides and the introduced NalI endonuclease restriction sites, respectively.

which were cloned in the pBluescript KS I (+/SK (+) series (Stratagene Cloning Systems, La Jolla, CA). Mutagenized fragments were reintroduced into pET149, from which the corresponding HinII–PstI fragment was removed, to yield pET23 (Figure 1) in which Met121, Met182 and Met300 have been replaced by Ala.

Replacement of Ala13 in proOmpA by Met was done by two-step PCR as described by Landt et al. (1990). A 0.3 kb HinII–EcoRI restriction fragment from pRD87 was cloned into the EcoRV site of pBluescript KS I (+). The resulting plasmid pET133 was used as a template to amplify a 171 bp DNA fragment corresponding to the 5′-end of the ompA gene using the oligonucleotide forKS (5′-TGAGTACCG-GGCCCTTTCTC-3′) as 5′-primer and OMW12 (Table I) as mismatch primer. The resulting DNA fragment was isolated and, together with revKS (5′-GAAGATTTAGGGCCCGCTCCCGG-3′), used in a second PCR as primer, in which pET133 again was used as a template. From the resulting 384 bp PCR product, a 225 bp HinII–PstI restriction fragment was isolated, and ligated into pET23, from which the corresponding HinII–PstI fragment was removed. The resulting plasmid pET033 was used to express the modified proOmpA3 protein (Figure 1). All mutagenized DNA fragments were sequenced on a Vistra DNA sequencer 725 (Amersham, Buckinghamshire, UK).

In vitro translocation of ProOmpA

In vitro translocation of [35S]proOmpA (in 50 µl) was performed at 37°C as described (Cunningham et al., 1989) with 20 µg/ml of SecA, 32 µg/ml of SecB, 1 µl of area-denatured [35S]proOmpA, 10 mM phosphocreatine and 50 µg/ml creatine kinase in buffer B [50 mM HEPES KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml bovine serum albumin (BSA)], 10 mM dithiothreitol and 2 mM Mg(OAc)2. Escherichia coli D10 or KM9 inverted IMVs were added to a final concentration of 300 µg/ml and, as indicated, the reactions were initiated by the addition of 2 µM or 2 mM ATP. At various time points, translocation was terminated by chilling on ice. Samples were treated with proteinase K (0.1 mg/ml) for 15 min on ice, precipitated with 7.5% (w/v) trichloroacetic acid, washed with ice-cold acetone and solubilized in SDS sample buffer. Samples were analysed by 16% High Tricine SDS–PAGE (Schägger and Von Jagow, 1987) to separate polypeptide fragments in the range of 3–30 kDa or, when indicated, by conventional 12% SDS–PAGE. Gels were dried and exposed to Kodak Biomax MR film. Autoradiograms were scanned densitometrically using a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and analysed using SigmaScan/Image Jandel Corp., San Rafael, CA).

In separate translocation reactions in which the [35S]proOmpA was replaced by unlabelled proOmpA, the concentrations of ATP and ADP were determined by including a trace amount of [γ-32P]ATP. Radiolabelled nucleotides were separated on thin-layer chromatography using Polygram gel300 P11UV254 plates (Machery-Nagel, Düren, Germany) and 0.65 M KH2PO4 as eluents. After air-drying, spots were UV-irradiated and visualized by autoradiography. Autoradiograms were scanned using a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and analysed using SigmaScan/Image Jandel Corp., San Rafael, CA).

Other techniques

Protein determination was performed according to Lowry et al. (1951) with BSA as standard. Generation of a Δψ (inside positive) and Δψ (inside negative) was followed with the fluorescent indicators bis(3-propyl-5-oxoisooaxazol-4-yl)pentamethine oxonol (Oxonol VI) (Ex/Em, 595 nm/634 nm) (Apell and Biersch, 1987) and 9-amino-6-chloro-2-methoxycridine (ACMA) (Ex/Em, 409 nm/474 nm) (Klionsky et al., 1984). The nucleotide occupancy of NBS-I and -II was calculated as described by Den Blaauwen et al. (1996) using Kd values of 0.15 and 340 µM, respectively. The insertion and de-insertion of SecA were monitored by following the formation of the 30 kDa trypsin-protected fragment of 125I-labelled SecA (Economou and Wickner, 1994; Den Blaauwen et al., 1997).

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References


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