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Precursor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive force

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The SecY/E protein of *Escherichia coli* was coreconstituted with the proton pump bacteriorhodopsin and cytochrome *c* oxidase yielding proteoliposomes capable of sustaining a protonmotive force (Δp) of defined polarity and composition. Proteoliposomes support the ATP- and SecA-dependent translocation of proOmpA which is stimulated by a Δp, inside acid and positive. Δp of opposite polarity, inside alkaline and negative, suppresses translocation while SecA-mediated ATP hydrolysis continues unabated. Δψ and ΔpH are equally effective in promoting or inhibiting translocation. Membrane-spanning translocation intermediates move backwards in the presence of a reversed Δp. These results support a model [Schiebel,E., Driessen,A.J.M., Hartl,F.-U. and Wickner,W. (1991) *Cell*, 64, 927-939] in which the Δp defines the direction of translocation after ATP hydrolysis has released proOmpA from its association with SecA. The polarity effect of the Δp challenges models involving Δp-dependent membrane destabilization and provides further evidence for a role of the Δp as driving force in precursor protein translocation.

Key words: protonmotive force/secretion/SecA/SecE/SecY

Introduction

Most proteins that reside in the periplasmic space or outer membrane of *Escherichia coli* are synthesized as precursors with an N-terminal extension, the signal peptide. Export across the inner membrane proceeds via the general secretion pathway (Wickner *et al.*, 1991) which involves components encoded by the *sec*(retion) genes (Schatz and Beckwith, 1990). SecB is a molecular chaperon that stabilizes preproteins for translocation (Kumamoto, 1991) and directs them to the membrane surface (Hartl *et al.*, 1990). SecA is a peripheral membrane protein and performs a pivotal role in translocation (Oliver *et al.*, 1990; Wickner *et al.*, 1991). SecA functions as a membrane receptor for the SecB-preprotein complex by virtue of its affinity for SecB (Hartl *et al.*, 1990) and both the signal and mature domain of preproteins (Cunningham and Wickner, 1989; Lill *et al.*, 1990; Akita *et al.*, 1990). Acidic phospholipids are required for translocation (De Vrije *et al.*, 1988; Lill *et al.*, 1990; Kusters *et al.*, 1991) and allow SecA to insert into the lipid bilayer (Breukink *et al.*, 1991). SecY (Ito, 1984) and SecE (Schatz *et al.*, 1989) are integral membrane proteins which physically interact (Brundage *et al.*, 1990; Bieker and Silhavy, 1990; Tokuda *et al.*, 1991) and provide a functional membrane binding site for SecA (Hartl *et al.*, 1990; Fandl *et al.*, 1988). Together they form an oligomeric membrane protein complex, the preprotein translocase of *E.coli* (Brundage *et al.*, 1990). SecA hydrolyses ATP when activated by its interaction with the SecY/E protein (Lill *et al.*, 1989), acidic phospholipids (Lill *et al.*, 1990) and preproteins (Cunningham and Wickner, 1989; Lecker *et al.*, 1989). SecD and SecF are inner membrane proteins (Gardel *et al.*, 1990) which may function at some late step in export (Schatz and Beckwith, 1990).

Translocation requires two energy sources, ATP and the protonmotive force (Δp) (Tai, 1990). ATP is an imperative energy source (Chen and Tai, 1985), while Δp affects the rate of the reaction (Geller *et al.*, 1986). Translocation occurs through consecutive steps each with distinct energy requirements (Tani *et al.*, 1989, 1990; Geller and Green, 1989; Schiebel *et al.*, 1991). ATP binding to SecA allows the translocation of a small domain of the preprotein, whereas hydrolysis releases the preprotein from its association with SecA and permits Δp-dependent translocation (Schiebel *et al.*, 1991). In the absence of a Δp, multiple cycles of rebinding and release promote membrane transit of the complete preprotein. Though the role of the Δp in translocation is not well understood, it is performed by the total Δp (Bakker and Randall, 1984; Driessen and Wickner, 1991) and is different from that of ATP hydrolysis (Schiebel *et al.*, 1991).

Translocation of preproteins has been reconstituted with purified soluble and membrane components (Brundage *et al.*, 1990; Akimara *et al.*, 1991) and now allows a quantitative analysis of the role of the Δp. The effect of Δp was studied with respect to its orientation, composition and magnitude. The results support a role of the Δp as driving force and indicate that ATP hydrolysis and translocation are uncoupled processes. A model of the energetics of intermediate stages of preprotein translocation is presented.

Results

Reversed Δp inhibits translocation

We have previously reported the reconstitution of SecA-dependent preprotein translocation into proteoliposomes bearing purified SecY/E protein (Brundage *et al.*, 1990). This reaction requires ATP and is stimulated by the Δp, inside positive and acid. To examine the role of the Δp in more detail, SecY/E protein was coreconstituted with the light-driven H^+-pump bacteriorhodopsin (bR). Upon illumination, a Δp was generated, inside acid and positive (data not shown), which stimulates the initial rate of proOmpA translocation up to 7-fold (Figure 1, lanes 2 and 3). Translocation required ATP (lane 1) and SecA protein (data not shown). The effect of light was prevented by the uncoupler 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S13) (lane 4). SecY/E protein was also coreconstituted with cytochrome *c* oxidase (CytOx), a redox-linked H^+-pump. Only CytOx molecules reconstituted with their

cytochrome *c* binding site exposed to the outer membrane surface are able to accept electrons from reduced cytochrome *c*. This guarantees the exclusive generation of a Δp, inside alkaline and negative (Driessen and Konings, 1991). Translocation was followed for a longer period to allow sufficient accuracy in the assessment of the effect of the reversed Δp. Translocation was almost completely suppressed in the presence of reduced cytochrome *c* (lanes 6 and 7). Suppression was alleviated by the protonophore S13 (lane 8). These data establish a critical polarity effect of the Δp on the translocation reaction.

Non-linear relation between Δp and rate of translocation

The role of the Δp was further analysed at a quantitative level. bR−SecY proteoliposomes were illuminated with a saturating light intensity in the presence of different amounts of S13 to vary the magnitude of Δp. In the absence of S13, the transmembrane electrical potential (Δψ) and transmembrane pH gradient (ΔpH) equalled +12 mV and −0.9 pH units, respectively. Complete dissipation of the Δp required 9 nM S13. The proOmpA translocation rate was determined by quantitative immunoblot analysis. The rate of translocation decreased with increasing S13 concentration, and varied in a non-linear manner with the Δp (Figure 2A). The Δp of reversed polarity was studied in an analogous manner using CytOx−SecY/E proteoliposomes. Instead of assessing the initial rate of translocation, the level of translocation was determined after 40 min. In the absence of S13, Δψ and ΔpH were equal to −76 mV and 0.2 pH units, respectively. Approximately 45 nM S13 was needed to collapse Δp. Efficient inhibition of translocation required high Δp values (Figure 2B).

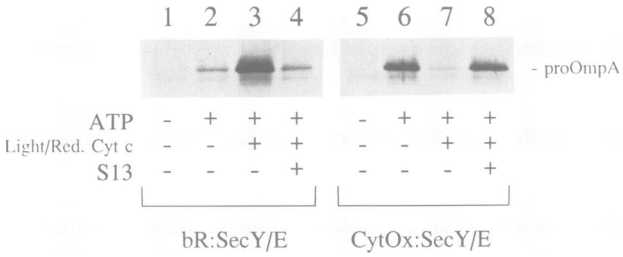

Fig. 1. Reversed Δp inhibits translocation. ProOmpA translocation into SecY/E proteoliposomes coreconstituted with bR (lanes 1−4) and CytOx (lanes 5−8) was assayed in the presence of purified SecA (10 μg/ml) and SecB (40 μg/ml) protein, and where indicated, 2 mM ATP. bR−SecY/E proteoliposomes were illuminated to generate a Δp, inside acid and positive (lanes 3 and 4). Control samples were wrapped in aluminum foil to prevent stimulation by stray light (lanes 1 and 2). CytOx−SecY/E proteoliposomes were energized with cytochrome *c*/ascorbate/TMPD to generate a Δp, inside alkaline and negative (lanes 7 and 8). Lanes 4 and 8 received 100 nM S13. Only the relevant part of the immunoblots is shown. Further experimental details are as described in Materials and methods.

Δψ and ΔpH are equally effective

Ionophores can be used to analyse the role of Δψ and ΔpH independently. With bR−SecY/E proteoliposomes, selective dissipation of Δψ and ΔpH was accomplished with the Na^+-ionophore non-actine and the Cl^-/OH^- exchanger triphenyltin, respectively. The magnitude of Δp was varied with S13. Δψ (Figure 2A, ●) and ΔpH (▽) exert similar effects on the rate of proOmpA translocation. With CytOx−SecY/E proteoliposomes, the ionophores valinomycin and nigericin were used to collapse Δψ and ΔpH, respectively. The inhibitory effect of a reversed Δp revealed no significant preference with respect to its composition (Figure 2B). These results suggest that Δψ and ΔpH are equivalent as to their effect on the rate of proOmpA translocation.

Coupling between ATP hydrolysis and translocation is affected by Δp

Translocation requires the hydrolysis of ATP. The enhancement of ATP hydrolysis by SecA upon interaction

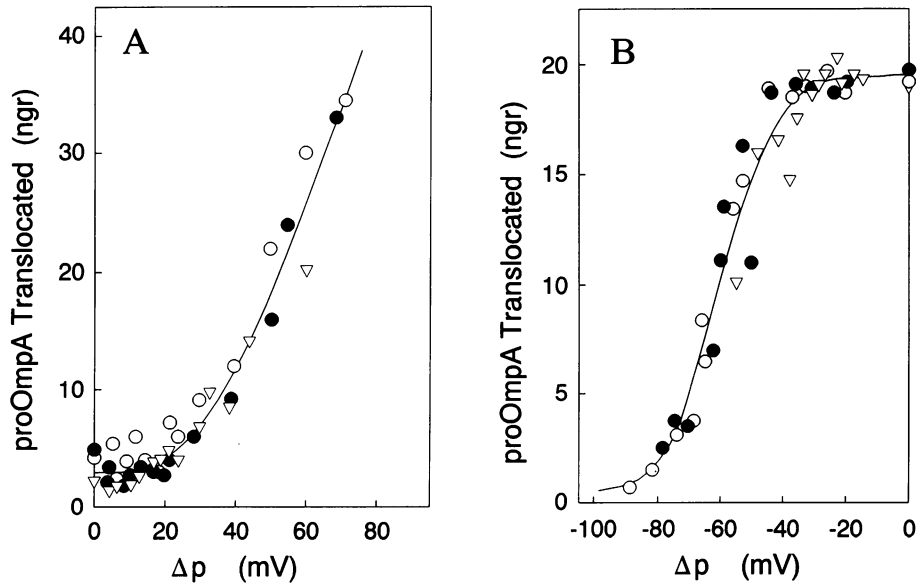

Fig. 2. Relation between the Δp and translocation. (**A**) bR−SecY/E proteoliposomes were illuminated with a saturating light intensity in the presence of S13 (0−20 nM) to vary the magnitude of the Δp, inside positive and acid. Translocation was determined in the absence (○; Δp) or presence of 25 nM triphenyltin (●; Δψ) or 100 nM non-actin (▽; −ZΔpH). Reactions were initiated by the addition of proOmpA (4 μg/ml) and arrested after 5 min. (**B**) CytOx−SecY/E proteoliposomes were energized with cytochrome *c*/ascorbate/TMPD. S13 (0−75 nM) was used to vary the magnitude of the Δp, inside alkaline and negative. ProOmpA translocation was assayed in the absence (○; Δp) or presence of 10 nM nigericin (●; Δψ) or 100 nM valinomycin (▽; −ZΔpH). Reactions were terminated after 40 min. Further experimental details are as described in Materials and methods.

with SecY/E, acidic phospholipids and preproteins is the 'translocation ATPase' (Lill *et al.*, 1989). Translocation ATPase was assayed in the absence and presence of a Δp.

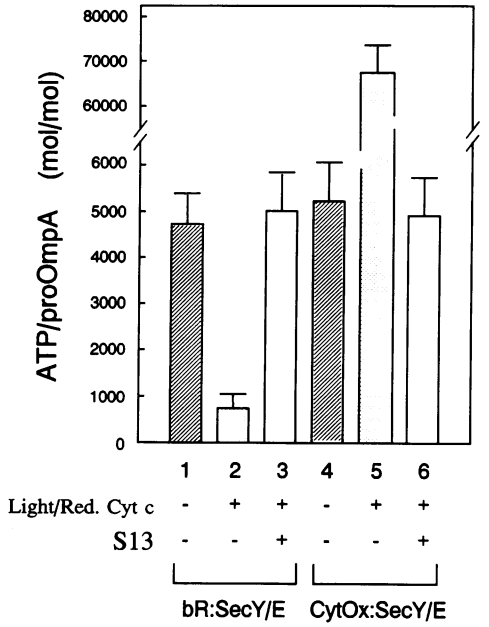

Fig. 3. Coupling between ATP hydrolysis and translocation is affected by Δp. The rate of proOmpA translocation and translocation ATPase activity of bR−SecY/E and CytOx−SecY/E proteoliposomes was assayed in the absence (lanes 1 and 4) and presence of light (lanes 2 and 3) or reduced cytochrome *c* (lanes 5 and 6). Lanes 3 and 6 received 100 nM S13. Reactions were performed as described in Materials and methods and started by the addition of proOmpA (20 μg/ml). Samples of 100 μl were removed after 1, 2, 3, 5 and 10 min, chilled on ice, and assayed for translocation ATPase (Lill *et al.*, 1989) and proOmpA translocation. Translocation ATPase activity is the difference in the amount of inorganic phosphate released in the presence and absence of proOmpA. The ATP/proOmpA ratio with indicated SE (n = 3) was calculated from the rate of ATP hydrolysis and proOmpA translocation.

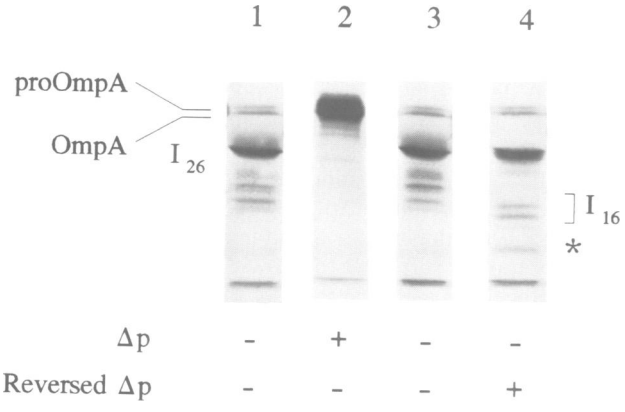

Fig. 4. Reversed translocation of membrane-spanning intermediates. [^{35}S]methionine-labelled proOmpA was translocated into *E.coli* membrane vesicles to yield I_{26} as described in Materials and methods. Membrane vesicles loaded with K-acetate were diluted into K-acetate (no Δp; lane 1) or Na-gluconate solution (Δp, inside negative and alkaline; lane 2) in the presence of valinomycin. In another experiment, Na-gluconate loaded membrane vesicles were diluted into Na-gluconate (no Δp; lane 3) or K-acetate solution (Δp, inside positive and acid; lane 4). Translocation reactions were carried out at 37°C for 15 min. I_{26} and I_{16} are major intermediates with an apparent molecular mass on SDS−PAGE after proteinase digestion of 26 and 16 kDa, respectively. * denotes a novel intermediate which is formed in the presence of a reversed Δp.

Δp of either polarity had a profound effect on the efficiency of translocation (see Figure 1) and only a minor effect on the translocation ATPase (data not shown). ProOmpA translocation and translocation ATPase activities were correlated to obtain an apparent coupling stoichiometry (Figure 3). Translocation was more efficiently coupled to the hydrolysis of ATP in the presence of a Δp, inside positive and acid, than in its absence (lanes 1 and 2). A Δp of opposite polarity strongly reduced the degree of coupling (lane 5). The effect of the Δp on the apparent coupling stoichiometry was reversed by S13 (lanes 3 and 6). Similar results were obtained with the $\Delta\psi$ or ΔpH as sole component of the Δp (data not shown). These results suggest that Δp allows a more efficient coupling between translocation and ATP hydrolysis.

Reversal of translocation of membrane-spanning intermediates

Translocation intermediates of proOmpA are readily detected at low ATP concentration (Schiebel *et al.*, 1991). Major intermediates formed at low ATP levels are I_{16} and I_{26} which can be chased to full-length translocated proOmpA or OmpA in the presence of a high concentration of ATP or a Δp (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991). Membrane vesicles bearing I_{26} were centrifuged through a sucrose solution to remove ATP. I_{26} (Figure 4, lane 1) and several earlier intermediates continued translocation upon imposition of an artificial Δp, inside negative and alkaline (lane 2). A Δp, inside positive and acid, causes the earlier intermediates (lane 3) to reverse translocate (lane 4) yielding I_{16} and a novel intermediate (designated as *) with an apparent molecular mass remaining after proteinase digestion of ~10 kDa. Most of the I_{26} remained at its position in the presence of a reversed Δp. I_{26} is stabilized by its association with SecA, and reverses its translocation when SecA is inactivated (Schiebel *et al.*, 1991). The transient nature of the imposed Δp, which is especially prominent with a reversed Δp (data not shown), and the binding of the membrane-spanning translocation intermediates to SecA or other components of the export system (Schiebel *et al.*, 1991) most likely precludes further reversal. All intermediates were chased to full-length proOmpA and OmpA upon the addition of ATP (data not shown). These results indicate that intermediate stages of translocation are reversible, while the direction of movement is determined by the polarity of the Δp.

Discussion

To define the mechanism of preprotein translocation across the inner membrane of *E.coli*, we developed a reconstituted preprotein translocation reaction using purified components (Brundage *et al.*, 1990). The focus of this study is to understand the role of the Δp at a quantitative level. Using different Δp-generating proteins, a reconstituted translocation reaction allows changes in polarity of the Δp and a quantitative assessment of its effects. The results suggest that Δp specifies the direction of translocation and acts as a driving force. The data confirm previous observations (Schiebel *et al.*, 1991) that hydrolysis of ATP and translocation are not coupled. The non-linear relation between Δp and proOmpA translocation has important implications for the comparison of *in vivo* and *in vitro*

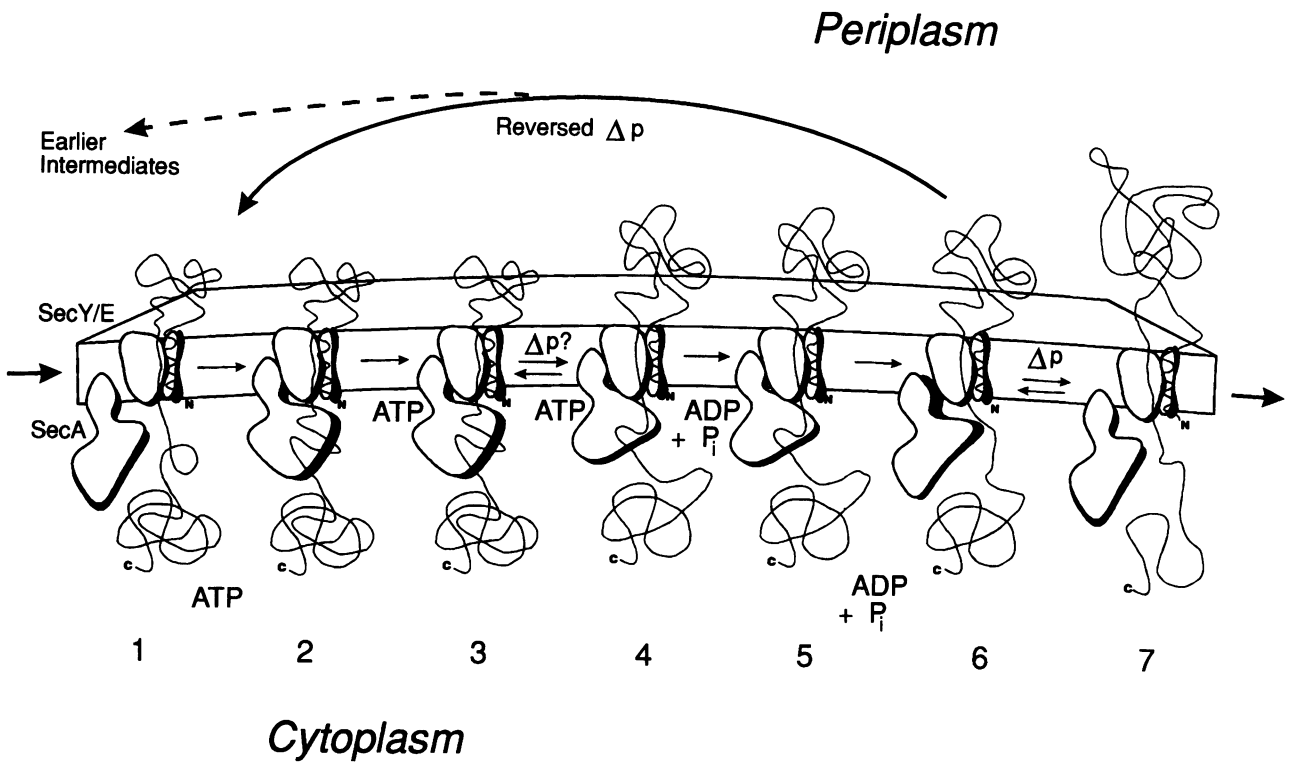

Fig. 5. Hypothetical model of the intermediate stages of Sec-mediated preprotein translocation. The translocase consists of the peripheral membrane protein, SecA (Lill *et al.*, 1989) which binds with high affinity to the membrane at the integral membrane protein SecY/E (Hartl *et al.*, 1990) and acidic phospholipids (Lill *et al.*, 1990; Kusters *et al.*, 1991). The translocation of precursor proteins from the SecA-bound state is initiated at the expense of ATP causing the *in vitro* appearance of membrane-spanning translocation intermediates under conditions that Δp is low or absent (Schiebel *et al.*, 1991). SecA rebinds to a translocation intermediate (step 1→2), and the binding of ATP to SecA allows limited translocation (step 3→4). Hydrolysis of ATP results in the dissociation of SecA from the preprotein (step 5→6), and permits forward translocation driven by Δp (step 6→7). The model assumes that membrane translocation events are rate-limiting in the overall cycle (steps 3→4 and 6→7). Δp may facilitate SecA-dependent translocation by promoting forward translocation (step 3→4) and preventing backward translocation (step 3→4). The rapid regeneration of free SecA at the translocation site in the presence of a Δp is predicted to result in a lowering of the apparent K_m for ATP. This kinetic parameter is determined by the relative distribution of SecA among its preprotein-associated state (at 2) and the other remaining states. In the absence of a Δp or in the presence of a reversed Δp, extensive backward translocation can take place once ATP hydrolysis has released SecA from its association with the preprotein (step 6→1 or earlier stages). A futile cycle is established when the translocation intermediate rebinds to SecA to undergo further ATP-dependent forward translocation and reversed Δp-dependent backward translocation. The role of the SecD and SecF proteins, and the associations of the translocation intermediates with lipids and the SecY/E protein are not known. Adapted from Schiebel *et al.* (1991).

energetics of translocation. Along with the dramatic effect of the intracellular pH on SecA ATPase activity (Driessen and Wickner, 1991), the high magnitude of the Δp *in vivo* may provide an explanation for the conflicting observations that Δp is essential for *in vivo* translocation and only stimulatory *in vitro* (Tai, 1990). The inhibitory effect of a reversed Δp and the thermodynamic equivalence of $\Delta\psi$ and ΔpH contradicts models that propose an indirect role of the Δp in preprotein translocation as to locally disrupt the membrane structure (Tai, 1990). Moreover, these *in vitro* results confirm previous *in vivo* studies on the energetics of precursor protein export (Bakker and Randall, 1984).

Model for translocation

We have previously shown that the initial stages of translocation involve the ATP-dependent relocation of the preprotein from the SecA-bound state to a membrane-inserted state (Schiebel *et al.*, 1991). *In vitro* translocation then proceeds though a series of transmembrane intermediates possibly by multiple cycles of the mechanism shown in Figure 5. A full-size discussion of this model is presented in Schiebel *et al.* (1991). This section is focused on the energetics of the intermediate stages of translocation as revealed by *in vitro* experiments. The catalytic cycle of the translocase at intermediate stages can be separated by its SecA dependency: SecA-dependent translocation which requires ATP (steps 1−6) and SecA-independent translocation driven by the Δp (step 7). ATP is essential for the initiation of translocation. *In vivo*, further translocation may be solely driven by Δp. However, *in vitro* experiments have not yet addressed the question of whether initiation of translocation is sufficient to allow Δp-driven translocation or whether first a critical mass of the preprotein has to be translocated at the expense of ATP before Δp suffices the energy requirements for translocation.

SecA-dependent translocation

SecA protein, bound to the membrane surface at the SecY/E protein and acidic phospholipids, binds a membrane-spanning intermediate of the preprotein (step 1→2). The energy of ATP binding to SecA permits forward translocation of a small segment of the preprotein (step 3→4). Hydrolysis of ATP (step 5→6) results in the dissociation of the preprotein−SecA complex to allow another cycle of ATP-dependent translocation (stepwise progress of the preprotein across the membrane by multiple repeats of steps 1−6) or Δp-driven translocation (step 7; see below). SecA may undergo major conformational

changes during these ATP-dependent binding and release steps (Shinkai *et al.*, 1991) and alternate between a membrane-inserted and membrane-bound state (Breukink *et al.*, 1991).

SecA-independent translocation

We have previously shown that Δp-driven translocation takes place when SecA has released the precursor protein (step 5→6) (Schiebel *et al.*, 1991). Δp drives most of the translocation process without SecA and ATP. The reconstituted translocation reaction involves only the SecA and SecY/E proteins (Brundage *et al.*, 1990). In this reaction, Δp accelerates the rate of translocation. The role of SecY/E may thus go beyond just serving as a receptor for SecA or the signal peptide (Wickner *et al.*, 1991). SecY/E protein may couple export of preproteins to an inward flux of protons according to a polypeptide/H^+ antiport (Driessen and Wickner, 1991). It should be emphasized, however, that a specific requirement for SecY/E during the intermediate stages of translocation has not yet been demonstrated. Support for the putative catalytic function of SecY/E is provided by observations that both $\Delta\psi$ and ΔpH are effective driving forces (Bakker and Randall, 1984; this paper) while Δp-driven translocation exhibits a major kinetic isotope effect (Driessen and Wickner, 1991). The latter is indicative for an essential protonation/deprotonation reaction in a rate-limiting step. Proton transfer events may be either scalar or vectorial (see below). Since translocation is readily reversible (Schiebel *et al.*, 1991; this paper), Δp may promote forward translocation (rate constant $k\times\beta$) and inhibit the reversed reaction (rate constant k/β) (step 6→7). k is a first order rate constant while β is a variable linear related to $e(-\Delta p/RT)$ (see Figure 2). This reaction may drive translocation of a large domain of the precursor protein before it reassociates with SecA (Schiebel *et al.*, 1991). Δp thereby lowers the amount of ATP required for translocation and coupling appears more efficient. Translocation resumes ATP dependence when SecA reassociates with the preprotein. In this model, translocation is reversed by an everted Δp as it favours the backward reaction over the forward reaction (Figure 5, step 6→1). These are conditions that render ATP hydrolysis unproductive as translocation is reversed in the SecA-independent cycle. Reversed translocation is observed when the preprotein is not bound to SecA or when Δp equals zero (Schiebel *et al.*, 1991). Backward translocation of some intermediates is possible in the presence of a reversed Δp (Figure 4). In the absence of a Δp, forward translocation results from the ATP-dependent dynamic association states of SecA with the preprotein (Figure 5, steps 1–6). In this scheme, membrane transit is thought to result from conformational changes (Schiebel *et al.*, 1991). Coupling between ATP hydrolysis and translocation mainly depends on the balance between forward and backward reactions. Forward translocation may be promoted by refolding or binding of translocated domains at the periplasmic face of the membrane, perhaps mediated by the SecD and SecF proteins (Schatz and Beckwith, 1990; Wickner *et al.*, 1991). Putative binding of translocated domains to SecD and SecF at the periplasmic side of the membrane may contribute to an efficient translocation as it will prevent backward translocation. On the cytoplasmic side, SecA may prevent excessive backward translocation by binding the preprotein, and promote forward translocation by unfolding local domains of the preprotein at the expense of ATP. However, refolding of translocated domains is not sufficient to release the protein into the periplasm as the final step requires Δp (Geller, 1990). As preproteins are thought to traverse the membrane in an extended conformation, translocation may be solely driven by Δp once the C-terminus of the preprotein has dissociated from SecA.

The involvement of Δp in translocation suggests that each (limited) translocation event which is driven by Δp is guided by vectorial or scalar H^+ transfer reactions. Since SecA requires the SecY/E protein for catalytic activity (Lill *et al.*, 1989), SecA-mediated translocation may also be guided by H^+ transfer reactions. Such a mechanism would explain observations in which Δp lowers the apparent K_m of the translocation reaction for ATP (Shiozuka *et al.*, 1990), assuming that the actual translocation event represents the rate-limiting step (Figure 5, step 3→4). It has been proposed that Δp directly modulates SecA by promoting the release of ADP (Shiozuka *et al.*, 1990). The observations that Δp functions in the absence of SecA (Schiebel *et al.*, 1991) whereas a reversed Δp promotes backward translocation are incompatible with this hypothesis. Therefore, a more indirect modulation of SecA by the Δp has to be assumed. In the model depicted in Figure 5, the apparent K_m for translocation is determined by the relative distribution of the different liganded SecA species bound at the translocator sites. Since translocation is reversible, Δp may promote the actual translocation step (step 3→4) by shifting the equilibrium towards the SecA–preprotein complex (and free SecA) species. If the membrane translocation event is the rate-limiting step in the catalytic cycle, SecA species with bound ATP will accumulate when Δp is absent. The model predicts that Δp lowers the apparent K_m of translocation for ATP while V_{max} increases as observed by Shiozuka *et al.* (1990). Imposition of a high phosphorylation potential ($\Delta G_p = \Delta G° - 2.3\ RT/F \log [ATP/ADP\times P_i]$), i.e. the use of an ATP regenerating system (Chen and Tai, 1985; Shiozuka *et al.*, 1990) or high levels of ATP (Chen and Tai, 1986a,b) exert comparable effects on the V_{max}. These are conditions at which the system exhibits characteristics of the high-affinity state, while ADP, which acts as a competitive inhibitor of ATP-dependent translocation (Chen and Tai, 1986a; unpublished data), is either removed or has little effect due to its low concentration. Nevertheless, also under these conditions the rate of translocation is further increased by the Δp in the SecA-independent cycle. It should be emphasized that it is difficult to envisage the lowering of the apparent K_m for ATP as a major mechanism by which Δp acts *in vivo*. The intracellular ATP concentration can be as high as 3–5 mM, and thus far in excess of the reported micromolar affinity of SecA. *In vivo* dissipation of the Δp nearly completely blocks translocation, whereas the intracellular ATP concentration remains unchanged. Under those conditions, ATP-driven translocation may be too slow to prevent tight folding of non-translocated domains which require Δp for translocation (see also below). In addition, the lowering of the intracellular pH elicited by uncouplers may have further adverse effects on translocation rates (Driessen and Wickner, 1991). *In vitro*, excess SecA suppresses the strict Δp requirement found for the translocation of the chimeric preprotein OmpF–Lpp (Yamada *et al.*, 1989a). Δp-driven translocation of proOmpA is inhibited by SecA and completely blocked by SecA plus non-hydrolysable ATP analogues (Schiebel *et al.*, 1991) (arrest at step 4). These observations imply that the

SecA-dependent and -independent cycles compete for preprotein translocation. Surplus SecA will promote the ATP-dependent pathway as rebinding of the preprotein becomes more likely once ATP hydrolysis has released the preprotein from its association from SecA (steps 5 and 6). Likewise, translocation is more dependent on Δp when SecA is limiting. These effects may vary for different preproteins as well as the number of SecA interacting sites and their affinities (Driessen and Wickner, 1991; Yamada *et al.*, 1989b). To test the model depicted in Figure 5, further kinetic studies are required in order to reveal rate-limiting steps in translocation.

Though Δp directs translocation, it functions as a force as it allows stably folded domains to translocate in the presence of ATP (Tani *et al.* 1990). *In vivo*, previously folded protein domains are exported (Freudl *et al.*, 1987; Reed and Cronan, 1991). Recent findings that point mutations in the linking region between the signal peptide and mature domain confer Δp dependency (Lu *et al.*, 1991) may suggest that Δp acts on the preprotein by affecting its conformation during membrane transit.

Δp acts throughout the translocation reaction (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991; Lu *et al.*, 1991; this paper) by a molecular mechanism that involves proton transfer reactions (Driessen and Wickner, 1991). Our current model of Δp-driven translocation does not discriminate between vectorial and scalar H^+-transfer reactions. The latter would result from the deprotonation of positively charged residues before translocation and their protonation afterwards. Electrogenicity is assumed to arise from an electrophoretic effect of $\Delta\psi$ on the transfer of negatively charged residues across the membrane. Unless deprotonation is an active event, such a mechanism seems unlikely as simple acid−base reactions exhibit kinetic isotope effects around unity (Driessen and Wickner, 1991). The significance of these reactions will depend on the primary and secondary structure of the membrane translocating domains as intramolecular ion-pairing may reduce the net charge (von Heijne, 1980). Likewise, the charge distribution along the polypeptide chain will determine the Δp dependency of the translocation of the preprotein. The requirement for Δp in translocation is not unique to the Sec-dependent translocation pathway of proteins across the cytoplasmic membrane of *E.coli*. Δp is also required for the secretion of haemolysin by the dedicated HlyB/HlyD transport system which facilitate secretion of HlyA across both the cytoplasmic and outer membrane (Koronakis *et al.*, 1991). The energetic parallel between both systems may suggest a common mechanism. It will be important to establish the nature of the translocation pathway and the domain structure of transmembrane intermediates to resolve the fundamental role of Δp in protein translocation.

Materials and methods

Materials and bacterial strains

Inverted membrane vesicles were prepared from *E.coli* strain KM9 (*unc*$^-$ Tn*10*, *rel*A1, *spo*T1, *met*B1; Klionsky *et al.*, 1984) as described by Chang *et al.* (1978). ProOmpA (Crooke *et al.*, 1988), SecA (Cunningham *et al.*, 1989) and SecB protein (Weiss *et al.*, 1988; Lecker *et al.*, 1989), bR (Danon and Stoeckenius, 1974; Driessen *et al.*, 1987) and beefheart CytOx (Yu *et al.*, 1975; Driessen *et al.*, 1985) were purified as described. SecY/E protein was extracted from *E.coli* UT5600 (F$^-$; *ara*14, *leu*B6, *azi*6, *lac*Y1, *pro*C14, *tsx*67, Δ(*omp*T-*fep*C)266, *ent*A403, *β*$^-$, *trp*E38, *rfb*D1, *rps*L109, *xyl*5, *mtl*1, *thi*1; Elish *et al.*, 1988) inner membranes and partially purified through the DEAE-cellulose step to allow coreconstitution experiments (Brundage *et al.*, 1990). [^{35}S]proOmpA was isolated from *E.coli* UH203 (*lac*, *sup*F, *omp*A, *rec*A, *pro*A or B, *rps*L/F', *lac*I^Q, *lac*Z, *M*15, *pro*AB$^+$) harbouring plasmid pRD87 (Freudl *et al.*, 1985) which carries the *OmpA* gene under the control of the *lac* promotor. Cells were grown in M9 supplemented with proline (40 μg/ml), thiamine (1 μg/ml) and glucose [0.5% (w/v)]. At an A_{660} of 1.0, cells were collected by centrifugation, suspended in glucose-deficient minimal medium as described above supplemented with ampicillin (50 μg/ml), isopropyl thiogalactoside (1 mM) and [^{35}S]methionine (0.5 mCi/100 ml of culture), and shaken for 3 h at 37°C. Cells were collected by centrifugation, resuspended in buffer [50 mM Tris−HCl, pH 7.5, and 10% (w/v) sucrose], and frozen as small nuggets by pipetting the suspension into liquid nitrogen. Thawed cells were disrupted and the sarkosyl-insoluble proOmpA aggregates were isolated as described by Freudl *et al.* (1986). [^{35}S]proOmpA was solubilized from this pellet fraction with 8 M urea as described by Crooke *et al.* (1988). Anti-OmpA antiserum was a gift of Dr W.Wickner (University of California at Los Angeles, USA).

Reconstitution

Liposomes were prepared from *E.coli* phospholipid (PL) supplemented with 10% (w/w) dioleoylphosphatidylglycerol. CytOx was reconstituted into liposomes by detergent dialysis (Driessen and Konings, 1991). PL [20 mg in chloroform−methanol (9:1, v/v)] was dried under N_2, lyophilized and hydrated in 1 ml of buffer A (50 mM HEPES−KOH, pH 7.3, 50 mM KCl) containing 1.25% (w/v) *n*-octyl β-D-glucopyranoside. The suspension was sonicated (5 min; cycle time 15 s; 4 μm peak-to-peak) (MSE Scientific Instruments, West Sussex, UK) at 4°C under N_2. CytOx (4.5 nmol heme *a*) was added and detergent was removed by dialysis at 4°C for 20 h against a 500-fold volume of buffer A with two changes. bR was reconstituted into liposomes by sonication (Driessen *et al.*, 1985; Driessen and Konings, 1991). PL (10 mg) and bR (105 nmol) were mixed in 1 ml of buffer B (50 mM HEPES−NaOH, pH 7.3, 50 mM NaCl) and sonicated (40 min; cycle time 30 s) as described above.

SecY/E protein was reconstituted into bR and CytOx proteoliposomes by rapid detergent-dilution (Brundage *et al.*, 1990). SecY/E protein [1.8 ml; 7.5−9 μg of protein in 15 mM Tris−HCl, pH 7.9, 0.5 M KCl, 5 mM *p*-aminobenzamidine, 40% (v/v) glycerol, 1.25% (w/v) octylglucoside, 0.5 mg/ml *E.coli* PL, 1 mM DTT] was mixed with CytOx proteoliposomes [360 μl; 0.9 nmol of CytOx and 6.5 mg of PL in buffer A containing 1.25% (w/v) octylglucoside], and rapidly diluted with 90 ml of buffer A. Liposomes were precipitated by the addition of 450 μl of 1 M $CaCl_2$, kept on ice for 1 h, and collected by centrifugation (120 000 g_{max}, 2 h, 4°C; Ti 35). Pellets were suspended in 0.9 ml buffer A containing 2.5 mM EGTA. SecY/E protein was reconstituted into bR proteoliposomes (32 nmol of bR) as described above. K^+-salts were substituted for Na^+-salts. Proteoliposomes were stored in liquid nitrogen, and before use thawed at room temperature and sonicated for 6 s with a microtip at 3 μm output.

Translocation assay

Translocation into CytOx−SecY/E proteoliposomes was assayed in buffer C (50 mM HEPES−KOH, pH 7.3, 50 mM KCl, 5 mM $MgSO_4$, 0.5 mg/ml fatty acid-free bovine serum albumin, 10 mM dithiothreitol) containing 10 μg/ml SecA, 40 μg/ml SecB, 2 mM ATP, 10 mM creatine phosphate and 10 μg/ml creatine kinase. Translocation mixtures (100 μl) included 10 μM horse-heart cytochrome *c*, 10 mM ascorbate−KOH, pH 7.3, and 100 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as energy source, and 10 μl of the stock proteoliposomes. Reactions at 37°C were initiated with proOmpA (4 μg/ml) diluted 100-fold from solution in 6 M urea, 50 mM Tris−HCl, pH 7.5. Reactions were terminated after 40 min incubation by chilling the sample on ice. Translocation into bR−SecY/E proteoliposomes was performed essentially as described above in buffers containing Na^+-salts. Samples were incubated for 5 min in uncapped tubes sealed with a cover glass. Light from a 150 W Xenon Arc Lamp (Oriel Co., Stratford, CT) equipped with a water filter was guided through a long-pass filter (>515 nm) and focused into the tubes by a collimating beam probe and glass fibre optics. Ionophores were added as ethanolic solutions [final concentration 1% (v/v)]. Samples were treated with proteinase K (1 mg/ml, 15 min, 0°C) and analysed immunochemically with anti-OmpA antibodies (Ito *et al.*, 1980; Cunningham *et al.*, 1989; Schiebel *et al.*, 1991) using the ECL Western blotting detection system (Amersham Ltd, UK). Fluorographs were quantified with an enhanced LKB UltraScan XL Laser Densitometer. Values were normalized to the amount of proOmpA translocated per reaction.

Translocation intermediates

E.coli KM9 membrane vesicles bearing I_{26} were prepared as described by Schiebel *et al.* (1990). [^{35}S]proOmpA−SecB (~4 × 10^6 c.p.m./ml) was preincubated with 0.1 mg/ml membrane vesicles, 40 μg/ml SecA, 10 mM creatine phosphate and 10 μg/ml creatine kinase for 2 min at 37°C.

Translocation reactions were performed either in buffer D [50 mM HEPES–KOH (pH 7.0), 100 mM K-acetate] or buffer E [50 mM HEPES–NaOH (pH 7.0), 100 mM Na-gluconate] supplemented with 5 mM $MgSO_4$ and 50 μg/ml BSA. The reaction was initiated by the addition of 2 μM ATP and arrested after 10 min by chilling the suspension on ice. Membrane vesicles were layered on a sucrose solution (0.2 M sucrose in buffer D or E) and centrifuged (250 000 g_{max}, 45 min, 0°C). The sediment was resuspended in buffer D or E supplemented with 5 mM $MgSO_4$ and 50 μg/ml BSA, and stored at 0°C. For the imposition of a Δp, inside negative and alkaline, K-acetate loaded membrane vesicles (5 μl) were diluted 1000-fold into buffer E supplemented with 20 nM valinomycin. For the imposition of a Δp, inside positive and acid, Na-gluconate loaded membrane vesicles were diluted into buffer D containing 20 nM valinomycin. Buffer contained 5 mM $MgSO_4$ and 50 μg/ml BSA. Samples were incubated for 10 min at 37°C, treated with proteinase and analysed by SDS–PAGE and fluorography.

Measurement of Δp

$\Delta\psi$, inside negative, was estimated from the transmembrane distribution of the lipophilic cation tetraphenylphosphonium ion monitored with an ion-selective electrode (Driessen *et al.*, 1985; Driessen and Konings, 1991). Values were corrected for probe binding to membranes (De Vrij *et al.*, 1986). $\Delta\psi$, inside positive, was measured with the same electrode using the lipophilic anion tetraphenylboron (Driessen *et al.*, 1987). Liposomes had a calceïn trapping volume of 5.2 ± 0.6 μl/mg of phospholipid (De Vrij *et al.*, 1986; Driessen and Konings, 1991). ΔpH was estimated from internal pH measurements using the fluorophore pyranine (Molecular Probes, Eugene, OR) (De Vrij *et al.*, 1986; Driessen and Konings, 1991; Driessen and Wickner, 1991). A Perkin Elmer LS-2 fluorometer was used equipped with a magnetically stirred, thermostatted microcurvet. Pyranine fluorescence (excitation, 460 nm; emission, 510 nm) was calibrated by adjusting the medium pH in the presence of 0.5 μM S13.

Other methods

Protein was assay by the micro BCA Protein method (Pierce, Rockford, IL) using BSA as a standard. PL phosphorus was determined as described (Rouser *et al.*, 1970).

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