Membrane deinsertion of SecA underlying proton motive force-dependent stimulation of protein translocation

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The proton motive force (PMF) renders protein translocation across the *Escherichia coli* membrane highly efficient, although the underlying mechanism has not been clarified. The membrane insertion and deinsertion of SecA coupled to ATP binding and hydrolysis, respectively, are thought to drive the translocation. We report here that PMF significantly decreases the level of membrane-inserted SecA. The *prlA4* mutation of SecY, which causes efficient protein translocation in the absence of PMF, was found to reduce the membrane-inserted SecA irrespective of the presence or absence of PMF. The PMF-dependent decrease in the membrane-inserted SecA caused an increase in the amount of SecA released into the extra-membrane milieu, indicating that PMF deinserts SecA from the membrane. The PMF-dependent deinsertion reduced the amount of SecA required for maximal translocation activity. Neither ATP hydrolysis nor exchange with external SecA was required for the PMF-dependent deinsertion of SecA. These results indicate that the SecA deinsertion is a limiting step of protein translocation and is accelerated by PMF, efficient protein translocation thereby being caused in the presence of PMF.

Keywords: *E.coli*/protein translocation/proton motive force/SecA cycle

Introduction

Protein translocation across the *Escherichia coli* cytoplasmic membrane is catalyzed by a machinery comprising six Sec factors, A, D, E, F, G and Y, and assisted by a molecular chaperone, SecB (for reviews see Schatz and Beckwith, 1990; Pugsley, 1993; Driessen, 1994; Tokuda, 1994; Ito, 1996; Duong *et al.*, 1997). SecY and SecE have been thought to form a protein-conducting channel in the membrane, through which translocation of the precursor takes place. Both ATP and proton motive force (PMF) are required for protein translocation, although the latter is not absolutely essential. SecA has been proposed to drive protein translocation by undergoing a cycle of membrane insertion and deinsertion, which take place upon ATP binding and hydrolysis, respectively (Economou and Wickner, 1994; Economou *et al.*, 1995). The domains of SecA inserted into the membrane have been identified (Chen *et al.*, 1996; Price *et al.*, 1996; Ramamurthy and

Oliver, 1997). The insertion of the C-terminal ~30 kDa and N-terminal ~65 kDa domains has been studied most extensively as to protein translocation (Economou and Wickner, 1994; Economou *et al.*, 1995; Chen *et al.*, 1996; Eichler and Wickner, 1997). Membrane insertion has been shown to protect these domains from protease digestion. SecG has been proposed to assist the SecA insertion (Duong and Wickner, 1997; Suzuki *et al.*, 1998) by undergoing a cycle of topology inversion in the membrane (Nishiyama *et al.*, 1996). A *secY* mutant was found to inhibit the SecA insertion, and a *secA* mutant that overcomes the inhibitory effect of this *secY* mutation has been isolated (Matsumoto *et al.*, 1997). A cold-sensitive SecA mutant was found to be defective in membrane insertion (Suzuki *et al.*, 1998). SecD and SecF were also found to be important for stabilization of SecA insertion (Kim *et al.*, 1994; Economou *et al.*, 1995; Duong and Wickner, 1997). These experiments also revealed that the efficiencies of SecA insertion and protein translocation are tightly correlated with each other. From these observations, it seems certain that the ATP-dependent membrane insertion of SecA is the direct driving force for protein translocation.

In contrast to the role of ATP in protein translocation, that of PMF remains unclear. However, various observations have been reported as to the effect of PMF on protein translocation. PMF remarkably stimulates the rate of protein translocation (Chen and Tai, 1985; Geller *et al.*, 1986; Yamane *et al.*, 1987). The late stage of protein translocation can be driven by PMF even in the absence of ATP hydrolysis (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991; Driessen, 1992). The ATP concentration required for maximal protein translocation decreases in the presence of PMF (Shiozuka *et al.*, 1990; Driessen, 1992). Precursor proteins having abnormal structures, such as disulfide bonds or a non-peptide moiety, in the mature region can be translocated only when PMF is generated (Tani *et al.*, 1990; Schiebel *et al.*, 1991; Tani and Mizushima, 1991; Kato and Mizushima, 1993). PMF stimulates the forward movement of a precursor protein (Driessen, 1992). The protein translocation defect observed with SecG-depleted membrane vesicles (Hanada *et al.*, 1996) or a cold-sensitive SecA mutant (Suzuki *et al.*, 1998) is partially suppressed by PMF *in vitro*. In spite of these observations, the molecular events underlying PMF-dependent energization of protein translocation, which has been proposed to involve proton transfer (Driessen and Wickner, 1991; Kawasaki *et al.*, 1993), remain open to question. Furthermore, depletion of SecD and SecF was found to partially uncouple the generation of PMF (Arkowitz and Wickner, 1994), although the reason for this uncoupling is unknown. It was found recently that protein translocation becomes less dependent on PMF if SecY carries the *prlA4* mutation (Nouwen *et al.*, 1996), which suppresses the translocation defect caused by a signal peptide mutation (Emr *et al.*,

1981; Bieker *et al.*, 1990). Although this finding suggests that SecY is the target of PMF, the detailed molecular mechanism remains obscure. The *prlA4* mutation was found recently to stabilize SecA–SecY interaction (Van der Wolk *et al.*, 1998).

The SecA cycle comprising membrane insertion and deinsertion has been largely examined in the absence of PMF for simplicity (Economou and Wickner, 1994; Economou *et al.*, 1995; Duong and Wickner, 1997; Eichler and Wickner, 1997). Although PMF has been reported to stimulate SecA insertion, this was only observed with a very low ATP concentration (Economou *et al.*, 1995). It is therefore unknown whether PMF has any effect on the SecA cycle with a physiological ATP concentration. Furthermore, deinsertion of membrane-inserted radioactive SecA was shown to be induced only when an excess amount of non-radioactive SecA was added externally (Economou and Wickner, 1994; Economou *et al.*, 1995). Therefore, it is not completely clear whether or not protein translocation proceeding at a constant rate always involves SecA deinsertion. Moreover, it is not known how excess non-radioactive SecA causes the deinsertion of labeled SecA.

We report here that PMF accelerates the deinsertion of SecA from the membrane, thereby stimulating the SecA cycle. A striking difference between the PMF-dependent

Fig. 1. PMF stimulates protein translocation and lowers the level of membrane-inserted SecA. Membrane vesicles prepared from K003 were washed with 4 M urea, and then incubated with 8 nM non-radioactive (A), ¹²⁵I-labeled (B and C) or ³⁵S-labeled (D) SecA. The SecA-loaded membrane vesicles were recovered by centrifugation and then subjected to *in vitro* proOmpA translocation at 37°C as described under Materials and methods. (A) The translocation of proOmpA into membrane vesicles loaded with non-radioactive SecA was examined with a mixture of [³⁵S]proOmpA (~1×10⁷ c.p.m./ml) and non-radioactive proOmpA (25 µg/ml) in the presence (●) and absence (○) of 5 mM succinate. After initiation of the assay, succinate (5 mM, Δ) or CCCP (10 µM, ■) was added to a portion of the reaction mixture as indicated by the arrows. At specified times, aliquots (25 µl) of the reaction mixture were withdrawn and digested with 1 mg/ml proteinase K on ice for 15 min. The translocated mature OmpA and proOmpA were analyzed by SDS–PAGE and fluorography, and then quantitated densitometrically. (B) The SecA insertion was examined with membrane vesicles loaded with [¹²⁵I]SecA in the presence of non-radioactive proOmpA (25 µg/ml) with (●) or without (○) 5 mM succinate. Where indicated by arrows, CCCP (10 µM, ■) or succinate (5 mM, Δ) was added to portions of the reaction mixture. Aliquots (100 µl) of the reaction mixture were withdrawn at the times indicated and then subjected to proteinase K digestion (1 mg/ml). The proteinase K-resistant 30 kDa fragment of SecA was detected by SDS–PAGE and autoradiography, followed by quantitation. The initial amount of SecA in 100 µl of the reaction mixture was taken as 100%. (C) The proteinase K-resistant 30 kDa fragment of SecA was examined as in (B) in the presence (●) and absence (○) of 5 mM succinate. As indicated by arrows, 10 µM CCCP (■), 50 mM KSCN (Δ), 20 mM (NH₄)₂SO₄ (▲) or 50 mM KSCN plus 20 mM (NH₄)₂SO₄ (□) was added to portions of the reaction mixture. The amounts of the proteinase K-resistant 30 kDa fragment of SecA were determined as in (B). (D) SecA insertion was examined with membrane vesicles loaded with [³⁵S]SecA as in (B) in the absence (○) and presence (●) of 5 mM succinate. The addition of 5 mM succinate (Δ) and 10 µM CCCP (▲) was performed at 15 min, as indicated by the arrows. Aliquots (100 µl) of the reaction mixture were withdrawn at the specified times and treated with 1 mg/ml proteinase K. The membrane-inserted 65 kDa fragment was detected by SDS–PAGE and fluorography, and then quantitated. The amount of the 65 kDa fragment is expressed in arbitrary units as the exact amounts of methionine and cysteine in the fragment are unknown.

and external SecA-dependent deinsertion was also revealed. Various effects on protein translocation brought about by PMF can now be understood from this stimulation of the SecA cycle.

Results

PMF stimulates protein translocation and lowers the level of membrane-inserted SecA

To determine whether PMF stimulates protein translocation by affecting the membrane insertion of SecA, proOmpA translocation and SecA insertion were analyzed in the presence and absence of PMF (Figure 1). The inverted membrane vesicles used here were prepared from K003 cells lacking F_0F_1 -ATPase and washed with 4 M urea to remove membrane-bound SecA. These membrane vesicles required external SecA for proOmpA translocation as reported previously (Suzuki *et al.*, 1998). The urea washing removed ~80% of the SecA initially associated with the membranes (see Figure 2A). Washing with 5 M or a higher concentration of urea further decreased the amount of SecA on the membranes, but irreversibly abolished the ability of membrane vesicles to generate PMF. In contrast, washing with 4 M urea had no effect on the ability of membrane vesicles to generate PMF through succinate oxidation (data not shown).

Generation of PMF on the addition of succinate significantly enhanced the rate of proOmpA translocation, and the collapse of PMF due to carbonylcyanide *m*-chlorophenylhydrazone (CCCP) inhibited the translocation (Figure 1A), indicating the non-essential but stimulatory features of PMF. The level of the membrane-inserted 30 kDa fragment of 125 I-labeled SecA, which was protected from proteinase K digestion, was significantly higher in the absence than in the presence of PMF (Figure 1B). The 30 kDa fragment level in the absence of PMF immediately decreased on the generation of PMF, and that in the presence of PMF increased on the collapse of PMF. These results indicate that the level of membrane-inserted SecA is regulated by PMF. PMF is composed of a membrane potential ($\Delta\psi$) and Δ pH, which are interchangeable. The collapse of $\Delta\psi$ on the addition of KSCN (Reenstra *et al.*, 1980; Shiozuka *et al.*, 1990) also increased the level of the 30 kDa fragment, albeit to a lesser extent than did CCCP (Figure 1C). The collapse of Δ pH on the addition of $(\text{NH}_4)_2\text{SO}_4$ (Reenstra *et al.*, 1980; Shiozuka *et al.*, 1990) did not cause an increase in the 30 kDa fragment. However, the addition of KSCN together with $(\text{NH}_4)_2\text{SO}_4$ increased the level of the 30 kDa fragment to essentially the same level as that observed with CCCP. These membrane vesicles were found to generate a $\Delta\psi$ (inside positive) of 154 mV and a Δ pH (inside acidic) of 86 mV through succinate oxidation. These values explain why the level of the 30 kDa fragment appeared to be more sensitive to $\Delta\psi$ than to Δ pH.

Not only the C-terminal 30 kDa domain but also the N-terminal 65 kDa domain is inserted into the membrane upon protein translocation (Chen *et al.*, 1996; Eichler and Wickner, 1997). To determine whether PMF also affects the insertion of the 65 kDa domain, ^{35}S -labeled instead of ^{125}I -labeled SecA was used for the assay (Figure 1D) since the 65 kDa domain cannot be labeled with ^{125}I (Price *et al.*, 1996). The level of the membrane-inserted 65 kDa

Fig. 2. The *prlA4* mutation lowers the level of membrane-inserted SecA. Membrane vesicles were prepared from CE1437 (Δ uncB-C) and CE1438 (Δ uncB-C *prlA4*). (A) The level of SecA in the two types of membrane vesicles (MV) before and after 4 M urea washing were determined by SDS-PAGE and immunoblotting with anti-SecA antibodies. The averages of three determinations carried out with various amounts of membrane proteins are expressed with error bars as percentages of the amount of SecA in the wild-type membrane vesicles (PrlA⁺) before urea washing. (B) Urea-washed membrane vesicles were loaded with [125 I]SecA, and then subjected to *in vitro* translocation of proOmpA as described in Figure 1B. The reaction was carried out in the presence (closed symbols) and absence (open symbols) of 5 mM succinate. The proteinase K-resistant 30 kDa fragment of SecA was quantitated and expressed as a percentage of the initial amount of SecA in 100 μ l of the reaction mixture.

fragment was lower in the presence of PMF than in its absence. Furthermore, the 65 kDa fragment levels in the presence and absence of PMF increased on the collapse and decreased on the generation of PMF, respectively. Taken together, these results indicate that the membrane insertion of the entire SecA molecule is maintained at a low level in the presence of PMF although the insertion of the 65 and 30 kDa domains exhibited different kinetics. Since the effect of PMF on the SecA insertion could be examined more sensitively as the level of the ^{125}I -labeled 30 kDa fragment, the following experiments were carried out with ^{125}I -labeled SecA.

SecA insertion is maintained at a low level by the *prlA4* mutation of SecY

The *prlA4* mutation of SecY is known to suppress the translocation defect caused by a signal peptide mutation

without PMF (Figure 3A). Stimulation of the translocation by PMF was remarkable at a lower SecA concentration. An Eadie-Hofstee plot of the data revealed that the amount of SecA required for half-maximal stimulation of translocation is lower by a factor of >5 in the presence of PMF than in its absence, whereas the $V_{\max}$ values are independent of PMF (Figure 3B).

To determine whether the PMF-dependent decrease in the membrane-inserted SecA alters the SecA-membrane interaction, membrane vesicles loaded with [125 I]SecA were subjected to proOmpA translocation in the absence of PMF at 37°C for 15 min. The reaction mixture was incubated further for 5 min with either no additions, 5 mM succinate or 40 μ M non-radioactive SecA. Each reaction mixture was then treated with proteinase K to determine the level of the 30 kDa fragment, or subjected to fractionation into membrane vesicles and supernatant to determine the level of [125 I]SecA (Figure 3C and D). A portion of each reaction mixture was treated with 0.1 M potassium acetate prior to fractionation. Approximately 10% of the labeled SecA was recovered in the supernatant when the translocation reaction was performed in the absence of PMF (lane 3 in Figure 3C and D). On the other hand, ~30% of the SecA was recovered in the supernatant when PMF was generated (Figure 3C and D, lane 9). Furthermore, the translocation reaction in the presence of PMF appreciably increased the amount of SecA extracted with 0.1 M potassium acetate (Figure 3C and D, compare lanes 5 and 11). The addition of non-radioactive SecA caused the release of $>60\%$ of the labeled SecA into the supernatant (Figure 3C and D, lane 15). These results qualitatively coincided with the decrease in the level of the proteinase K-resistant 30 kDa fragment (Figure 3C and D, lanes 2, 8 and 14). Taken together, these results indicate that PMF increases the amount of soluble SecA, which is critical for efficient protein translocation at a low SecA concentration.

PMF-dependent SecA deinsertion requires neither ATP hydrolysis nor exchange with soluble SecA

SecA is thought to be inserted into the membrane upon ATP binding and deinserted from the membrane upon ATP hydrolysis. The PMF-dependent decrease in the 30 kDa fragment could result from inhibition of the insertion or stimulation of the deinsertion. If the insertion is inhibited, then most of the SecA molecules should remain deinserted all the time during protein translocation in the presence of PMF or in PrlA4 membrane vesicles. However, since the initiation of protein translocation absolutely requires ATP, the ATP-dependent SecA insertion seems to be a central driving force irrespective of the presence or absence of PMF. Although inhibition of the insertion by PMF or PrlA4 seems to be unlikely, the effect of PMF on the level of membrane-inserted SecA was examined in more detail under various conditions where ATP hydrolysis is inhibited.

Adenylylimidodiphosphate (AMP-PNP), a non-hydrolyzable ATP analog, is known to cause SecA insertion, which does not require proOmpA (Economou *et al.*, 1995). Irrespective of the presence (not shown) or absence (Figure 4A) of proOmpA, PMF generated either before or after the start of the insertion assay had no effect on the level of the 30 kDa fragment. The AMP-PNP-dependent

Fig. 4. Effect of AMP-PNP on the membrane insertion of SecA. (A) The membrane insertion of [125 I]SecA was examined in the presence (▲) and absence (○) of 5 mM succinate as in Figure 1B, except that AMP-PNP (1 mM) was used instead of ATP and proOmpA was omitted. At 10 min, 5 mM succinate (●) or 0.4 μ M non-radioactive SecA (■) was added to a portion of the reaction mixture with no PMF. (B) Membrane vesicles loaded with [125 I]SecA were subjected to *in vitro* proOmpA translocation as described in Figure 1B. At 15 min, 10 mM AMP-PNP plus 10 mM MgSO₄ (●) or nothing (○) was added to a portion of the reaction mixture. At 20 min, 0.4 μ M non-radioactive SecA (△), 5 mM succinate (▲), 0.4 μ M non-radioactive SecA plus 5 mM succinate (□) or nothing (●) was added further to a sample containing AMP-PNP. The proteinase K-resistant 30 kDa fragment was analyzed and quantitated as in Figure 1B.

insertion of SecA, which causes only the partial translocation of proOmpA (Schiebel *et al.*, 1991), is therefore insensitive to PMF. In contrast, the addition of non-radioactive SecA caused a slow decrease in the level of the 30 kDa fragment. We then examined the effects of AMP-PNP and PMF after initiation of the ATP-dependent SecA insertion. The level of the 30 kDa fragment in the absence of PMF increased ~2-fold on the addition of AMP-PNP (Figure 4B). Both the addition of non-radioactive SecA and the generation of PMF decreased the level of the 30 kDa fragment even in the presence of AMP-PNP. The further decrease induced on the addition of non-radioactive SecA with the generation of PMF was marginal. These results indicate that the translocation-coupled SecA insertion is sensitive to PMF even in the

Fig. 5. Membrane-inserted D209N SecA is deinserted by PMF but not external SecA. Membrane insertion of the wild-type SecA (circles) and D209N SecA (triangles and squares) was examined. The step of membrane loading with [125 I]SecA was omitted. The assay was started, in the absence of PMF, in a reaction mixture comprising 4 nM [125 I]-labeled SecA, 0.2 mg/ml urea-washed membrane vesicles, 5 mM MgSO₄, 0.2 mg/ml BSA, 1 mM DTT, 1 mM ATP, 2.5 mM creatine phosphate, 5 μ g/ml creatine kinase and 50 μ g/ml SecB in 50 mM potassium phosphate (pH 7.5). At 15 min, 5 mM succinate was added to a portion of the reaction mixture (●, ▲). Non-radioactive wild-type SecA (0.4 μ M) was also added to the reaction mixture containing [125 I]D209N SecA at 15 min (■). The amounts of the membrane-inserted 30 kDa fragment were determined as described in Figure 1B and expressed as percentages taking the value at 15 min as 100%. The 30 kDa fragment level at 15 min was similar to the wild-type and D209N SecA.

presence of AMP-PNP (Figure 4B), whereas the insertion induced by AMP-PNP is insensitive (Figure 4A).

A SecA mutant, D209N SecA (Mitchell and Oliver, 1993), has a single amino acid substitution (Asp to Asn) at position 209, which is located in the Walker motif B of the N-terminal high affinity ATP-binding site. The mutant SecA is inactive with regard to protein translocation (Mitchell and Oliver, 1993), but can be inserted into the membrane in the presence of ATP and proOmpA (Economou *et al.*, 1995; Figure 5). Non-radioactive wild-type SecA did not decrease the membrane-inserted [125 I]D209N SecA (Figure 5; Economou *et al.*, 1995). Strikingly, however, generation of PMF significantly decreased the level of the 30 kDa fragment of D209N SecA as well as that of the wild-type SecA (Figure 5). The response of the mutant SecA to PMF is therefore normal. Taken together, these results revealed that the decrease in the membrane-inserted SecA induced by external SecA and that by PMF are mechanistically different. These results also indicate that ATP hydrolysis is not required for the PMF-dependent decrease in the membrane-inserted SecA. However, SecA insertion occurs in the presence of AMP-PNP and with D209N SecA. Therefore, these data did not reveal whether the PMF-dependent decrease is caused by inhibition of the insertion or stimulation of the deinsertion.

EDTA is known to inhibit the binding of ATP to various ATPases by preventing the binding of magnesium to ATP (Walker *et al.*, 1982; Müller and Schulz, 1992; Story and

Steitz, 1992; Van der Wolk *et al.*, 1995). Neither SecA insertion (Figure 6A) nor proOmpA translocation (not shown) occurred in the presence of EDTA even though ATP and proOmpA were present. The addition of succinate caused the generation of PMF in the presence of EDTA (not shown) but did not cause SecA insertion (Figure 6A). When magnesium was added, SecA insertion (Figure 6A) and proOmpA translocation (not shown) immediately took place. When EDTA was added after the initiation of protein translocation, the 30 kDa fragment level remained unchanged for at least 25 min (Figure 6B), indicating that not only insertion (Figure 6A) but also deinsertion of SecA is inhibited. Both the addition of non-radioactive SecA and the generation of PMF decreased the level of the 30 kDa fragment. Therefore, the decrease in the 30 kDa fragment due to PMF is caused by stimulation of the deinsertion. The decrease leveled off when about two-thirds of the inserted SecA had been deinserted in both cases, indicating that the same fraction of inserted SecA is competent with regard to both PMF-induced and external SecA-induced deinsertion. The external SecA-dependent deinsertion represents either displacement of the membrane-inserted radioactive SecA with non-radioactive SecA or exchange of spontaneously deinserted radioactive SecA with non-radioactive SecA. Approximately one-third of the inserted SecA, which was incompetent with regard to deinsertion, became competent for the external SecA-dependent deinsertion upon the addition of 4 mM MgSO₄ (Figure 6B), indicating that the deinsertion-incompetent SecA is converted to the competent form by ATP hydrolysis.

Intermediates of proOmpA translocation have been detected in the absence of PMF (Tani *et al.*, 1989; Schiebel *et al.*, 1991). They migrate faster than the intact OmpA molecule on SDS-PAGE after proteinase K treatment since a portion of these molecules is not translocated and is therefore proteolysed. When PMF is generated, the translocation of the intermediates is completed even in the presence of AMP-PNP, indicating that the late stage of protein translocation can be driven by PMF without ATP hydrolysis (Tani *et al.*, 1989; Schiebel *et al.*, 1991). To determine whether PMF has the same effect in the presence of EDTA, proOmpA translocation was started in the absence of PMF with the same amount of SecA as used in Figure 6B. The bands migrating to positions corresponding to approximate molecular masses of 14, 18 and 29 kDa (Figure 6C) probably correspond to the previously identified translocation intermediates (Tani *et al.*, 1989; Schiebel *et al.*, 1991). Upon the addition of EDTA (Figure 6C) or AMP-PNP (not shown), the 14 kDa band disappeared with the concomitant appearance of an 18 kDa band. The amount of intact OmpA did not increase on EDTA addition (Figure 6D). When PMF was generated, the 29 kDa band decreased with an appreciable increase in intact OmpA. The 18 kDa band also decreased, albeit at a slower rate. Taken together, these results suggest that the PMF-dependent deinsertion of SecA can complete the translocation of the intermediates even in the presence of EDTA.

Discussion

We have revealed here that the PMF-dependent stimulation of protein translocation is coupled to the deinsertion of

SecA from the membrane (Figure 1). The membrane deinsertion due to PMF caused an increase in the amount of SecA recovered in the supernatant (Figure 3C and D). The specific activity of SecA at low concentration was significantly higher in the presence than in the absence of PMF (Figure 3A and B). Taken together, these results most likely indicate that not only insertion but also deinsertion of SecA is a limiting step of protein translocation. PMF-dependent deinsertion of membrane-inserted SecA occurred in an external SecA-independent manner and the net amount of membrane-inserted SecA decreased (Figure 3C and D). In contrast, external SecA-dependent deinsertion (Figure 6) does not cause any alteration in the SecA-membrane interaction or the net amount of membrane-inserted SecA. The difference between PMF-dependent and external SecA-dependent deinsertion (Figure 7) is critically important for understanding the role of PMF in protein translocation.

Promotion of the SecA deinsertion by PMF most likely causes efficient re-insertion of SecA, thereby rendering protein translocation efficient. On the other hand, for the SecA insertion to be repeated efficiently in the absence of PMF, a high concentration of external SecA is required. Protein translocation into PrlA4 membrane vesicles recently was found to take place efficiently even at low SecA levels (Van der Wolk *et al.*, 1998). This observation is consistent with our observation that PMF decreases the amount of SecA required for maximum translocation activity (Figure 3) since the *prlA4* mutation renders the translocation less dependent on PMF (Nouwen *et al.*, 1996). It was also reported that SecA binds to PrlA4 membrane vesicles with higher affinity than to wild-type membrane vesicles, thereby causing more efficient initiation of the translocation (Van der Wolk *et al.*, 1998). However, since the translocation activity is substantially higher with PrlA4 membrane vesicles than with wild-type membrane vesicles even at saturating SecA concentrations (figure 6 of Van der Wolk *et al.*, 1998), the elevated affinity of SecA binding does not seem to be the sole

Fig. 6. PMF deinserts SecA from the membrane and causes the completion of OmpA translocation. (A) The membrane insertion of [¹²⁵I]SecA in the absence of PMF was started in a reaction mixture containing 1 mM EDTA and no MgSO₄ (○). At 10 min, 2 mM MgSO₄ (●) or 5 mM succinate (▲) was added to a portion of the reaction mixture. (B) Membrane vesicles loaded with [¹²⁵I]SecA were subjected to *in vitro* proOmpA translocation in the absence of PMF. The reaction mixture contained 1 mM, instead of 5 mM, MgSO₄. At 15 min, 2 mM EDTA was added to the reaction mixture (●). At 20 min, 0.4 μM non-radioactive SecA (Δ), 5 mM succinate (□) or nothing (●) was added further. At 30 min, 4 mM MgSO₄ was then added to a portion of a sample containing non-radioactive SecA (▲). The level of the membrane-inserted 30 kDa fragment of SecA was determined as described in Figure 1B. (C) The translocation of proOmpA into 4 M urea-washed membrane vesicles was examined as in Figure 1A. EDTA (2 mM) and succinate (5 mM) were added successively to the reaction mixture at 15 and 25 min, respectively. Aliquots (25 μl) of the reaction mixture were withdrawn at the specified times and treated with 1 mg/ml proteinase K on ice for 15 min. The samples were then analyzed by SDS-PAGE and fluorography. The translocation intermediates, mature OmpA (m) and proOmpA (p), are indicated on the right. The left lane represents 2% of the input proOmpA. (D) The amounts of the bands detected in (C) were determined with an ATTO densitograph and expressed as percentages of the amount of proOmpA plus OmpA at 35 min. The results are averages for four experiments. The standard deviation is indicated by an error bar.

Fig. 7. Schematic model of the PMF-dependent deinsertion of [125 I]SecA. The Sec factors are represented by the respective upper case letters, and A* represents [125 I]SecA. YajC is also encoded by the *secD* operon (Gardel *et al.*, 1990) and constitutes the translocation machinery (Duong and Wickner, 1997). The membrane-inserted SecA exists in the deinsertion-competent and -incompetent states. Inhibition of ATP hydrolysis by AMP-PNP or EDTA blocks the conversion of the deinsertion-incompetent SecA to the competent form. Only competent SecA is deinserted by PMF and non-labeled SecA. A portion of the membrane-deinserted SecA dissociates from the membrane and is recovered in the supernatant after centrifugation. For details, see the text.

reason for the efficient translocation into PrlA4 membrane vesicles.

As far as translocation-coupled SecA insertion was concerned, PMF and external SecA induced similar extents of SecA deinsertion (Figures 4B and 6B), indicating that the same species of inserted SecA molecules are competent with regard to deinsertion by PMF and external SecA (Figure 7). This deinsertion-competent SecA fraction is estimated to comprise about two-thirds of the inserted SecA (Figure 6). When MgSO_4 was added to induce ATP hydrolysis, the deinsertion-incompetent SecA was converted to the competent form, and then completely deinserted by external SecA (Figure 6B). The SecA deinsertion step per se is, therefore, independent of ATP hydrolysis. It should be noted that both competent and incompetent SecA molecules generate the identical proteinase K-resistant 30 kDa fragment.

The requirement for excess SecA for deinsertion immediately explains why an excess amount of SecA is required for efficient protein translocation in the absence of PMF (Yamada *et al.*, 1989a). Stimulation of SecA deinsertion by PMF is likely to compensate partly for the less efficient SecA insertion caused by the ΔsecG (Duong and Wickner, 1997; Matsumoto *et al.*, 1997; Suzuki *et al.*, 1998) or *secAcsR11* (Suzuki *et al.*, 1998) mutation, thereby making the translocation defect less significant (Hanada *et al.*, 1996; Suzuki *et al.*, 1998). SecD and SecF are involved in the late stage of proOmpA translocation (Matsuyama *et al.*, 1993), and stabilize the membrane-inserted SecA (Kim *et al.*, 1994; Economou *et al.*, 1995; Duong and

Wickner, 1997). Premature deinsertion of SecA caused by the depletion of SecD and SecF may be the reason why PMF generation is uncoupled in a mutant lacking the *secD* operon (Arkowitz and Wickner, 1994). Protein translocation activity reconstituted from purified SecY, SecE and SecA is stimulated by PMF (Brundage *et al.*, 1990). Moreover, both protein translocation (Nouwen *et al.*, 1996) and SecA deinsertion (Figure 2) are less dependent on PMF with membrane vesicles containing PrlA4. These observations strongly suggest that SecY is the main target of the PMF action. PMF presumably induces a conformational change of SecY, which forms a protein-conducting channel in the membrane, and thereby accelerates the SecA deinsertion. The conformational change of SecY induced by PMF may also allow the translocation of precursors with abnormal structures (Tani *et al.*, 1990; Schiebel *et al.*, 1991; Tani and Mizushima, 1991; Kato and Mizushima, 1993; Van der Wolk *et al.*, 1998).

In contrast to translocation-coupled SecA insertion, AMP-PNP-dependent SecA insertion was insensitive to PMF regardless of the presence or absence of proOmpA, although it was deinserted slowly by external SecA (Figure 4A). The state of a SecA molecule inserted in an AMP-PNP-dependent manner is unknown. It may be similar to the state of SecA which is inserted in a translocation-coupled manner and exists in a deinsertion-incompetent state. On the other hand, the insertion of D209N SecA was sensitive to PMF but resistant to deinsertion by external SecA (Figure 5). The deinsertion and protein translocation are therefore uncoupled in the mutant SecA.

The results we present here raise questions that remain to be answered. It will be important to demonstrate more directly the PMF-dependent conformational change of SecY and/or the translocation machinery. As has been postulated for flagella motor rotation (Eisenbach, 1990; DeRosier, 1998), transmembrane proton movement may be involved in the conformational change. The structures of the PrlA4 protein and the translocation machinery containing PrlA4 are important since they may resemble those of the wild-type SecY and the machinery containing SecY under the influence of PMF. SecA possessing two ATP-binding sites is known to function as a dimer (Akita *et al.*, 1991; Cabelli *et al.*, 1991; Driessen, 1993), but little is known about how the dimer undergoes the SecA cycle, i.e. whether both subunits or only one subunit of the dimer is inserted into the membrane. What is different between the deinsertion-competent and -incompetent SecA molecules remains to be clarified. The D209N mutation appears to fix SecA in the deinsertion-competent state since the PMF-dependent deinsertion of the mutant SecA is similar to that of the wild-type SecA even though the mutant exhibits no ATPase activity at the high affinity ATP-binding site (Mitchell and Oliver, 1993).

It has been reported that SecA insertion into membrane vesicles washed with 6 M urea is stimulated by PMF only with a non-physiologically low ATP concentration (Economou *et al.*, 1995). The magnitude of PMF generated by these membrane vesicles was not reported. From the data presented here, PMF was found to accelerate the SecA cycle even with a physiological ATP concentration.

Materials and methods

Materials

SecA (Akita *et al.*, 1990), SecB (Weiss *et al.*, 1988) and proOmpA (Tani *et al.*, 1990) were purified from cells overproducing the respective proteins. [³⁵S]ProOmpA was synthesized *in vitro* in the presence of Trans³⁵S-label and partially purified by means of gel filtration to remove small molecules (Tani *et al.*, 1989). ATP, AMP-PNP and creatine kinase were purchased from Boehringer Mannheim. Proteinase K was from Merck. Succinate, creatine phosphate and CCCP were from Sigma. Na[¹²⁵I] (629 GBq/mg I) and Trans³⁵S-label (37 TBq/mmol as [³⁵S]-methionine) were from ICN. [¹⁴C]KSCN (0.56 GBq/mmol) and [¹⁴C]-methylamine (1.9 GBq/mmol) were obtained from Moravex.

Preparation of D209N SecA

D209N SecA was overproduced in *E. coli* BL21.19 [*secA(am) supFts trp(am) zch::Tn10 recA::cat clpA::kan*] harboring pD209N and then purified from the membrane fraction of the cells as reported (Mitchell and Oliver, 1993). A mixture of protease inhibitors comprising 5 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, 5 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml antipain and 10 µg/ml phosphoramidon was present throughout the purification procedures. The membrane fraction was suspended in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol, 8.5% sucrose and the protease inhibitor mixture, and then treated with 5 M urea on ice for 1 h to extract D209N SecA. The supernatant obtained on centrifugation at 150 000 *g* for 2 h was dialyzed against 50 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol (DTT) and the protease inhibitor mixture. After removal of the insoluble materials by centrifugation, the supernatant was subjected to column chromatography to purify D209N SecA as described for the wild-type SecA (Akita *et al.*, 1990).

Preparation of inverted membrane vesicles

Inverted membrane vesicles were prepared from *E. coli* K003 (HfrH *pnp-13 tyr met RNaseI⁻ Lpp⁻ ΔuncB-C::Tn10*) (Yamane *et al.*, 1987), CE1437 (F⁻ *ΔlacU169 araD139 rpsL thi relA ptsF25 deoC1 lamBD60 ΔuncB-C::Tn10*) and CE1438 (CE1437 *prlA4*) (Nouwen *et al.*, 1996) as described previously (Yamada *et al.*, 1989b), and washed with 4 M urea as reported (Hanada *et al.*, 1994).

Membrane insertion of SecA

SecA was iodinated with [¹²⁵I]NaI and iodogen (Pierce) as described (Economou and Wickner, 1994). ³⁵S-labeled SecA was synthesized *in vitro* in the presence of Trans³⁵S-label using an *E. coli* S-30 extract system (Promega) and plasmid pMAN400 (Kawasaki *et al.*, 1989) carrying the *secA* gene under the control of the *tac* promoter. SecA insertion was examined as reported (Economou and Wickner, 1994) with a minor modification. Briefly, membrane vesicles (0.4 mg/ml) washed with 4 M urea were mixed with [¹²⁵I]- or ³⁵S-labeled SecA (8 nM) in 50 mM potassium phosphate (pH 7.5) containing 5 mM MgSO₄, 0.2 mg/ml bovine serum albumin (BSA) and 1 mM DTT (buffer A). After incubation on ice for 30 min, membrane vesicles loaded with [¹²⁵I]SecA or [³⁵S]SecA were overlaid on an equal volume of buffer A containing 0.2 M sucrose, recovered by centrifugation (170 000 *g* for 30 min) at 4°C, and then suspended in the original volume of buffer A. To examine the effect of CCCP (10 µM), DTT was omitted from the reaction mixture. When the effect of EDTA was examined, the MgSO₄ concentration in the reaction mixture was reduced to 1 mM. Where specified, PMF was generated by the addition of succinate at 5 mM. The membrane suspension was mixed with an equal volume of a solution comprising 2 mM ATP, 5 mM creatine phosphate, 10 µg/ml creatine kinase and 0.1 mg/ml SecB in buffer A. After pre-incubation for 2 min at 37°C, proOmpA (25 µg/ml) was added to initiate the translocation reaction. At the indicated times, aliquots (100 µl) were withdrawn and treated with proteinase K (1 mg/ml) on ice for 15 min. The proteinase K-resistant 30 and 65 kDa bands detected on autoradiography and fluorography, respectively, after SDS-PAGE were quantitated densitometrically and expressed as percentages of the initial amount of radio-labeled SecA. In some experiments, the membrane insertion of [¹²⁵I]SecA was examined without the membrane-loading step described above.

Protein translocation into urea-washed membrane vesicles

The reaction mixture, comprising 4 M urea-treated membrane vesicles (0.2 mg/ml), SecA at a specified concentration, SecB (50 µg/ml), 1 mM ATP, 5 mM MgSO₄, 1 mM DTT, an ATP-generating system (5 µg/ml creatine kinase plus 2.5 mM creatine phosphate) and 50 mM potassium

phosphate (pH 7.5), was pre-incubated at 37°C for 2 min. The effects of CCCP, EDTA and succinate were examined as described for SecA insertion. The reaction was initiated by the addition of a pre-warmed mixture of non-radioactive proOmpA (25 µg/ml) and [³⁵S]proOmpA (2.0×10⁶ c.p.m./ml). Aliquots (25 µl) of the reaction mixture were withdrawn at various times and mixed with proteinase K (1 mg/ml) to terminate the reaction. After proteinase K digestion on ice, the OmpA materials were recovered by trichloroacetic acid precipitation (final, 10%), successively washed with acetone and ether, and then analyzed by SDS-PAGE and fluorography. The translocation activity was determined by densitometric quantitation of the OmpA materials with an ATTO densitograph and expressed as pmol of OmpA translocated per mg of membrane vesicles.

Other methods

The membrane potential (inside positive) and ΔpH (inside acidic) were examined at 37°C by monitoring the fluorescence quenching of oxonol V (1 µM) and quinacrine (1 µM), respectively, as described (Yamada *et al.*, 1989b). The distribution of [¹⁴C]KSCN (20 µM) and [¹⁴C]methylamine (15 µM) in membrane vesicles was measured using flow dialysis (Reenstra *et al.*, 1980) to quantitate the membrane potential and ΔpH, respectively. Protein was determined by the method of Lowry *et al.* (1951). SDS-PAGE was performed according to Laemmli (1970).

Acknowledgements

We thank J. Tommassen for *E. coli* CE1437 and CE1438, and D. Oliver for *E. coli* BL21.19 harboring pD209N. The ¹²⁵I-labeling of SecA was carried out at the Radioisotope Center with the help of N. Nogawa. We also thank S. Nishikawa for secretarial support and the Asahi Glass Foundation for financial support. This work was also supported by grants to H.T. from CREST of Japan Science and Technology Corporation, and the Ministry of Education, Science, Sports and Culture of Japan.

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Received October 20, 1998; revised and accepted December 15, 1998