A High Concentration of SecA Allows Proton Motive Force-independent Translocation of a Model Secretory Protein into Escherichia coli Membrane Vesicles*

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The in vitro translocation of OmpF-Lpp, a model secretory protein, into inverted membrane vesicles of Escherichia coli obligatorily requires the proton motive force (ΔμH+) in the conventional assay system (Yamada, H., Tokuda, H., and Mizushima, S. (1989) J. Biol. Chem. 264, 1723-1728). The translocation, however, took place efficiently, even in the absence of ΔμH+, when the system was supplemented with additional SecA. With the stripped membrane vesicles, which are permeable to protons, or in the absence of NADH, the supplementation of SecA remarkably stimulated the translocation activity. The further addition of NADH did not significantly enhance the translocation activity under the SecA-enriched conditions. OmpF-Lpp thus translocated could be recovered from the vesicular lumen by sonication, indicating that complete translocation occurred in the absence of ΔμH+. It is suggested that ΔμH+ is required for high affinity interaction of SecA with the presumed secretory machinery in the cytoplasmic membrane and that a high concentration of SecA modulates the ΔμH+ requirement.

The translocation of proteins across the cytoplasmic membrane, the first step of protein secretion in prokaryotic cells, requires two sources of energy, ATP and the proton motive force (ΔμH+). ATP is absolutely essential for the translocation, no in vitro translocation of secretory proteins being detected at all in its absence (1-7). On the other hand, the role of ΔμH+ has been the subject of controversy. Some in vitro studies demonstrated the involvement of ΔμH+ in the translocation reaction (4-7), whereas others did not (1, 2). Very recently, we quantitatively and kinetically studied the ΔμH+ requirement using purified membrane vesicles of Escherichia coli and found that the degree of the requirement differs with the secretory protein species (8). For example, the translocation of OmpF-Lpp, a model secretory protein, absolutely requires ΔμH+, whereas that of OmpA takes place appreciably, although at a slower rate, even in the complete absence of ΔμH+. On the other hand, the ΔμH+ requirement has been consistently demonstrated in vivo (9-13).

The existence of a secretory machinery in the cytoplasmic membrane has been assumed. As possible candidates for the protein components of the machinery, SecY and SecA have been studied rather extensively in E. coli. SecY is a membrane protein required for protein export (14, 15). It is embedded in the cytoplasmic membrane through its several transmembrane segments (16). Its essentiality in the export of several periplasmic and outer membrane proteins has been demonstrated both in vivo (14, 17) and in vitro (18, 19). SecA is another essential component for protein export (20, 21). SecA has been found in both cytoplasmic membrane and cytosol preparations, suggesting that it is a peripheral membrane protein (21). This protein was overproduced in E. coli cells with the aid of high expression: vector plasmids and almost purified to homogeneity (22). The purified SecA stimulated the in vitro translocation of secretory proteins into membrane vesicles (22, 23).

Despite this accumulated knowledge, little is known about how these two sources of energy are involved in the functioning of the translocation machinery that includes SecA and SecY. We show here that the in vitro translocation of OmpF-Lpp, which absolutely requires ΔμH+ under the conventional assay conditions, can take place appreciably in the absence of ΔμH+ when a substantial amount of SecA is added to the reaction mixture. OmpF-Lpp, thus translocated into membrane vesicles, can be recovered from the vesicular lumen in a soluble form, suggesting that the translocation can be completed in the absence of ΔμH+. The results suggest that the roles of ΔμH+ and SecA in the protein translocation reaction are related.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains and Plasmids—E. coli K002 (Lpp uncB C*) (26), and K003 (Lpp-ΔuncB C*-Tn10) (5) were used. Plasmid pNM31 contains an ompF-Lpp chimeric gene (24) and plasmid pK107 carries a mutant ompF-Lpp chimeric gene that codes for an uncleavable signal peptide-containing OmpF-Lpp chimeric protein (uncleavable OmpF-Lpp) (6).

Materials—ATP and other high energy phosphate compounds, pyruvate kinase (rabbit muscle), creatine kinase (rabbit muscle), and a tRNA mixture (E. coli) were obtained from Boehringer Mannheim. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was from Sigma and N,N'-dicyclohexyl-carbodiimide (DCCD) from TAKARA SHUZO CO., LTD. The purified F$_{1}$ complex of F$_{1}$-ATPase and the anti-F$_{1}$-ATPase antiserum were gifts from M. Futai (Osaka University).

Preparation of Purified SecA—SecA was purified from SecA-overproducing strains (RR1/pMAN400) by means of ammonium sulfate.
fractionation and gel filtration, as described previously (22). The purity of the final preparation was about 90%. The purified SecA was dissolved in 50 mM potassium phosphate, pH 7.6, 5 mM magnesium sulfate.

Preparation of a Cell Extract for in Vitro Protein Synthesis—A membrane-free cell extract for protein synthesis, the S100 fraction, was prepared from E. coli K003 by the method described previously (8).

Preparation of Inverted Membrane Vesicles and Stripped Membrane Vesicles for Protein Translocation Experiments—E. coli K003 (ΔuncC-B-C) was used as the source of inverted membrane vesicles (SDV) and K002 (uncB-C) as the source of stripped membrane vesicles. The latter were prepared by stripping the F1 subunit off Ff,Ff-ATPase through treatment with 2.5 mM urea as described (8). The amount of membrane vesicles was expressed as that of protein, which was determined by the method of Lowry et al. (20).

Transcription and Translation Reactions—In vitro transcription of the ompF-Lpp chimeric genes was performed with SP6 RNA polymerase as described (25). The translation reaction was then carried out in the presence of L-[35S]methionine by the method described (8).

Removal of Small Molecules from the Reaction Mixture after Translation—Removal of small molecules, including ATP and NADH, from the translation mixture was carried out as described previously (5).

Translocation Reactions—Post-translational translocation reactions were carried out with either the translation reaction mixture containing proOMP-F-Lpp or proOMP-F-Lpp partially purified from the reaction mixture by gel filtration. The methods used were basically those described previously (6, 8).

For the direct translocation reaction, membrane vesicles (5 μg) suspended in 8 μl of 50 mM potassium phosphate, pH 7.6, 5 mM magnesium sulfate were mixed with 2 μl of the SecA solution (0.4 μg) on ice, and then the mixture was preincubated at 37°C for 3 min. It was then mixed with 15 μl of the preincubated translation mixture to initiate the translocation reaction.

For the translocation reaction with the partially purified proOMP-F-Lpp, the mixture containing membrane vesicles and SecA mentioned above was further mixed with 5 μl of 10 mM ATP, 30 mM NADH on ice, followed by preincubation at 37°C for 3 min. To the mixture was added 10 μl of the preincubated partially purified proOMP-F-Lpp to initiate the translocation reaction. The final concentrations of ATP, NADH, and MgSO4 were, therefore, 2, 6, and 5 mM, respectively.

The reaction was terminated on ice. The reaction mixture was then treated with proteinase K, and the translocated protein, which was proteinase K-resistant, was detected on sodium dodecyl sulfate-polyacrylamide gel by means of fluorography as described (3). The amounts of protein in the individual bands on fluorograms were determined densitometrically with a Shimadzu CS-930 chromatogram scanner. The efficiency of translocation was estimated from the intensities, on fluorograms, of protein bands before and after the translocation reaction. The reaction mixture was then analyzed as described previously (5).

Determination of Δψ and ΔpH—The generation of Δψ (inside positive) and ΔpH (inside acidic) in the stripped membrane vesicles was monitored as described previously (8) by following the fluorescence quenching of oxonol V and quinacrine, respectively. The reaction mixtures, at 37°C, contained, in 2 ml, 50 mM potassium phosphate, pH 7.5, 5 mM MgSO4, stripped membrane vesicles (50 μg) and 1 μM oxonol V or quinacrine. The fluorescence emission of oxonol V was measured at 655 nm with excitation at 580 nm and that of quinacrine at 500 nm with excitation at 420 nm. Where specified, the stripped membrane vesicles were pretreated in the presence of DCCD as described previously (8).

Recovery of Translocated Proteins from the Vesicular Lumen—A translation mixture (180 μl), which contained either proOMP-F-Lpp or uncleavable OmpF-Lpp, was directly mixed with either intact membrane vesicles (60 μg) or the equivalent amount of stripped membrane vesicles (5 μg) and then sonicated in 125 mM potassium phosphate, pH 7.6, 5 mM magnesium sulfate. When indicated, 4 μg of SecA was added. The translocation reaction was then carried out at 37°C for 20 min. A portion (50 μl) of the reaction mixture was stored as the total fraction and remainder was centrifuged at 50,000 rpm for 30 min in a Beckman TLS-100 rotor to obtain supernatant and pellet fractions. The latter was then suspended in 250 μl of 10 mM potassium phosphate, pH 7.6, and 50 μl of the suspension was stored as the membrane vesicles fraction. The remainder (200 μl) was sonicated with a bath-type sonicator four times (30 s each) with 30 s intervals. The sonicates were separated by centrifugation at 50,000 rpm for 2 h into supernatant (sonic sup) and pellet (sonicated membrane) fractions, and the latter was suspended in 200 μl of 10 mM potassium phosphate, pH 7.6. 25 μl each of all the fractions was treated with or without proteinase K, followed by analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described (5). Portions of the sonicated membrane fractions were further sonicated in the presence of proteinase K or treated with proteinase K in the presence of 1% Triton X-100 before gel electrophoretic analysis.

Immunoblotting and Quantitation of SecA—Proteins in the cytosol and membrane fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then the bands were transferred to Durapore sheets (Millipore) using a semidyry transfer cell (Bio-Rad) according to the method recommended by the manufacturer. The blots were incubated in TBS (50 mM Tris-HCl, 0.15 M NaCl, pH 7.5) containing 10% horse serum for 1 h and then with the anti-SecA antiserum in TBS, 3% horse serum for 12 h. The blots were washed with TBS, 0.05% Tween 20, and then incubated with the alkaline phosphatase-associated anti-rabbit antibody in TBS. After washing with TBS, 0.05% Tween 20, the blots were visualized with 0.1% 5-bromo-4-chloro-3-indolylphosphate in 0.1 M sodium carbonate, pH 9.6, for 20 min at 37°C, washed with water, and finally dried.

For quantitation of SecA, the blots were scanned with a Dual-wavelength Chromato Scanner CS-930 (Shimadzu Co.). The purified SecA was used as a standard.

RESULTS

In Vitro Translocation of OmpF-Lpp Can Take Place in the Absence of Δψ* when SecA Is Supplemented at a High Concentration—The in vitro translocation of OmpF-Lpp absolutely requires Δψ*- (8). When stripped membrane vesicles, which are permeable to protons through Ff-channels, were used no in vitro translocation was observed (8 and Fig. 1). Upon the addition of SecA at a high concentration, however, the membrane vesicles exhibited considerable translocation activity (Fig. 1A). The addition of SecA also enhanced, to

FIG. 1. Stripped membrane vesicles exhibit efficient translocation of OmpF-Lpp in the presence of a high concentration of SecA. A. Translocation of OmpF-Lpp was carried out with stripped membrane vesicles (A, Δ) and intact membrane vesicles (●, O) in the presence (A, ●) and absence (Δ, O) of externally added SecA (16 ng/μl). The translocation reaction was directly used as the source of proOMP-F-Lpp. At the indicated times, the translocation reaction was terminated on ice. The reaction mixtures were then treated with proteinase K and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The x-ray film was further subjected to densitometric scanning to determine the degree of translocation. 100% translocation means that all the proOMP-F-Lpp became mature Omp-Lpp resistant to proteinase K. B, The translocation reaction was carried out with stripped membrane vesicles in the presence of the indicated amounts (ng/μl) of SecA for 15 min. The reaction was terminated on ice and then the mixtures were analyzed as described in A. A fluorogram is presented as an inset. The positions of ProOMP-F-Lpp (p) and mature Omp-F-Lpp (m) are indicated.
some extent, the translocation activity of intact membrane vesicles as reported previously (8) (Fig. 1A).

The translocation activity of stripped membrane vesicles was increased as the increase in the amount of SecA added. The addition of 16 ng/μl of SecA was required for the maximum activation (Fig. 1B). The amounts of SecA originally contained in the membrane vesicles and the S100 fraction, in 1 μl of reaction mixture, were roughly estimated by means of Western blotting using the anti-SecA antiserum. These were about 0.15, 0.8, and 2.2 ng for the stripped membrane vesicles, the intact membrane vesicles prepared from K003 (Δunc), and the S100 fraction, respectively.

The stripped membrane vesicles restore the ΔμH⁺ generating activity and thereby restore the translocation activity when the F₁ subunit of FoF₁-ATPase is added (3, 4, 8). The anti-F₁ antiserum inhibited the F₁-dependent restoration of the translocation activity, whereas it did not inhibit the activity observed upon addition of SecA (data not shown). We conclude, therefore, that the restoration of the translocation activity by the SecA preparation was not due to contamination by the F₁ subunit.

We also examined the possibility that the stripped membrane vesicles might have become capable of generating ΔμH⁺ upon the addition of SecA. The addition of SecA, however, resulted in the generation of neither the membrane potential (Δφ) nor ΔμH⁺, which together constitute ΔμH⁺, in the presence of NADH, whereas DCCD, which binds to the F₁ complex and thereby inhibits proton movement through the F₁ channels, did (Fig. 2).

The SecA-stimulated Translocation into Stripped Membrane Vesicles Is ATP Dependent and NADH Independent—In order to examine more closely the relationship between the requirements for ΔμH⁺ and SecA, small molecules, including ATP and NADH, were removed from the reaction mixture after the translational synthesis of proOmpF-Lpp. The translocation of the partially purified precursor absolutely required ATP, irrespective of whether the membrane vesicles were stripped or intact, or whether SecA was added or not (data not shown). With the stripped membrane vesicles, translocation was not observed even after the addition of NADH, whereas translocation took place efficiently when SecA (16 ng/μl reaction mixture) was added (Fig. 3). The SecA-stimulated translocation was almost completely independent of contamination by intact membrane vesicles.

![Fig. 2. Effect of SecA on the generation of Δφ and ΔμH⁺ by stripped membrane vesicles. The generation of Δφ (inside positive) (A) and ΔμH⁺ (inside acidic) (B) by stripped membrane vesicles (stripped) in the presence or absence of SecA was monitored by means of fluorescence quenching as described under "Experimental Procedures." Where indicated (stripped/SecA), 4 μg of purified SecA was added to 50 μl of the stripped membrane vesicles, followed by incubation at 37 °C for 3 min prior to starting the assay. As a control, stripped membrane vesicles treated with DCCD as described (8) were used (stripped/DCCD).](image)

![Fig. 3. The SecA-stimulated translocation into stripped membrane vesicles is NADH-independent. ProOmpF-Lpp partially purified by gel filtration was used as a substrate, and translocation reactions were performed with the stripped membrane vesicles. The additions were ATP (△), ATP+NADH (○), ATP+SecA (▲) and ATP+NADH+SecA (●). The final concentrations of ATP, NADH, and SecA were 2, 6 mM, and 16 ng/μl, respectively. At the indicated times, the translocation reaction was terminated on ice. All samples were then treated with protease K and analyzed as described in the legend to Fig. 1.](image)

![Fig. 4. SecA also remarkably stimulates the translocation into intact membrane vesicles in the absence of NADH. ProOmpF-Lpp, partially purified by gel filtration, was used as a substrate and translocation reactions were performed with intact membrane vesicles. The additions were ATP (△), ATP+NADH (○), ATP+SecA (▲), and ATP+NADH+SecA (●). The final concentrations of ATP, NADH, and SecA were 2 mM, 6 mM, and 16 ng/μl, respectively. At the indicated times, the translocation reaction was terminated on ice. All samples were then treated with protease K and analyzed as described in the legend to Fig. 1.](image)

SecA Also Remarkably Stimulates Translocation into Intact Membrane Vesicles in the Absence of NADH—A similar kinetic study was performed with intact membrane vesicles (Fig. 4). The very low translocation activity observed in the absence of NADH was enhanced remarkably upon the addition of the purified SecA. More than 10-fold stimulation was observed. The activity was comparable with that of the
stripped membrane vesicles supplemented with SecA. Thus, the result supports the view that efficient translocation can take place in the absence of δψHt when a sufficient amount of SecA is added.

Fig. 4 also shows that the stimulation by NADH in the presence of a high concentration of SecA was not as remarkable as that without externally added SecA. It is probable, therefore, that SecA modulates the δψHt requirement for translocation.

Effect of SecA on Translocation in the Presence of CCCP—
CCCP is a protonophore which collapses δψHt across membranes. CCCP at 10 μM almost completely inhibited the translocation of OmpF-Lpp into intact membrane vesicles (8, see also Fig. 5A). The translocation was, however, enhanced significantly upon the addition of SecA, although the enhanced activity level was still lower than that with SecA but without CCCP. With the stripped membrane vesicles, SecA-stimulated translocation was also observed in the presence of CCCP (Fig. 5B). It was noticeable, however, that the SecA-stimulated translocation activity in the presence of CCCP was not as high as that with the stripped membrane vesicles or that in the absence of NADH, both of which also result in δψHt collapse. In particular, the translocation activity considerably decreased upon the addition of CCCP, even with the stripped membrane vesicles, in which case δψHt had already been abolished. These results suggest that CCCP has another target, i.e. in addition to δψHt, in the translocation machinery.

In the experiments shown in Fig. 5, membrane vesicles were first treated with CCCP and then with SecA. When SecA was added first to the membrane vesicles and then CCCP was added, on the other hand, no stimulation by SecA was observed at all (data not shown). It is probable, therefore, that SecA is another target of CCCP. The rather weak inhibitory effect of CCCP observable in Fig. 5 may be due to strong binding of this inhibitor to membrane vesicles when both are mixed first, so that SecA added later is not inhibited completely by this compound.

OmpF-Lpp Can be Completely Translocated across the Membrane and Ejected into the Vesicular Lumen in the Absence of δψHt When a Sufficient Amount of SecA Is Added—In all the experiments so far described, the translocation activity was determined in terms of protease K resistance. It was possible, therefore, that incompletely translocated OmpF-Lpp might have given the same result. To determine whether or not OmpF-Lpp can be completely translocated into the lumen of stripped membrane vesicles, membrane vesicles were recovered by centrifugation and then sonicated to separate materials in the vesicular lumen and the membrane.

First, an experiment was carried out with intact membrane vesicles in the presence of both ATP and δψHt (Fig. 6A). Almost all of the mature form of OmpF-Lpp formed was recovered with the membrane vesicles in a protease K-resistant form. Upon sonication, most of it was released from the membrane vesicles and became proteinase K-sensitive, indicating that it was translocated across the membrane and ejected into the vesicular lumen. A small portion of OmpF-Lpp, which was recovered with the pellet even after the sonication, was protease K resistant. The resistance did not disappear even after sonication in the presence of protease K.

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K unless Triton X-100 was added to the mixture, suggesting that a portion of the protein was retained inside the membrane bilayer.

As a control experiment, uncleavable OmpF-Lpp (6) was used as a substrate for translocation (Fig. 6B). Since this protein contains an uncleavable signal peptide, the translocated protein is assumed to be held on the membrane through the signal peptide. Almost all of the uncleavable OmpF-Lpp was recovered with the membrane vesicles as a proteinase K-resistant form. The protein was not released even on sonication. This suggests that the fractionation procedures employed here were satisfactory and that the recovery in the supernatant fraction of mature OmpF-Lpp after the sonication was not due to fragmentation of the membrane vesicles into very fine particles which cannot be recovered on centrifugation.

Essentially the same result as that with intact membrane vesicles was obtained when stripped membrane vesicles were used with a substantial amount of SecA (Fig. 6C); namely, a larger portion of the mature OmpF-Lpp formed was recovered as a proteinase K-resistant form with the membrane vesicles and released from them upon sonication. We conclude, therefore, that the complete translocation, including ejection of the translocated protein into the vesicular lumen, can take place in the complete absence of ΔpH, when a sufficient amount of SecA is added.

DISCUSSION

The role of ΔpH in the in vitro translocation of secretory proteins has been the subject of controversy (1, 2, 4–7). In a previous study (8), we showed that the degree of the requirement of ΔpH for in vitro protein translocation differs with the secretory protein species: some obligatorily require ΔpH, while the translocation of others takes place rather efficiently in the absence of ΔpH. We further found in the present study that even the translocation of a protein which obligatorily requires ΔpH with the conventional assay method took place efficiently in the complete absence of ΔpH when a substantial amount of SecA was added. On the other hand, the requirement of ΔpH for in vivo protein translocation across the cytoplasmic membrane has been reported (9–13). It would be interesting to determine, therefore, whether or not the in vivo ΔpH requirement can be modulated by the amount of SecA in cells, and this is currently being investigated in this laboratory. It is also interesting, in this respect, that SecA is overproduced when the cellular translocation process is inhibited (21, 27–29).

In contrast to the requirement for ΔpH, the requirement for ATP is highly obligatory (1–7). Furthermore, the present work showed that the protein translocation did not take place at all without ATP, even in the presence of a large amount of SecA. It is probable, therefore, that ATP is the energy source that is directly involved in protein movement across the cytoplasmic membrane.

What are the roles of ΔpH in protein translocation and how does SecA apparently replace ΔpH then? At the moment, we do not have a definite answer to these questions. A simple explanation is that ΔpH is directly involved in a high affinity interaction of SecA with the secretory machinery, which is presumed to exist in the membrane. In the presence of a higher concentration of SecA, the requirement of ΔpH would, therefore, be suppressed. Our recent finding, which is described below, may not support this view, however. The collapse of ΔpH resulted in the transient accumulation of a translocation intermediate of proOmpA in vitro, and the accumulation was rather enhanced, not suppressed, by the addition of SecA.2 If ΔpH directly participates in the SecA-secretory machinery interaction, SecA would rather suppress the accumulation.

Alternatively, ΔpH may be required for a proper conformation of the presumed machinery for protein translocation as discussed previously (5, 13), and changes in the conformation occurring upon deenergization would lower the affinity to SecA on one side and enhance the accumulation of the translocation intermediate on the other side. The requirement of ΔpH for the maintenance of functional structures in membranes has been reported as to several membrane proteins (30, 31). The SecY protein, which is assumed to play an important role in protein translocation, is certainly a possible candidate for this kind of protein. Suppression of the SecY24(Ts) defect for protein translocation by additional SecA was reported recently (32). It would be interesting, in these respects, to determine whether or not some SecY mutations result in alteration in the ΔpH requirement for protein translocation or in accumulation of the translocation intermediate.

Acknowledgments—We thank I. Sugihara for the secretarial support.

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