Reconstitution of an Efficient Protein Translocation Machinery Comprising SecA and the Three Membrane Proteins, SecY, SecE, and SecG (p12)*

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The protein translocation machinery of Escherichia coli has been reconstituted from independently purified SecY, SecE, and SecA (1) or the purified SecY-SecE complex and SecA (2). The direct involvement of these three Sec proteins in protein translocation has thus been established. The SecY-SecE complex, whether isolated chromatographically (2) or immunoprecipitated with an anti-SecY antibody (3), contained another protein, termed band 1, however. The activity of the translocation machinery reconstituted from SecY, SecE, and SecA is significantly lower than that of everted membrane vesicles, whereas the translocation machinery reconstituted from the SecY-SecE band 1 complex and SecA has been reported to be comparable to that of membrane vesicles (4).

A novel membrane protein, p12, of E. coli was discovered as a new factor that stimulates the protein translocation reconstituted with SecA, SecY, and SecE (Nishiyama, K., Mizushima, S., and Tokuda, H. (1993) EMBO J. 12, 3409-3415). Direct involvement of p12 in protein translocation was subsequently demonstrated in vivo by genetic studies, and the name SecG has been proposed for p12 (Nishiyama, K., Hanada, M., and Tokuda, H. (1994) EMBO J. 13, 3272-3277). To elucidate the role of SecG in protein translocation and to characterize the translocation apparatus comprising these four Sec proteins, the activity of reconstituted proteoliposomes was examined in detail as a function of the amount of each component. SecG markedly stimulated the translocation activity over wide ranges of amounts of the other three Sec proteins, indicating that none of the other three Sec proteins substitutes for the SecG function. Detailed kinetic analyses indicated that the activity of proteoliposomes was dependent on the amount of the SecY-SecE complex when SecG was absent and the amount of the SecY-SecE-SecG complex when the proteoliposomes contained SecG. The translocation activity of the latter complex was significantly higher than that of the former one. Binding of SecA to liposomes appreciably increased when they contained both SecY and SecE, whereas the further presence of SecG did not enhance the binding. On the other hand, the ATPase activity of SecA, which was dependent on proOmpA and SecY-SecE-containing proteoliposomes, was significantly enhanced when the proteoliposomes contained SecG. Taken together, these results indicate that SecG enhances the translocation activity of the apparatus after the step of SecA targeting to SecY-SecE.

The amino acid sequences of the subunits of SecG exhibit some similarity to those of SecY and SecE, whereas the further presence of SecG did not enhance the binding. On the other hand, the ATPase activity of SecA, which was dependent on proOmpA and SecY-SecE-containing proteoliposomes, was significantly enhanced when the proteoliposomes contained SecG. Taken together, these results indicate that SecG enhances the translocation activity of the apparatus after the step of SecA targeting to SecY-SecE.

Our previous reconstitution studies involving SecA, SecY, and SecE revealed that the activity of proteoliposomes did not exhibit saturation even with an excess amount of SecE (1), suggesting that SecE is present in excess over SecY in functional stoichiometry. These observations appeared to support the proposal by Bieker and Silhavy (10), who assumed SecE functioned as a shuttle between SecA and SecY in the protein translocation pathway. On the other hand, the isolation of the SecY-SecE band 1 (SecG) complex suggests that these proteins function together. Our previous reconstitution studies were carried out without SecG, which plays an important role in an efficient protein translocation reaction. More detailed and careful reconstitution studies are, therefore, required.

In this study, the activity of the translocation machinery reconstituted from the four Sec proteins and phospholipids was examined as a function of the amount of each component.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli W3110 M25 (ompT) was transformed with pMAN809 and pMAN510 for the overproduction of SecE and SecY (11). E. coli K002 (lpp-) was transformed with pDG1, which carries the tac-secG gene, for the overproduction of SecG. The

The translocated protein was detected as a proteinase K-resistant band after SDS-PAGE by fluorography. The amount of translocated protein was determined by densitometric scanning of the fluorograms with a Shimadzu CS-930 chromatocorder. The initial rates of translocation were determined from at least four different time points.

**SecA Binding to Proteoliposomes—**Proteoliposomes were reconstituted as described above except that 125 μg of a phospholipid mixture was used instead of 1.25 μg of *E*. *coli* phospholipids. The phospholipid mixture contained *E*. *coli* phospholipids and NBD-PE, a fluorescent analogue of phosphatidylethanolamine, at a weight ratio of 49:1. The amount of phospholipids in proteoliposomes was determined from the fluorescence intensity of NBD-PE (excitation at 480 nm and emission at 526 nm) with a Shimadzu RF-5000 spectrofluorometer. The assay mixture comprised in 75 μl, the indicated amount of proteoliposomes or liposomes, 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, 2 mM ATP, 2 mM MgSO₄, and 60 μg/ml (0.6 μM) SecA. After a 10-min incubation at 30 °C, the assay mixture was centrifuged at 170,000 × g for 1 h. The precipitates were analyzed by SDS-PAGE, followed by Coomassie Brilliant Blue staining. The assay was also carried out in the absence of phospholipids for correction of the nonspecifically precipitated SecA.

The amounts of precipitated SecA and SecY were determined by densitometric scanning of the Coomassie Brilliant Blue-stained gels using purified SecA and SecY as standards, respectively.

**ATPase Activity of SecY—**SecY (0.45 nmol) and SecE (1.44 nmol), with or without SecG (0.9 nmol), were mixed with 250 μg of *E*. *coli* phospholipids, followed by reconstitution into proteoliposomes. The reconstituted proteoliposomes were resuspended in 210 μl of 50 mM potassium phosphate (pH 7.5) containing 150 mM NaCl. ATPase activity was determined by a coupled assay using creatine kinase and luciferase, and was expressed as picomoles of ATP hydrolyzed per minute per milligram of protein.

**RESULTS**

**SecE and SecY Function Together—**We previously showed that the activity of proteoliposomes reconstituted with various amounts of SecY and a fixed amount of SecE became maximum at a certain amount of SecY, whereas the activity of one reconstituted with various amounts of SecE and a fixed amount of SecY did not level off even with an excess amount of SecE (1). The SecE preparation used in these experiments was, however, found to still contain a considerable amount of phospholipids, which had been added together with octyl glucoside to solubilize the membranes (14). It was also found that the membrane filtration method employed to concentrate SecY resulted in the simultaneous concentration of octyl glucoside. These factors might have interfered with the accurate determination of the activity in relation to the amounts of these components. As described under "Experimental Procedures," the reaction methods for SecY and SecE were improved to overcome these problems. SecY and SecE purified by the improved methods did not contain detectable amounts of phospholipids, as judged by thin layer chromatography. Moreover, the concentration of octyl glucoside in the final preparation was readjusted by means of gel filtration chromatography. The following reconstitution studies were carried out using these preparations. The initial

The abbreviations used are: octyl glucoside, n-octyl-p-D-glucopyranoside; NBD-PE, N'-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-1,2-dihexadecanoyl-sn-glycero-3-phosphothanolamine; PAGE, polyacrylamide gel electrophoresis.
When the amount of SecY was fixed at various amounts of SecE, the amount of SecE required to maximize the activity exceeded that of SecY (Fig. 2). However, the activity reconstituted with 20 pmol of SecY did not level off with 300 pmol of SecE. Taken together, these results most likely indicate that SecY and SecE function together as a SecY-SecE complex and that the reconstituted activity represents the amount of the SecY-SecE complex, which is in equilibrium with dissociated SecY and SecE, in proteoliposomes. When the activity becomes maximum, most of the functionally reconstituted molecules of one component, the amount of which is fixed, are assumed to become part of the complex upon the addition of the other component. The amount of functional SecY molecules in proteoliposomes reconstituted with equal amounts of SecY and SecE seems to be, therefore, sufficiently high for most SecE molecules to become part of the complex (Fig. 1). The amount of functional SecE molecules in proteoliposomes is assumed to reach a level that is required for most SecE molecules to become part of the complex when an amount of SecE more than 20-fold that of SecY is added (Fig. 2). The significant difference in the SecE:SecY molar ratio for maximum activity observed in Figs. 1 and 2 seems to be due to the fact either that the fraction of functionally reconstituted molecules is smaller with SecE than with SecY or that the SecE:SecY stoichiometry of the complex is more than 1 or both. It is noteworthy that the functional amount of SecY or SecE in proteoliposomes is proportional to the amount if it added since the reconstituted activity increased proportionally with an increase in the amount of the component added until the activity became maximum. The recoveries of SecY (80%) and SecE (90%) in proteoliposomes were constant, irrespective of the amount added.

SecG Stimulates the Activity Even in the Presence of Large Amounts of SecY and SecE—SecG (p12) was identified as a factor that stimulates the translocation activity of proteoliposomes reconstituted from fixed amounts of SecY and SecE (5). The translocation activity upon reconstitution from various amounts of SecY and SecE was determined in the presence and absence of SecG. Analysis of the reconstituted proteoliposomes by SDS-PAGE revealed that SecG did not affect the recoveries of SecY and SecE under the conditions employed in this study (data not shown).

The activity of proteoliposomes reconstituted from 50 pmol of SecE and various amounts of SecY, with or without 150 pmol of SecG, was examined (Fig. 3). SecY was essential irrespective of the presence or absence of SecG, as observed previously (5). SecG significantly enhanced the reconstituted activity over the entire range of amounts of SecY examined. Eadie-Hofstee plots of the data revealed that SecG caused an approximately 5-fold enhancement of the activity upon SecY addition.
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Furthermore, SecG significantly stimulated the translocation activity over the entire range of amounts of SecY (Fig. 3) or SecE (Fig. 4). The SecG-dependent stimulation of the translocation activity over a wide range of amounts of SecY indicates that SecG has a function different from those of SecY and SecE and that it renders the protein translocation machinery more efficient.

Both SecY and SecE, but Not SecG, Are Required for SecA Binding—Proteoliposomes were reconstituted from SecY and SecE, with or without SecG, and then assayed for translocation in the presence of various concentrations of SecA (Fig. 5A). Although proteoliposomes containing SecG exhibited higher translocation activity than those without SecG over the entire SecA concentration range examined, both types of proteoliposomes exhibited essentially the same $K_v$ value for SecA (Fig. 5B), suggesting that SecG does not affect the affinity of SecA for protein translocation.

It has been shown that the binding of SecA to membranes was inhibited by an anti-SecY antibody (22), suggesting that SecY constitutes a part of the receptor for SecA. However, the interaction of SecA with SecY and/or SecE has not been directly demonstrated. Since SecA binds with high capacity to liposomes of *E. coli* phospholipids (23), the effect of phospholipids on the detection of SecY-dependent SecA binding was examined. Proteoliposomes were reconstituted with the normal amount and one-tenth of the normal amount of phospholipids and then assayed for SecA binding in the presence of ATP, which has been shown to decrease the SecA binding to phospholipids (24). Proteoliposomes containing the same amount of phospholipids were used for each assay (Fig. 6A and B). SecA bound to liposomes or proteoliposomes was recovered by ultracentrifugation and then analyzed by SDS-PAGE, followed by Coomassie Brilliant Blue staining. The amount of SecA was then determined by densitometric scanning of the gel. The addition of 0.6 μM SecA to liposomes caused about 0.1 μM to be bound, leaving about 0.5 μM unbound. These values were essentially the same as those reported by Hendrick and Wickner (23) for the SecA binding to phospholipids. When reconstitution was carried out with the normal amount of phospholipids, the amount of SecA bound to proteoliposomes containing SecY and SecE was only marginally higher than that bound to liposomes, irrespective of the presence or absence of SecG (Fig. 6A). On the other hand, proteoliposomes reconstituted with the 10-fold lower amount of phospholipids bound an appreciably higher amount of SecA than liposomes (Fig. 6B). However, SecG did not affect the amount of SecA bound. Although ATP has been reported to decrease the binding of SecA to phospholipids (24), the SecY-SecE-dependent binding of SecA was not appreciably affected by the omission of ATP (data not shown).

We further examined whether SecA binding requires both SecY and SecE or only one of them. Proteoliposomes were reconstituted with SecY, SecE, or both and then assayed for SecA binding (Fig. 6C). The amount of SecA bound to SecY-SecE-containing proteoliposomes was larger than that bound to liposomes, whereas the amount of SecA bound to proteoliposomes containing SecY or SecE alone was similar to that bound to liposomes.
liposomes. The binding of SecA, therefore, required both SecY and SecE, most likely suggesting that SecY and SecE form a complex and then serve as a SecA receptor.

The proteoliposomes used in the experiments shown in Fig. 6 (A and B) were analyzed by SDS-PAGE, followed by Coomassie Brilliant Blue staining. The amounts of SecY, SecE, and SecG recovered in the proteoliposomes were then densitometrically determined using the respective purified proteins as standards. Reconstitution with the 10-fold lower amount of phospholipids was found to decrease the amount of SecY recovered in proteoliposomes by a factor of 2–3, whereas the recoveries of SecE and SecG were only marginally affected. Proteoliposomes reconstituted with the reduced amount of phospholipids were, however, found to contain an approximately 4-fold higher amount of SecY per unit of phospholipids than ones reconstituted with the normal amount of phospholipids. The translocation activity of these proteoliposomes was examined with proOmpA D26 as a substrate, and the initial rates of translocation per unit of SecY in proteoliposomes were estimated (Fig. 7). The activity reconstituted with the reduced amount of phospholipids did not differ significantly from that reconstituted with the normal amount of phospholipids, irrespective of the presence or absence of SecG. These results indicate that the SecY-SecE-dependent binding of SecA detected with the proteoliposomes reconstituted with the reduced amount of phospholipids is functional.

From the amount of SecY in SecY-SecE-containing proteoliposomes reconstituted with the reduced amount of phospholipids and that of SecA bound to the proteoliposomes, the SecA:SecY stoichiometry of the SecA binding was estimated. Although the estimated stoichiometry was rather variable, depending on the experiment, a value of 2±1 was obtained as an average of more than 10 determinations. The SecY content of the SecY-SecE-containing proteoliposomes reconstituted with the reduced amount of phospholipids was 1.1% (w/w), which was considerably higher than the value (0.05–0.1%) reported for membrane vesicles (25). Hartl et al. (22) reported that the number of high affinity binding sites for SecA, which is assumed to be dependent on SecY and/or SecE, is about 0.1 nmol per mg of membrane protein or per mg of membrane lipid, since the amounts of protein and phospholipid in membranes are similar. The SecY-SecE proteoliposomes bound 1.4 nmol of SecA/mg of phospholipids (Fig. 6C). This value seems reasonable since the amount of SecY in these proteoliposomes was 10–20-fold that in membrane vesicles. A smaller amount (0.3 nmol/mg of phospholipids) of SecA bound was estimated from the results shown in Fig. 6B, however.

SecG Enhances the ProOmpA-dependent ATPase Activity of SecA—The ATPase activity of SecA increases upon the addition of proOmpA in the presence of membrane vesicles (26). This proOmpA-dependent ATPase activity, called translocation ATPase activity (26), is significantly higher with membrane vesicles containing overproduced SecY-SecE than with normal membrane vesicles (15). The effect of SecY-SecE with or without SecG on the proOmpA-dependent ATPase activity of SecA was examined in proteoliposomes reconstituted with the reduced amount of phospholipids. ATPase activity stimulated by the addition of proOmpA was slightly higher with proteoliposomes containing SecY and SecE than with liposomes (Fig. 8). Reconstitution of SecG with SecY and SecE significantly enhanced the proOmpA-dependent ATPase activity. These results are consistent with SecG-dependent stimulation of the translocation activity. Taken together, these results indicate that SecG stimulates the protein translocation reaction after SecA binds to the machinery.

DISCUSSION

It has been proposed on the basis of genetic studies that SecE functions as a shuttle between SecA and SecY (19). Our previous reconstitution studies indicating the requirement of an excess amount of SecE as compared with SecY for the maximum activity appeared to support this proposal (1). On the other hand, more critical and detailed reconstitution studies described in this paper revealed that the amount of one component, which was fixed, determines the amount of the other component required to maximize the activity (Figs. 1 and 2), indicating that SecY and SecE function together, most likely as a SecY-SecE complex. The requirement of both SecY and SecE for the binding of SecA also supports this conclusion. Furthermore, in the presence of a fixed amount of one component, the reconstituted activity increased linearly with an increase in the amount of the other component, i.e. either SecY (Fig. 1) or SecE (Fig. 2), until the activity became maximum. This simple relationship indicates that the activity reconstituted with SecY and SecE is determined by the amount of a single factor, the SecY-SecE complex. These results also suggest that the SecY-SecE complex has a definite subunit stoichiometry. However, the subunit stoichiometry of the complex has not been clarified yet.
SecG stimulated the activity even in the presence of large amounts of SecY (Fig. 3), SecE (Fig. 4), and SecA (Fig. 5), indicating that none of these Sec proteins can substitute for the SecG function. Stimulation of the activity by SecG is most likely caused by the formation of a SecY-SecE-SecG complex, which possesses significantly higher activity than does the SecY-SecE complex. SecG not only stimulated the activity but also increased the affinity of SecE for complex formation (Fig. 4).

The SecY-SecE-SecG (band 1) complex has been isolated from membrane extracts and reconstituted into proteoliposomes (2). These proteoliposomes exhibit appreciable translocation ATPase activity (2). We found that SecG plays a role in the stimulation of this ATPase activity (Fig. 4). It has been shown that the solubilized SecY-SecE-SecG complex easily dissociates into subunits, causing a significant decrease in the reconstituted activity (3). These observations and the results shown in this paper, taken together, indicate that the SecY-SecE-SecG complex represents the fundamental unit of the translocation machinery in membranes.

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The addition of a large amount of SecG as well as of SecE was required to maximize the activity reconstituted with a fixed amount of SecY or the SecY-SecE-SecG complex. On the other hand, E. coli cells contain SecY, SecE (25), and SecG in similar molecular amounts. It seems possible, therefore, that some other factors are involved in the formation and stabilization of the complex in vivo. These factors, if present, may enhance the reconstituted translocation activity by facilitating complex formation. It has been reported that neither SecD nor SecF has an appreciable effect on the activity obtained upon reconstitution with SecY and SecE (27). It would be interesting to examine the effects of SecD and SecF on the reconstituted activity in the presence of SecG.

SecA binding was found to require both SecE and SecY, but not SecG (Fig. 6). This most likely indicates that SecA is targeted to the machinery through its recognition of the SecY-SecE portion of the SecY-SecE-SecG complex. Since a com-

2 K. Nishiyama and H. Tokuda, unpublished observation.
Reconstitution of an Efficient Protein Translocation Machinery

siderable amount of SecA bound to liposomes, as reported (23), reconstitution with the 10-fold lower amount of phospholipids was required to detect the SecY-SecE-dependent SecA binding (Fig. 6). SecA has been reported to interact with acidic phospholipids (28) and to penetrate the lipid bilayer (29). These steps are assumed to initiate the insertion of a precursor protein together with SecA into the membrane in an ATP-dependent manner (29, 30). Interaction of SecA with SecY-SecE has been speculated to cause the translocation of the precursor protein across the membrane (30). Both ATP hydrolysis and the proton motive force drive the translocation (20, 30). The SecY-SecE-dependent binding of SecA demonstrated here is likely to represent this step of the protein translocation pathway. It is not clear, however, whether or not any functional or structural difference exists between lipid-bound and SecY-SecE-bound SecA. Attempts to distinguish them by kinetic examination were unsuccessful because the amount of the latter was not sufficiently high for detailed analysis. In any case, it is important to clarify the difference in function or structure between these two types of SecA. Kim and Oliver (31) have reported that more than 50-fold overproduction of SecY and SecE caused only a 50% increase in the number of high affinity SecA-binding sites in membrane vesicles, suggesting that SecY and SecE are insufficient to constitute the SecA receptor. Although the results shown here indicate the importance of both SecY and SecE for the SecA targeting, the participation of some unknown components in this step cannot be completely excluded.

SecG had little effect on the SecY-SecE/SecA interaction, whereas it significantly stimulated the translocation ATPase activity as well as the proOmpA D26 translocation. Taken together, these results indicate that SecG plays a role in protein translocation after the SecA-precursor complex has been targeted to the SecY-SecE SecG complex.

The conditions for the reconstitution of an efficient translocation machinery with SecA and the three membrane proteins, SecY, SecE, and SecG, were thus established. On the other hand, the exact function of each membrane subunit and the subunit stoichiometry of the complex remain to be clarified. The role of the proton motive force in relation to the SecG function is currently under investigation in everted membrane vesicles prepared from the ΔsecG cells.

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REFERENCES