Volume 279, number 2, 233–236 FEBS 09412 February 1991

ADONIS 001457939100120F

Purification of SecE and reconstitution of SecE-dependent protein translocation activity

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Received 14 December 1990

SecE was solubilized from SecE-overproducing *E. coli* cells and purified through ion exchange and size exclusion chromatographies. When the solubilized membrane containing overproduced amounts of SecY and SecE was fractionated by means of size exclusion chromatography, the two proteins were eluted in different fractions with slight overlapping. Proteoliposomes active in protein translocation were reconstituted from these fractions only when both SecE and SecY were present. When reconstitution was carried out with the purified SecE and fractions containing SecY but only a small amount of SecE, the resultant proteoliposomes exhibited appreciable translocation activity, indicating that SecE is essential for protein translocation. The translocation activity of proteoliposomes was proportional to the amount of purified SecE used for reconstitution. SecE-dependent protein translocation absolutely required ATP and SecA.

Overproduction; Protein secretion; Reconstitution; SecE; SecY

1. INTRODUCTION

Genetic studies have revealed that several factors are involved in protein translocation across the cytoplasmic membrane of *Escherichia coli* [1,2]. Among them, SecE, SecY, SecD and SecF have been assumed to constitute the translocation machinery in the cytoplasmic membrane. Biochemical characterization of their functions, therefore, requires their solubilization and purification, and their reconstitution into liposomes. Reconstitution of the protein translocation system of *E. coli* has been reported [3–5]. None of the studies dealt with purified proteins, however. Brundage et al. [6] recently isolated a fraction containing SecE, SecY and an uncharacterized protein as major components. The fraction exhibited protein translocation activity when it was reconstituted into liposomes. Although this suggested the importance of SecE and/or SecY for protein translocation, it was not yet clear which component(s) in the fraction is essential for protein translocation.

We recently succeeded in the overproduction of both SecE and SecY [7]. In this paper, we report the purification of SecE from SecE-overproducing cells and present evidence that SecE is essential for protein translocation.

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Abbreviations: Octylglucoside, *n*-octyl-β-D-glucopyranoside; SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli W3110 M25 (OmpT⁻) [8] was transformed with pMAN809 for the overproduction of SecE or with pMAN809 and pMAN510 for the simultaneous overproduction of SecE and SecY [7]. The overproduction was induced by IPTG as reported [7]. pOAD26 carries the *ompA-D26* gene encoding proOmpA D26, which is a derivative of proOmpA and lacks about 250 amino acid residues at its C-terminus (Kanamaru, K., Yamada, H. and Mizushima, S., unpublished data). The gene is under the control of SP6 promoter.

2.2. *Preparation of the cytoplasmic membrane, SecA and phospholipids*

The cytoplasmic membrane was prepared from SecE- or SecE/SecY-overproducing cells as reported [9]. SecA was purified as described [10]. *E. coli* phospholipids were prepared as reported [5].

2.3. *Purification of SecE*

Cytoplasmic membrane fractions prepared from SecE-overproducing cells were solubilized at 1 mg protein/ml on ice for 10 min with 2.5% octylglucoside containing 20 mM Tris-HCl (pH 7.5), 10% (w/v) glycerol and 2.5 mg/ml *E. coli* phospholipids. After ultracentrifugation at 140 000 × *g* for 30 min in a Beckman TLA 100.3 rotor, the supernatant containing 23.8 mg of protein was applied on a Mono Q, an anion exchanger, column (1 cm × 10 cm; Pharmacia), which had been equilibrated with 2.5% octylglucoside and 20 mM Tris-HCl (pH 7.5). The column was then developed at the flow rate of 4 ml/min with a linear gradient of 0–1 M NaCl in the same buffer. The amount of SecE in each fraction was determined by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-SecE antiserum. The pass-through fraction (36.5 ml), which contained most of the SecE, was concentrated to 5 ml by means of membrane filtration. An aliquot (0.5 ml) of the concentrated fraction was further purified by size exclusion chromatography on a Superose 12 HR column (1 cm × 30 cm; Pharmacia), which had been equilibrated with 2.5% octylglucoside containing 50 mM potassium phosphate (pH 6.95), 10% glycerol and 150 mM NaCl. The column

was developed with the same buffer at the flow rate of 0.4 ml/min. The amount of SecE in each fraction was determined by densitometric scanning of the Coomassie brilliant blue-stained gels. Size exclusion chromatography was performed ten times, and fractions containing SecE with a purity of about 70% were combined and concentrated.

2.4. *Reconstitution of proteoliposomes exhibiting protein translocation*

The reported conditions [5] were slightly modified. Aliquots (100 μl) of fractions obtained on size exclusion chromatography of the solubilized membrane fraction derived from SecE/SecY-overproducing cells were mixed with 1.25 mg *E. coli* phospholipids in 2.5% octylglucoside. Where specified, purified SecE was also added to the mixture. After 20-min incubation on ice, the mixture was rapidly diluted with 4.6 ml of 50 mM potassium phosphate (pH 7.5) containing 150 mM NaCl, and then incubated at room temperature for 5 min with stirring. The proteoliposomes formed were recovered, suspended in 100 μl of 50 mM potassium phosphate (pH 7.5) and 150 mM NaCl, frozen, thawed and sonicated as described [5].

2.5. *In vitro transcription and translation*

In vitro transcription of the *ompA-D26* gene was performed as in [11]. The translation reaction was carried out in the presence of Tran^{35}S-label (0.46 mCi/ml) as described [12]. [^{35}S]Methionine-labeled proOmpA D26 was partially purified as reported [13].

2.6. *Protein translocation*

Aliquots (15 μl) of the reconstituted proteoliposomes were mixed with 1 μl of 1.5 mg/ml SecA and 5 μl of 50 mM potassium phosphate (pH 7.5) containing 10 mM $MgSO_4$, 10 mM ATP, 150 mM NaCl and an ATP generating system composed of 50 mM creatine phosphate and 1.25 mg/ml of creatine kinase. After 3-min preincubation at 37°C, the assay was started by the addition of 4 μl of [^{35}S]methionine-labeled proOmpA D26 (2×10^5 cpm). The translocated protein, which was proteinase K-resistant, was detected on an SDS-polyacrylamide gel by means of fluorography, as described [14]. Densitometric quantification of band materials was carried out with a Shimadzu CS-930 chromatoscanner. The amounts of translocated proOmpA D26 were expressed as percentages of the input precursor protein.

2.7. *Preparation of anti-SecE and anti-SecY antisera*

Peptides corresponding to the Lys^{64}-Lys^{81} region of SecE [15] and the Met^{1}-Arg^{22} region of SecY [16] were synthesized and used to raise antisera against the peptides as described [5].

2.8. *SDS-Polyacrylamide gel electrophoresis, and immunoblotting analysis of SecE and SecY*

A gel containing 13.5% acrylamide/0.36% *N,N'*-methylenebisacrylamide was used according to Laemmli [17]. All samples were applied to the gel without boiling. Immunoblot analysis was carried out as described [18].

2.9. *Materials*

n-Octyl-β-D-glucopyranoside was purchased from Dojindo Laboratories. Tran^{35}S-label was obtained from ICN.

3. RESULTS

3.1. *Purification of SecE from SecE-overproducing cells*

The cytoplasmic membrane prepared from SecE-overproducing cells was solubilized with 2.5% octylglucoside, and the supernatant containing more than 95% of the membrane protein was subjected to anion exchange chromatography. Most of the SecE was recovered in the pass-through fraction, whereas the majority of other proteins were retained by the resin. The pass-through fractions enriched with SecE were combined, concentrated and further fractionated by size exclusion chromatography. Fractions corresponding to an apparent molecular mass of 30–50 kDa were combined and concentrated as the final SecE preparation. SecE having a molecular mass of 13.6 kDa seemed to be oligomeric under these conditions.

The SecE-containing fractions obtained throughout the entire purification steps were analysed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The SecE contents were then determined by densitometric scanning of the Coomassie brilliant blue-stained gels (Table I). The purify of the final SecE preparation was about 70%.

The N-terminal 20 amino acid sequence of the purified SecE was found to be the same as that deduced from the DNA sequence of the *secE* gene [19] except for the absence of N-terminal methionine.

3.2. *SecE is an essential component of the protein translocation machinery*

The cytoplasmic membrane prepared from SecE/SecY-overproducing cells was solubilized with octylglucoside. More than 95% of SecY and SecE were recovered in the supernatant. The supernatant was then subjected to size exclusion chromatography. The amounts of SecE and SecY in each fraction were deter-

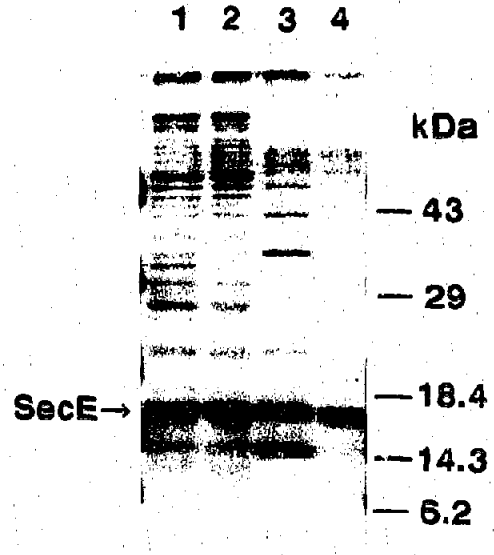

Fig. 1. Purification of SecE, as revealed by SDS-polyacrylamide gel electrophoresis. Fractions obtained at each purification step were analysed on 13.5% polyacrylamide gel and stained with Coomassie brilliant blue R-250. The positions of SecE and molecular weight markers are also indicated. (Lane 1) The cytoplasmic membrane fraction derived from SecE-overproducing cells (7.3 μg); (lane 2) octylglucoside supernatant of cytoplasmic membrane vesicles (7 μg); (lane 3) Mono Q pass-through fraction (3.5 μg); (lane 4) Superose 12 HR fraction (1.0 μg).

Table I

Purification of SecE

Sample	Protein (mg)	SecE (mg)*	Purity (%)	Recovery (%)
Membrane	25.0	3.4	13.6	100
Octylglucoside supernatant	23.8	3.2	13.4	94
Mono Q	2.40	0.88	36.7	26
Superose 12HR	0.97	0.66	68.0	19

*The amount of SecE in each sample was determined by densitometric scanning of the Coomassie brilliant blue-stained gel shown in Fig. 1

mined by immunoblotting with antibodies raised against SecE and SecY, respectively (Fig. 2A). SecE and SecY were eluted in different fractions with slight overlapping. Judging from the elution profile, SecY, which has a molecular mass of 49 kDa [20], seems to be oligomeric as well.

Proteoliposomes were reconstituted from various fractions obtained on the chromatography shown in Fig. 2A and assayed for translocation activity using [^{35}S]methionine-labeled proOmpA D26 as a presecretory protein (Fig. 2B). The translocated protein was only found at the position of the presecretory protein, indicating that the signal peptidase was not properly reconstituted into the proteoliposomes. High translocation activity was obtained in fractions 15 and 16, which also had high SecE contents. No fractions devoid of SecE exhibited the reconstituted translocation activity, irrespective of the presence or absence of SecY.

The purified SecE was then mixed with the individual fractions and reconstitution was performed (Fig. 2B). SecE caused remarkable enhancement of the activity when fraction 13 or 14, that contained only a low level of SecE (Fig. 2A), was used, indicating the essential requirement of SecE for the translocation reaction. No translocation took place in the absence of ATP.

The time course of protein translocation by proteoliposomes reconstituted from pure SecE and a SecE-deficient fraction (fractions 13 plus 14 in Fig. 2A) was examined (Fig. 3A). SecE or the SecE-deficient fraction alone did not exhibit translocation after reconstitution. When both fractions were reconstituted together on the other hand, the resultant proteoliposomes took up proOmpA D26 as a function of the incubation time.

In the experiment shown in Fig. 3B, proteoliposomes were reconstituted from the SecE-deficient fraction with various amounts of pure SecE. The translocation activity of the resultant proteoliposomes linearly in-

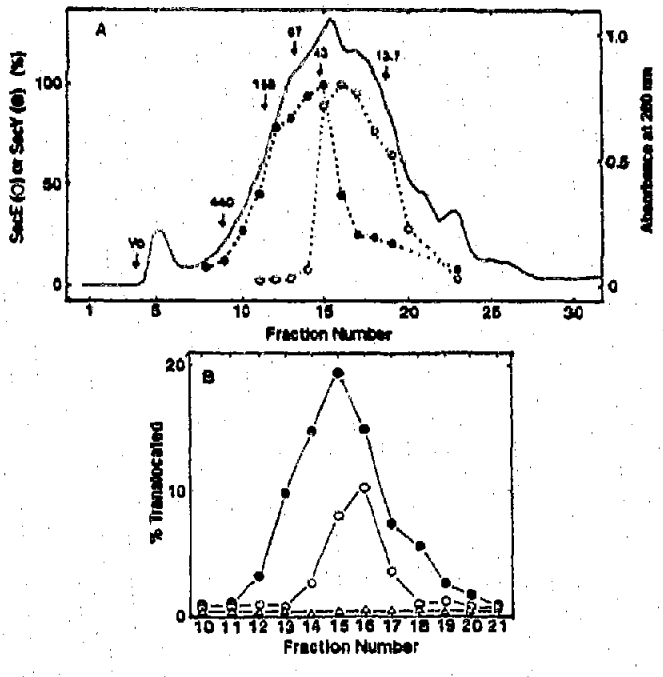

Fig. 2. Separation of SecY and SecE by size exclusion chromatography (A) and subsequent reconstitution of protein translocation activity (B). (A) The cytoplasmic membrane prepared from SecY/SecE-overproducing cells was solubilized at 1 mg protein/ml with 2.5% octylglucoside in the presence of 50 mM potassium phosphate (pH 6.95), 150 mM NaCl, 10% glycerol and 2.5 mg/ml *E. coli* phospholipids. An aliquot (500 μl) of the supernatant was subjected to size exclusion chromatography as described in the purification of SecE. The amounts of SecY and SecE (broken line) in each fraction (0.5 ml) were determined by immunoblotting with anti-SecY and anti-SecE antisera, respectively, followed by densitometric scanning. The maximum values for the amounts of SecE and SecY were taken as 100%. The column was standardized with ferritin (440 kDa), aldolase (158 kDa), thyroglobulin (167 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). The void volume (V_0) was determined with Blue dextran 2000. (B) Aliquots (100 μl) of individual fractions obtained on the size exclusion chromatography shown in A were mixed (●, ▲) or not mixed (○) with 1.9 μg of purified SecE and then subjected to reconstitution. Translocation activity of resultant proteoliposomes was assayed for 20 min with (○,●) or without (▲) 2 mM ATP.

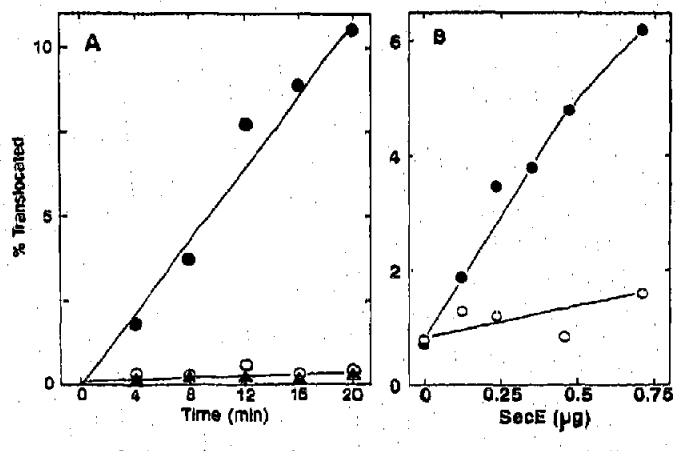

Fig. 3. SecE-dependent translocation of proOmpA D26. (A) Aliquots (100 μl) of a SecY-containing but SecE-deficient fraction (fractions 13 plus 14 in Fig. 2A) and 1.9 μg of purified SecE were used for reconstitution. Time course of the translocation was assayed using proteoliposomes reconstituted from the SecY-containing but SecE-deficient fraction alone (▲), purified SecE alone (○) or both (●). (B) The SecY-containing but SecE-deficient fraction was mixed with various amounts of purified SecE and reconstituted into liposomes. The translocation was assayed with (●) or without (○) SecA.

creased with an increase in the amount of SecE. When the translocation was assayed in the absence of SecA, the activity was only marginal, irrespective of the amount of SecE.

4. DISCUSSION

Participation of the *secE* gene product in protein translocation was demonstrated genetically [21,22]. Very recently, it was proposed that SecE functionally interacts with SecY [23]. It was also reported that proteoliposomes reconstituted from a membrane fraction containing SecY, SecE and an uncharacterized protein exhibited protein translocation [6]. None of these proteins has been purified to homogeneity, however, and hence their essentiality for the translocation has not been proved biochemically. In this study, we purified SecE and showed that the reconstitution of proteoliposomes active in protein translocation required SecE. The SecE-dependent protein translocation required both ATP and SecA, as the translocation into native membrane vesicles does. Since proteoliposomes reconstituted with SecE alone did not take up the presecretory protein, it is clear that other membrane component(s) are also required for the formation of the translocation machinery.

An observation of Brundage et al. [6], i.e. that translocation activity was reconstituted from a partially purified complex containing SecY, SecE and an uncharacterized protein suggests the importance of SecY for protein translocation. The participation of SecY in the translocation reaction has also been supported by the results of genetic [20] and immunochemical [24] studies. The SecE-dependent overproduction of SecY also suggests the involvement of SecY in the translocation reaction [7]. Contrary to these, Watanabe et al. [4] reported that SecY was dispensable for the reconstituted translocation activity. It is essential to perform experiments with purified SecY. SecY purification from SecY-overproducing cells is currently in progress in this laboratory.

Acknowledgements: We thank K. Sugimura for providing *E. coli* W3110 M25, H. Yamada and K. Kanamaru for pOAD26, and I. Sugihara for excellent secretarial support. This work was supported by grants from the Ministry of Education, Science and Culture of Japan (Nos 61060001, 02404013 and 02680153).

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