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SecA, AN ESSENTIAL COMPONENT OF THE SECRETORY MACHINERY OF ESCHERICHIA COLI, EXISTS AS HOMODIMER

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SUMMARY: Size exclusion chromatography of the cytosolic fraction of SecA-overproducing cells of Escherichia coli suggested that SecA, an essential component of the secretory machinery, exists as an oligomer. The subunit structure of SecA was then studied using a purified specimen. Estimation of the molecular mass by means of ultracentrifugation and chemical cross-linking analysis revealed that SecA exists as a homodimer. The purified SecA was denatured in 6 M guanidine-HCl and renatured to a dimer, which was fully active in terms of translocation, even in the presence of 1 mM dithiothreitol. It is suggested that the dimeric structure is not critically maintained by disulfide bonding between the two subunits, each of which contains four cysteine residues.

SecA plays an essential role in the translocation of secretory proteins across the cytoplasmic membrane of Escherichia coli by interacting with almost all of the components involved in the translocation reaction, such as ATP (1, 2), presecretory proteins (3, 4), SecE[1], SecY (1, 5), SecB (6) and phospholipids (7). In addition, the implication of the proton motive force in the SecA function has also been suggested (8). Recent studies involving protease digestion revealed that the SecA molecule undergoes conformational changes upon interaction with these components[2]. To gain an insight into the SecA function in the translocational movement of presecretory proteins, it is important, therefore, to elucidate the tertiary and quarternary structures

[1] E. Kimura, S. Matsuyama, J. Akimaru, H. Tokuda, and S. Mizushima, unpublished observation.
[2] A. Shinkai, H. M. Lu, H. Tokuda, and S. Mizushima, unpublished observation.

The abbreviations used are: EDAC, 1-ethyl-3-(3-dimethlaminopropyl) carbodiimide; DSS, disuccinimidylsuberate; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

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of the SecA molecule and their changes during the translocation reaction. Multiple forms, including a cytoplasmic form that appeared to be monomeric, have been suggested for SecA (9). We have performed extensive studies on the subunit structure of SecA isolated from the cytosol. Here we report that the cytosolic SecA is a homodimer of the 102 kDa subunit protein and that the bonding through disulfide bridges is not critically involved in dimerization.

MATERIALS AND METHODS

Purification of SecA - SecA was purified from a SecA-overproducing strain (RR1/pMAN400) (10). After ammonium sulfate fractionation of the cytosol, a SecA-enriched fraction was subjected to successive chromatographies on a Sephacryl S-300 column (10) and a hydroxylapatite column (3), as described previously.

Sucrose gradient centrifugation of SecA - Purified SecA (200 μg/500 μl) was subjected to centrifugation at 200,000 x g for 20 hr at 4 °C, in a Beckman SW50.1 rotor, through 4.4 ml of a 5-20 % (w/w) linear sucrose gradient.

Chemical cross-linking of SecA - SecA was crosslinked with 25 mM EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (3) or 2.5 mM DSS (disuccinimidylsuberate) (11) at 25 °C and the reaction was terminated with 50 mM Tris-HCl (pH7.5). When 0.1 % glutaraldehyde was used, cross-linking was carried out at 25 °C and then terminated with 0.2 $NaBH_4$ in 0.1 M NaOH for 20 min (12). After TCA precipitation and acetone washing, samples were analyzed by SDS-polyacrylamide gel electrophoresis as described (13).

Denaturation and Renaturation of SecA - SecA was denatured and then renatured as described (2) with some modifications. 50 mM potassium phosphate (pH 7.5) was used in place of 50 mM Tris-acetate(pH 7.5) and 1 mM dithiothreitol was added throughout the reaction.

Translocation Reaction - In vitro protein translocation of [^{35}S]-labeled proOmpF-Lpp was performed as described previously (14) with some modifications. Inverted membrane vesicles were treated with 6 M urea to remove SecA (15).

RESULTS

During SecA purification by means of size exclusion chromatography, we noticed that the migration of SecA was much faster than would be expected for the SecA monomer, whose molecular mass is 102 kDa. Therefore, we performed gel chromatography of the cell lysate obtained from SecA-overproducing cells on a Sephacryl S-300 column in the presence of several molecular mass standards. SecA was eluted between ferritin (440 kDa) and catalase (232 kDa) (Fig. 1). Its molecular mass was roughly estimated to be about 250 kDa. This suggests that SecA exists as a dimer. We then determined the molecular mass of SecA by means of sucrose gradient centrifugation (Fig. 2). The purified SecA

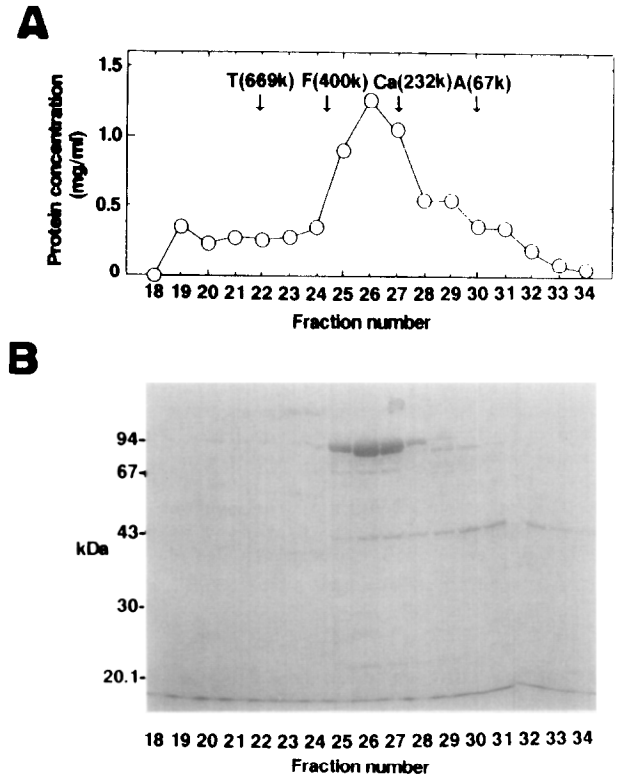

Fig. 1. Size exclusion chromatography of SecA on a Sephacryl S-300 column. The cytosolic fraction (50 mg protein) from SecA-overproducing cells was purified first by ammonium sulfate fractionation and then applied on a Sephacryl S-300 column (100 x 1.6 cm) as described (10). The total protein concentration (A) and SDS-polyacrylamide gel profile (B) of each fraction (3.5 ml) are shown. The standard proteins, denoted as T (bovine thyroid thyroglobulin), F (horse spleen ferritin), Ca (bovine liver catalase) and A (bovine serum albumin), and their positions are indicated with their molecular masses.

was sedimented between catalase (232 kDa) and aldolase (158 kDa), supporting the homodimer structure of SecA.

To further elucidate the dimeric structure of SecA, chemical cross-linking studies were performed using three different cross-linkers (Fig. 3). In all cases, the amount of the cross-linked dimer was increased with the reaction time, eventually amounting to 70-80 % of the total SecA. Although both

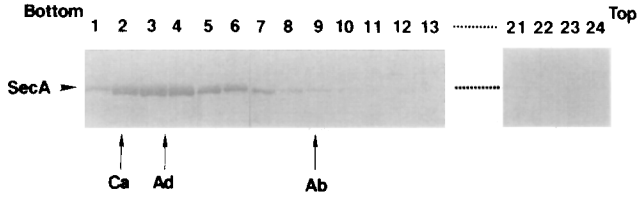

Fig. 2. Centrifugal analysis of SecA on a sucrose density gradient. SecA was subjected to centrifugation on a 5-20 % (w/w) linear sucrose gradient (4.4 ml). Each fraction (0.2 ml) collected from the gradient was analyzed by SDS-polyacrylamide gel electrophoresis. The standards used were bovine liver catalase (Ca), rabbit muscle aldolase (Ad) and bovine serum albumin (Ab).

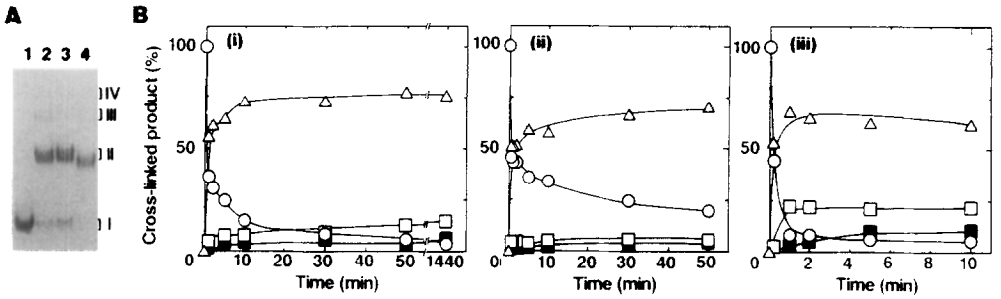

Fig. 3 Cross-linking of SecA. SecA was cross-linked with EDAC, DSS and glutaraldehyde for the times indicated. The cross-linked products were analyzed by SDS-polyacrylamide gel electrophoresis (A) and then subjected to densitometric scanning to determine their amounts (B). (A) Cross-linking of SecA was carried out without (lane 1) or with EDAC for 50 min (lane 2), DSS for 50 min (lane 3) or glutaraldehyde for 2 min (lane 4). The positions of the monomer (I), dimer (II), trimer (III) and tetramer (IV) of SecA are indicated. (B) The relative amounts of the monomer (○), dimer (△), trimer (□) and tetramer (■) of SecA are expressed as percentages of the total SecA: (i) EDAC, (ii) DSS and (iii) glutaraldehyde.

a trimer and a tetramer, that were cross-linked, were also observed, their amounts did not increase significantly, even on very prolonged incubation.

To exclude the possibility that the formation of the cross-linked dimer was due to the accidental collision of SecA monomers, the effect of the SecA concentration on the cross-linking with EDAC was examined (Fig. 4). In the presence of the same concentration of EDAC, the ratio of the cross-linked SecA dimer to the total SecA was almost the same, irrespective of the SecA concentration. On the other hand, the lower the concentration of SecA was, the smaller the fraction of the cross-linked trimer and probably that of the cross-linked tetramer were.

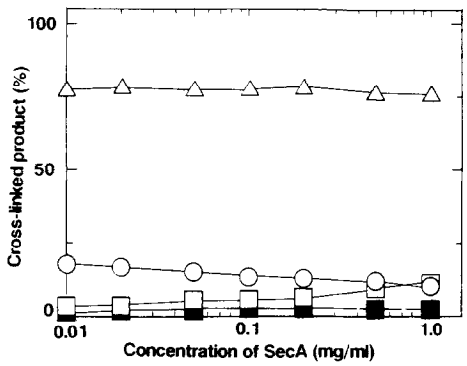

Fig. 4. Effect of the SecA concentration on the cross-linking. The cross-linking reaction was performed in the presence of EDAC with the indicated concentrations of SecA. The cross-linked products were then analyzed by SDS-polyacrylamide gel electrophoresis, and the relative amounts of monomer (○), dimer (△), trimer (□) and tetramer (■) were densitometrically determined and expressed as percentages of the total SecA.

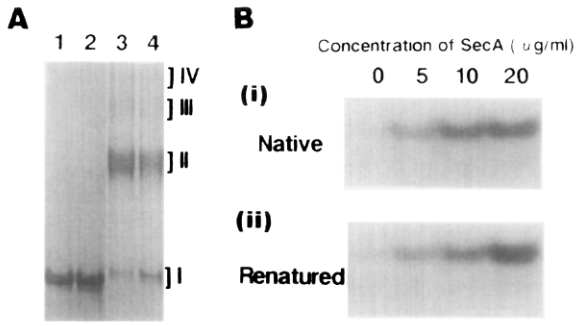

Fig. 5. Cross-linking (A) and translocation activity (B) of the native and renatured SecA. (A) Native SecA (lanes 1 and 3) and SecA denatured in 6 M guanidine-HCl and then renatured in the presence of 1 mM dithiothreitol (lanes 2 and 4) were cross-linked with (lanes 1 and 2) or without (lane 3 and 4) EDAC. (B) In vitro protein translocation was carried out in the presence of the indicated concentrations of native (i) and renatured (ii) SecAs.

Taking all these results together, we conclude that SecA is a homodimer of the 102 kDa subunit protein, and that the trimer and tetramer are artifacts of the cross-linking reaction.

The SecA monomer contains four cysteine residues (16). The possible involvement of disulfide bonding in the dimeric structure was, therefore, considered. The presence of 5 mM dithiothreitol did not affect the cross-linking of SecA, suggesting that disulfide bonding does not play a critical role in the dimeric structure (data not shown). Recently, we succeeded in reconstituting (renaturing) fully functionally active SecA from the 6 M guanidine-denatured state (17). Using this system, the effect of dithiothreitol on the reconstitution was examined. Dithiothreitol had no effect on the reconstitution, as judged from the dimer formation (Fig. 5A) and translocation activity (Fig. 5B). We conclude, therefore, that disulfide bonding is not required for SecA to take on the dimeric form, which is functionally active.

DISCUSSION

SecA is a peripheral membrane protein, and is found in both the cytoplasmic membrane and the cytosol (18). Recently, Oliver et al. (9) reported that the cytosolic SecA is most likely monomeric. On contrary, we showed in the present work that SecA in the cytosol and that purified from it exist as a homodimer. This conclusion was reached after experiments involving gel filtration on a Sephacryl S-300 column, sucrose density gradient centrifugation and chemical cross-linking. Although the profile on gel filtration and that on centrifugation were somewhat broad, there was no significant amount of SecA at the position for monomeric SecA. Furthermore, the results of cross-linking studies clearly indicated that SecA mainly exists as a homodimer.

SecA takes on the dimeric form in a wide concentration range (10 μg - 1 mg per ml) (Fig. 4). Since the concentration of SecA in the cytosol of intact cells has been estimated to be about 1.5 mg/ml and since SecA exhibits maximum *in vitro* translocation activity when its concentration is higher than about 10 μg/ml (8), it is likely that SecA exists as a dimeric form in the cytosol and that the dimeric form is functional.

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