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The Carboxyl-Terminal Region Is Essential for Sec-A Dimerization

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SecA, comprising 901 amino acid residues, exists as a dimer. By means of size exclusion chromatography and chemical cross-linking analysis, five truncated SecA derivatives were examined to identify the region of SecA essential for dimer formation. Among them, only N95 (Δ832-901) retained SecA activity. N95 existed as a dimer, indicating that the carboxyl-terminal three cysteine residues are dispensable for physiological dimerization. Both N76 (Δ675-901) and N66 (Δ583-901) existed as monomers. Monomeric N76 was able to bind to ATP, indicating that the dimerization of SecA is not a prerequisite for ATP binding. However, the rate of ATP hydrolysis by N76 was 25% of that by SecA. C53 (Δ1-437) and C28 (Δ1-661) formed dimers irrespective of the presence or absence of 2-mercaptoethanol. C28, but not C53, also existed as an oligomer in the absence of 2-mercaptoethanol, suggesting that the 438-661 region present in C53 prevents intermolecular disulfide bond formation at the carboxyl-terminal cysteine residue. From these results, the region essential for the physiological dimer formation was concluded to be located in the 662-831 region of SecA.

SecA plays a critical role in protein translocation across the cytoplasmic membrane of *E. coli* by undergoing the cycle of membrane-insertion and deinsertion (1). The SecA cycle seems to be facilitated by the topology inversion cycle of SecG, a membrane component of the protein translocation apparatus (2).

It is thought that SecA functions as a dimer (3, 4), although the dimerization of SecA has not been proved to be essential for its function. The functional regions of SecA have been revealed with various truncated derivatives. The amino-terminal region of SecA has been shown to be involved in ATP binding (5, 6), precursor binding (7), and membrane interaction (8). Deletion of the carboxyl-terminal 70 amino acid residues does not abolish the SecA function in vitro (5), although this region has been reported to be important for both SecB binding and lipid binding (9). Substitution of the cysteine residues in the carboxyl-terminal region with serine residues has also been reported to reduce the SecA activity in a precursor-specific manner (10). These observations, however, did not reveal the region which is essential for the SecA dimer formation.

MATERIALS AND METHODS

Materials. Restriction endonucleases, exonuclease III and T4 DNA polymerase were obtained from Takara Shuzo Co., Ltd. IPTG was from Nacalai Tesque. Oligonucleotides were synthesized using a Beckman Oligo 1000 DNA synthesizer. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was from Sigma. [α-^{32}P] ATP (111 GBq/μmol) was from ICN Radiochemicals.

Construction of SecA derivatives. The truncated SecA derivatives examined are depicted in Fig. 1. Plasmids encoding N95, N66, C53 and C28 were constructed from pMAN400, which carried the entire *secA* gene under the control of

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Abbreviations: CBB, Coomassie Brilliant Blue; EDAC, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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the *tac* promoter and *lac* operator, as described (5). To construct pMAN765-N76, the *Sna*BI-*Sac*I fragment of pMAN400 was replaced with synthetic termination linkers (5′-GTAGCATGCATAACTAACTAACTAAGAGCT-3′ and 3′-CATCGTACGTATTGATTGATTGATTC-5′). The *Sna*BI and *Sac*I sites are located about 210 bp upstream and 300 bp downstream of the *secA* termination codon, respectively. For pMAN789-N76 encoding N76, pMAN765-N76 was digested with *Eco*T22I, for which the site is located within the termination linker, and *Sna*B1. The digested plasmid was deleted with exonuclease III and mung bean nuclease from the *Sna*BI site, and then treated with T4 DNA polymerase, followed by self ligation. To confirm the truncation, the region covering the deletion end point was cloned into pUC19 and sequenced.

Purification of SecA and its derivatives. Overproduction of SecA and its derivatives was induced by IPTG as described (5). Intact SecA, N95 and C28 were purified from the cytosolic fraction by means of ammonium sulfate fractionation and hydroxylapatite chromatography as described (5). The other SecA derivatives, N76, N66 and C53, were isolated as aggregates, solubilized with 6 M guanidine-HCl, and renatured as described (5).

Chemical cross-linking of SecA derivatives. The purified SecA derivatives (10 μg) were incubated with a specified concentration of EDAC in 50 mM potassium acetate (pH 7.5) at 25°C for 50 min as described (11). Cross-linking of C28 was carried out with 50 mM EDAC using an aliquot (40 μl) of each fraction obtained on size exclusion chromatography. The cross-linking reaction was terminated by the addition of 50 mM Tris-HCl (pH 7.5). After TCA precipitation, samples were analysed by SDS-PAGE and then stained with CBB.

Size exclusion chromatography. TSK-gel G3000SW (Tosoh) or Superose 12 (Pharmacia) was used to analyze 80 μg of each of the purified SecA derivatives. The column had been equilibrated with 50 mM potassium acetate (pH 7.5) and was developed at the flow rate of 0.8 ml/min with the same buffer. Where specified, the buffer contained 5% 2-mercaptoethanol. Fractions of 0.2 ml were collected. Ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) were used as molecular mass markers.

Photoaffinity cross-linking. The intact SecA and truncated SecA derivatives were cross-linked with 0.1 μM [α-^{32}P] ATP as described (5). Where specified, the truncated SecA derivatives mixed in different combinations in 6M guanidine-HCl were renatured through dilution and dialysis, and then subjected to photoaffinity cross-linking as described (5). After the reaction, the samples were analysed by SDS-PAGE, followed by autoradiography.

RESULTS AND DISCUSSION

Deletion of the carboxyl-terminus prevents the dimer formation of SecA. N95 lacks the carboxyl-terminal 70 amino acid residues (832-901) but is active in protein translocation in vitro (5). Moreover, the *secAts(Am)* mutation of *E. coli* BA13 was complemented by the expression of N95 (data not shown). In contrast, all the other derivatives examined in this study did not complement the mutation. These results indicate that the carboxyl-terminal 70 amino acids including three cysteine residues are not essential for the SecA function, although the deletion of this region or replacement of these cysteine residues with serine residues has been reported to reduce the efficiency of SecB-dependent protein translocation (9, 10).

The purified N95 was eluted in the fraction corresponding to a molecular mass of about 180 kDa on size exclusion chromatography (Fig. 2A), suggesting that N95 possessing a molecular mass of 95 kDa exists as a dimer. When N95 was subjected to cross-linking with EDAC, the major cross-linked product was found to be a dimer (Fig. 2B). In marked contrast, both N76 (Fig. 2C) and N66 (data not shown), which lack the 675-901 and 583-901 regions (Fig. 1), respectively, were recovered in fractions corresponding to monomers. Furthermore, the two derivatives migrated to the respective monomer positions on SDS-PAGE even after the cross-linking reaction (Fig. 2D). These results indicate that deletion of the carboxyl-terminal region from residue at 675 prevents the dimerization.

Deletion of a large amino-terminal region does not prevent the dimer formation. When size exclusion chromatography was carried out in the absence of 2-mercaptoethanol, the purified C28, lacking a large amino-terminal region (1-661), was eluted as two broad peaks. The faster eluting peak corresponding to a molecular mass of about 410 kDa disappeared when the chromatography was carried out in the presence of 2-mercaptoethanol (Fig. 3A), indicating that C28 forms an oligomer through disulfide bonding at the carboxyl-terminal cysteine residue. Aliquots of fractions 11 to 21 obtained in the absence of 2-mercaptoethanol were subjected to cross-linking with EDAC, followed by SDS-PAGE analysis (Fig. 3B). A cross-linked product having a molecular mass of about 55 kDa was observed for all the fractions examined. Since

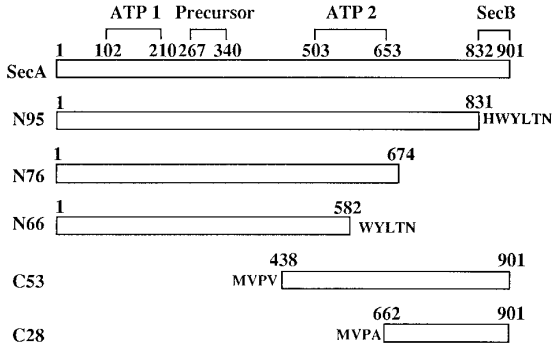

FIG. 1. SecA and its truncated derivatives. The construction of N95, N66, C53 and C28 has already been reported (5). N76 was constructed as described under Materials and Methods. The open boxes represent regions derived from SecA. Letters adjacent to the open boxes indicate amino acid residues derived from the vector sequence. N76 has no extra amino acid residue at its carboxyl-terminus. Regions of SecA involved in the binding of ATP (6), the precursor (7), and SecB (9) are indicated at the top.

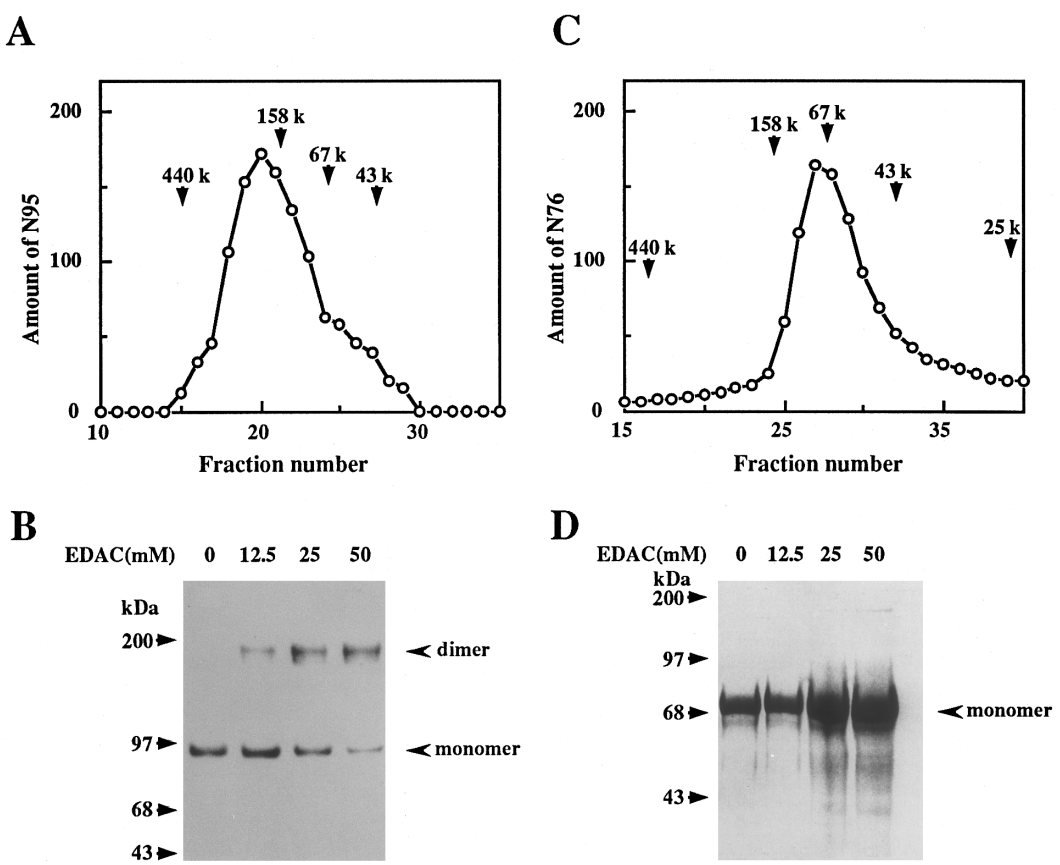

FIG. 2. Deletion of the carboxyl-terminal region prevents dimer formation. Purified N95 (A and B) and N76 (C and D) were analysed by size exclusion chromatography (A and C), or cross-linked with various concentrations of EDAC, followed by SDS-PAGE (B and D) as described under Materials and Methods. Except for the sample (5 μg) at 0 μM EDAC, 10 μg each of the derivatives was analysed by SDS-PAGE. The gel contained acrylamide at 5.5% (B) or 8% (D).

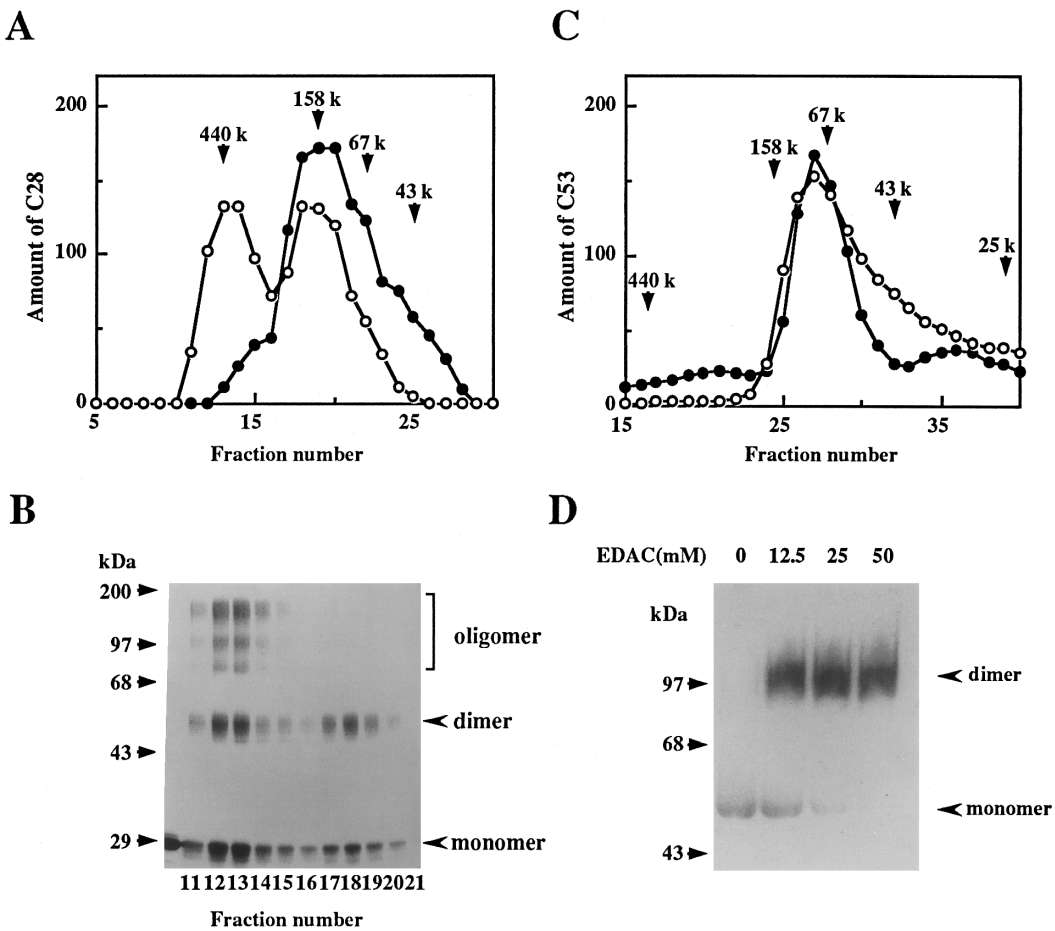

FIG. 3. The carboxyl-terminal region is involved in the dimerization of SecA. Purified C28 (A) and C53 (C) were analysed by size exclusion chromatography in the presence (●) and absence (○) of 2-mercaptoethanol. Aliquots (40 μl) of the specified fractions obtained on size exclusion chromatography carried out in the absence of 2-mercaptoethanol with C28, were subjected to cross-linking with 50 mM EDAC, and then analysed by SDS-PAGE (B). The sample in the left lane was analysed without cross-linking. Cross-linking of C53 was also examined, as described in the legend to Fig. 2 (D). Gels containing acrylamide at 11% (B) and 6.5% (D) were used.

C28, which was not subjected to cross-linking, exhibited a molecular mass of about 29 kDa on SDS-PAGE (left lane in B), the 55 kDa product most likely represents the C28 dimer. In addition to the 55 kDa band, cross-linked products larger than the dimer were observed in fractions 11 to 15. Judging from their mobilities on SDS-PAGE, these bands most likely represent the trimer, tetramer and pentamer of C28. In contrast, only the C28 dimer was detected in fractions 16 to 21 as a cross-linked product. Moreover, essentially all the C28 molecule were recovered in these fractions in the presence of 2-mercaptoethanol (Fig. 2A), indicating that the dimer formation is not dependent on disulfide bonding. Despite the large amino-terminal deletion, C28 therefore seems to possess the region essential for the SecA dimer formation. The C28 dimer may have an unusually elongated shape, since it is eluted in fractions corresponding to a molecular mass of about 150 kDa (Fig. 3A).

Irrespective of the presence or absence of 2-mercaptoethanol, C53, lacking the 1-437 region, was eluted in the fraction corresponding to a molecular mass of about 100 kDa (Fig. 3C).

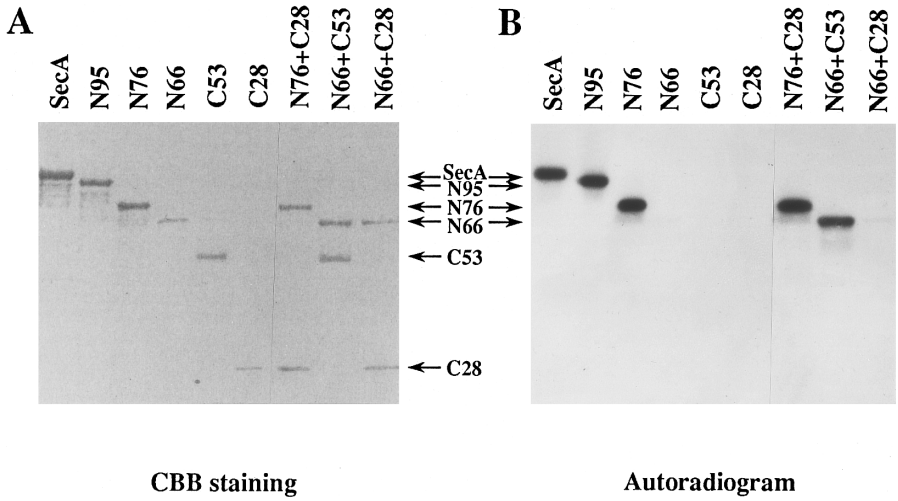

FIG. 4. Monomeric N76 is able to bind to ATP. Photoaffinity cross-linking of [α-^{32}P] ATP to either a single derivative or the given combinations of two derivatives was carried out and then analysed by SDS-PAGE, followed by CBB staining (A) or autoradiography (B), as described under Materials and Methods.

Cross-linking of the purified C53 revealed that it mostly exists as a dimer (Fig. 3D). The 438-661 region, which is present in C53 but absent from C28 (Fig. 1), therefore seems to prevent the disulfide bond formation between C53 dimers.

Taking all the results together, we conclude that the 662-831 region, that is present in N95, C53 and C28, but absent from N76 and N66, is responsible for the physiological dimer formation of SecA.

Monomeric N76 is able to bind to ATP. SecA possesses two ATP binding sites (6). The higher affinity site is located near the amino-terminus (Fig. 1) and can be photoaffinity cross-linked with ATP (5). Truncated SecA derivatives, which retain the amino-terminal binding site but lose the binding ability upon truncation, recover the binding ability when they are subjected to denaturation-renaturation treatment together with another derivative which complements the truncated region (5). The photoaffinity cross-linking assay revealed that N76, as well as intact SecA and N95, is capable of ATP binding (Fig. 4B), despite that N76 exists as a monomer. N66 alone did not bind to ATP whereas, after denaturation-renaturation treatment with C53 but not with C28, N66 was able to bind to ATP (Fig. 4). C28 did not appreciably enhance the ATP binding to N76. It has been shown that a derivative, which is slightly larger than N76, exhibits elevated ATP hydrolyzing activity (9). N76 also exhibited ATPase activity (0.04 μmole/min/mg), but it was considerably lower than that of intact SecA (0.16 μmole/min/mg). Taken together, these results indicate that dimerization is not essential for ATP binding but important for ATP hydrolysis.

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