Characterization of a Potential Catalytic Residue, Asp-133, in the High Affinity ATP-binding Site of Escherichia coli SecA, Translocation ATPase*

Ken Satoš, Hiroyuki Morit, Masasuke Yoshida, and Shoji Mizushima†

From the Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Yokohama 226 and School of Life Science, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

The high affinity ATP-binding site of SecA is located in its amino-terminal domain possessing amino acid sequences, the Walker A (GXXXXXGKT) and B (ZZZZD) motifs, that are characteristic of a major class of nucleotide-binding sites (Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945-951). Recently, we proposed that proteins possessing a typical set of Walker A and B motifs contain a conserved Glu or Asp between the two motifs. This Glu or Asp acts as a "catalytic residue" that activates a water molecule for an in-line attack on the γ-phosphate of ATP (Amano, T., Yoshida, M., Matsuo, Y., and Nishikawa, K. (1995) FEBS Lett. 359, 1-5). In the present study, the aspartate residue at position 133 in Escherichia coli SecA, which could be the "catalytic residue," was mutated to an asparagine. The mutant SecA (SecA D133N) protein was expressed in E. coli CK4706, encoding a duplication of the secA gene, and purified to homogeneity. The in vitro protein translocation activity and membrane vesicle-stimulated ATPase activity of SecA D133N were drastically reduced. Proteolytic studies indicated that the conformational changes of the mutant SecA occurring on interaction with ATP, presecretory proteins, phospholipids, and membrane vesicles, were similar to those of wild-type SecA. The mutant SecA allowed the signal peptide cleavage of proOmpA during translocation, indicating that the mutant retains the ability to bind ATP to perform the initial step of the translocation reaction. These data indicate that the carboxyl group of Asp-133 plays a role as a catalytic carboxylate, which activates a water molecule to attack γ-phosphate of ATP, and the mutant lacking this residue cannot perform the total translocation but can still perform the initial step of the protein translocation.

The translocation of secretory proteins across the cytoplasmic membrane in prokaryotic cells requires several protein factors (1–4). Among them, SecA is a peripheral cytoplasmic membrane protein, which plays an essential role in the translocation of secretory proteins across the cytoplasmic membrane of Escherichia coli. Biochemical studies, in addition to genetic evidence, clearly showed that SecA is involved in protein translocation (2, 4). SecA interacts with both ATP and presecretory proteins (5–7). Interactions between SecA and membrane vesicles/liposomes have also been demonstrated (5, 8). These interactions resulted in a conformational change of the SecA molecule (9). The possible interaction of SecA with SecY (5, 10) has also been suggested. The function of SecA was proposed to be directly related to a cycle of ATP binding and hydrolysis, which is essential for the translocation reaction (8).

The amino terminus of SecA contains typical ATP-binding motifs, the so-called Walker A and B sequences (11), that are commonly found in many nucleotide-binding proteins. The Walker A motif (also called the P-loop) consists of a consensus sequence, GXXXXXGKT (X varies), and the β- and γ-phosphates of ATP are liganded by Lys and Thr (12). The residues of motif A form a loop that contributes to the formation of a binding cavity for the phosphoryl groups of Mg2+-ATP (13–17). The Walker B motif, ZZZZD (Z is a hydrophobic residue), is responsible for the Mg2+-interaction among phosphates and proteins liganded to its Asp residue. A high affinity nucleotide-binding site, located within the first 217 amino acid residues from the amino terminus of E. coli SecA (7), contains these amino acid sequences. Replacement of Lys-108, located in motif A, blocks the translocation ATPase activity of SecA and interferes with the in vitro protein translocation of proOmpA (18, 19). Substitution of Asp-217 of E. coli SecA prevents the growth of cells and interferes with the translocation of precursor proteins in vivo (20).

A typical set of consensus sequence motifs A and B is likely to have a common structure, six parallel β-strands surrounding a central α-helix, and to catalyze ATP-triggered reactions. Recently, we found the existence of a conserved Glu or Asp, which may act as a general base that activates a water molecule for an in-line attack on the γ-phosphate of ATP (21). When the amino acid sequences of the region covering motifs A and B are aligned for several protein families, it became clear that the occurrence of a Glu or Asp at 24 ± 2 residues from the Lys of motif A is a common feature (22). This Glu or Asp residue is well conserved in each of the protein families. Therefore, it is likely that the carboxyl group of the Glu or Asp residue plays a functional role as a "catalytic carboxylate," which activates a water molecule that attacks the γ-phosphate of ATP in each of the proteins, as is proposed from the x-ray structural studies on the RoA protein (13) and Fα-ATPase (17) or biochemical studies on the Fα-ATPase β subunit (23, 24).

As for SecA, such a residue is found at amino acid residue 133. To determine whether the conserved Asp acts as an essential residue or not, we constructed a site-directed mutant as to Asp-133 of E. coli SecA. Our results support the validity of

* This work was supported by Grants 02404013, 04251902, and 07408015 from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Deceased on March 8, 1996.
§ Supported by a research fellowship of the Japanese Society for the Promotion of Science for Young Scientists. To whom correspondence should be addressed: Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan. Tel.: 81-426-77-7496; Fax: 81-426-77-7497.

17439
the catalytic carboxylate hypothesis for the SecA function and confirm the Asp-133 residue to be the "catalytic residue" for SecA ATPase activity as in the case of F$_1$-ATPase $\beta$ subunit. They also suggest that the ATP hydrolysis at this site is not essentially involved in the early step of protein translocation.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains—E. coli strains M109 (recA1, endA1, gyrA96, thi-), hsDr17, relA1, supE44, lacI(q) (lac-proAB, lacI, traD36), and lacI(q) (lac-proAB, lacI, traD36, lacIV, ampR) (25). Cj 236 (du1, ung1, thi-1, relA1, lacI (105"F' camR") (26), K003 (Lpp$^+$, unclc-B) (10) (27), MM666 (F $\Lambda$ araD139, $\Delta$argF-lac, lacI, aakA1, flaB5301, devC1, pufS25, genes37 (28), and CK4706 (F $\Lambda$, araD, lacI, rE, rplB, thi, secB $^+$, zah-T10, secA853$^+$) (29) were used.

Materials—EXPRE$^{35}$S Protein Labeling Mix, a mixture of 80% [35S]methionine and 20% cysteine (37 TBq/mmol), and [14C]formaldehyde (370 MBq/mol) were obtained from DuPont NEN. AMP-PNP and creatine kinase were from Boehringer Mannheim. ATP, GTP, and creatine phosphate were from Sigma. Staphylococcal V8 protease was from CalBioChem. ADP, ATP, AMP, and dithiothreitol), as described previously (45). $^{[35S]}$Methionine and 20% cysteine (37 MBq/mmol), and $^{[14C]}$formaldehyde (370 MBq/mmol) were purchased from DuPont NEN.

**RESULTS**

Construction and Expression of the Mutant secA Gene—As shown in Fig. 1, when the amino acid sequences of the region covering Walker A and B motifs were aligned for several SecA proteins, it became clear that the occurrence of Glu or Asp at position 24 is a common feature. To reveal the functions of these carboxyl groups and ATP hydrolysis in E. coli, SecA-133 was mutated to Asp at position 24 and the mutant SecA (Asp-133) was used as a mismatch primer for the replacement of Asp-133 by Asn in E. coli SecA, the changed bases being indicated by bold letters. The underline indicates the new (HpaI) restriction site. Mutations were screened as an appearance of the restriction site and confirmed by DNA sequencing. A 0.5-kilobase BamHI-Sphl fragment of pMAN400 was ligated with pUC18, which had been treated with BamHI and Sphl, to create pD133N. An oligonucleotide primer, 5'-GGGTTTCTCTTTATTGGC-3', was used as a mismatch primer for the replacement of Asp-133 by Asn in E. coli SecA, the changed bases being indicated by bold letters. The underline indicates the new (HpaI) restriction site. Mutations were screened as an appearance of the restriction site and confirmed by DNA sequencing. A 0.5-kilobase BamHI-Sphl fragment of this plasmid was subcloned into the BamHI-Sphl-digested expression vector, pDT-secA, to construct plasmid pDT-D133N encoding secA D133N.

Expression of the Mutant secA—E. coli strain CK4706 (29) harboring plasmids pDT-D133N and pGPI-2 (38) was used to express the secA D133N gene. After incubation of the cultures in L broth supplemented with 100 $\mu$g/ml ampicillin and 50 $\mu$g/ml kanamycin at 30°C. The cells were then diluted 10-fold with fresh L broth containing ampicillin and kanamycin, and at A$_{660}$ = 1.0 the temperature was raised to 42°C. After 30 min, the temperature was lowered to 37°C. After 120 min, the cells were harvested and washed with 50 mM Tris acetate, pH 7.8, 0.1$\times$(SOD) sucrose, and then SecA was purified as described previously (36). About 7 mg of purified SecA D133N was obtained from 3 liters of culture.

Preparation of Urea-Treated Inverted Membrane Vesicles—Inverted membrane vesicles were prepared from E. coli K003 (uncB-C) or W3110 M25 harboring both pM2809 and pM2510 (39). The membrane vesicles were treated with 6 M urea, 50 $\mu$g/ml potassium phosphate, pH 7.5, for 1 h on ice, recovered by centrifugation, and then suspended in 50 mM potassium phosphate, pH 7.5.

**Acknowledgments**

The abbreviations used are: AMP-PNP, adenosine 5'-($\beta$- $\gamma$-imino) triphosphate; ATP-$\gamma$S, adenosine 5'-($\beta$-thiotriphosphate).
The SecA D133N protein exhibited no translocation activity. These results demonstrate that the carboxyl group of Asp-133 of E. coli SecA is an essential residue for SecA-dependent protein translocation activity. Consistent with the in vitro results, SecA D133N protein could not complement the secretion defect of E. coli MM66 at the nonpermissive temperature (data not shown), suggesting that the Asp-133 is critical for SecA function in vivo.

SecA D133N Can Interact with ATP, Presecretory Protein, Everted Membrane Vesicles, and Phospholipids—

The sensitivities of SecA to protease V8 of S. aureus were marked by the presence of either ATP, presecretory proteins, membrane vesicles, or phospholipids (9). A similar experiment was performed with SecA D133N (Fig. 4). The sensitivities of the wild-type SecA and SecA D133N proteins to V8 were the same as each other (Fig. 4A). ATP, ADP, and ATPγS rendered the amino-terminal 95-kDa portion of the wild-type SecA highly resistant to V8. This is consistent with the previous finding that ATP interacts with the amino-terminal 25-kDa portion, the high affinity nucleotide-binding site of the SecA molecule (7). In the presence of ATP, in analogy with the wild-type SecA, the mutant SecA became resistant to V8 digestion over a wide concentration range of ATP, as in the case of the wild-type SecA (Fig. 4, B and C). ATPγS and ADP were as active as ATP in rendering the mutant resistant to V8 digestion (data not shown). In the presence of proOmpA or E. coli phospholipids, on the other hand, SecA became more sensitive to V8 digestion. SecA D133N was also digested as rapidly as the wild-type SecA was digested (Fig. 4, D and E). Therefore, the conformational changes of the mutant SecA induced by these nucleotides, preproteins, everted membrane vesicles, or phospholipids were essentially the same as those of the wild-type SecA.

The direct interaction of SecA D133N with secretory proteins was also demonstrated by means of chemical cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide using proOmpF-Lpp (49). Cross-linking with the mutant SecA to the same extent as that with the wild-type SecA was observed (data not shown). Our previous report (9) demonstrated that the V8 digestion profile after successive treatment with ATP and proOmpA was essentially the same as that with proOmpA alone, and we concluded that this is due to the release of ADP/ATP from the SecA molecule upon interaction with proOmpA. SecA D133N behaved in a similar manner (Fig. 4F). In the presence of urea-treated membrane vesicles, the digestion patterns of the wild-type SecA and SecA D133N were about the same as each other (Fig. 4G). Taken together, these data suggest that SecA D133N can interact with ATP, pres-
secretory proteins, everted membrane vesicles, and phospholipids, in the same fashion as the wild-type SecA.

Binding of SecA D133N and Wild-type SecA to Cytoplasmic Membranes—SecA binds to E. coli inverted membrane vesicles at the site comprising SecY/E/G, the membrane-embedded portion of the translocation machinery, and acidic phospholipids (45, 50). SecA also binds to membrane phospholipids. This may be due to the low affinity association with phospholipids (8). Using 14C-labeled wild-type SecA, which is fully active (51), and SecA D133N, the binding of SecA to urea-treated everted membrane vesicles of E. coli was assayed. The mutant and wild-type SecA showed similar binding affinities to the membrane (Fig. 5). These data show that SecA was not affected in its binding ability as to SecY/E/G by the replacement of Asp-133 with Asn.

SecA D133N Is Functional in the Initial Step of Translocation, Including Processing of the Signal Peptide—SecA undergoes ATP-modulated cycles of membrane insertion and deinsertion (52). In the early stage of presecretory protein translocation, SecA is assumed to use the energy of ATP binding to insert a precursor protein into the membrane so as to expose the signal cleavage site to the signal peptidase (53). 

35S-Labeled proOmpA was mixed with inverted membrane vesicles in the presence or absence of SecA and various nucleotides (Fig. 6). Proteolytic processing of proOmpA to OmpA due to the signal peptidase took place in the presence of AMP-PNP as well as ATP, demonstrating that at least a small N-terminal part of the proOmpA molecule can be translocated across the membrane, thereby becoming accessible to the signal peptidase, without using the energy of ATP hydrolysis, as reported previously (53). When samples were further incubated with proteinase K at 0 °C to digest polypeptide domains that had not been translocated into the membrane vesicles, translocation intermediates of proOmpA were detected only in the presence of ATP but not in that of a nonhydrolyzable analog (53) (Fig. 6B). Similar experiments were performed with SecA D133N. The processing of proOmpA by the signal peptidase is similar to that with the wild-type SecA (Fig. 6A). On the other hand, neither ATP nor its analog could support the translocation (Fig. 6B). These data clearly demonstrate that SecA D133N is able to bind ATP and thereby support the initial step of translocation to facilitate the processing of proOmpA by the signal peptidase. However, it retains no ability to promote the translocation reaction further.

SecA D133N Interferes with in Vitro Protein Transloca-

![Fig. 4. The V8 sensitivities of the wild-type SecA and SecA D133N in the presence of several cellular components.](image)

![Fig. 5. Binding of the wild-type and mutant SecA to urea-treated everted membranes.](image)
To determine the functional impact of SecA D133N on the membrane during protein translocation, the effect of SecA D133N on the SecA-dependent translocation of 35S-labeled proOmpA into everted membrane vesicles was examined in vitro in the presence of 100 \( \mu M \) (Fig. 7A) or 10 \( \mu M \) ATP (Fig. 7B). The translocation of proOmpA was efficiently inhibited as the amount of mutant SecA was increased (Fig. 7, A and B). It seems likely that the mutant SecA competes with the wild-type SecA for binding to the SecY/E/G translocation complex.

When a higher concentration of ATP (1 mM) was added to the reaction mixture, the interference described above was not observed at all (Fig. 7C).

**DISCUSSION**

The N-terminal region of SecA contains two nucleotide-binding sequence motifs that are found in a wide range of nucleotide-binding proteins; they are motif A (GXXXXGKT) and motif B (ZZZZD). Between the two motifs, there is a conserved Glu or Asp, which we proposed as a "catalytic residue" (22). We demonstrated here that the replacement of Asp-133, the putative catalytic residue of *E. coli* SecA, with Asn resulted in nearly complete inactivation of the in vitro presecretory protein translocation reaction (Fig. 3). This indicates the essentiality of the carboxyl group of SecA Asp-133 in translocation. SecA D133N exhibited about 50% of the endogenous and membrane ATPase activities, respectively, and about 20% of the translocation ATPase activity (Fig. 2). SecA has been shown to possess three (48) or two (19) ATP-binding sites. The remaining ATPase activity may be due to the hydrolytic reaction catalyzed by other ATP binding site(s). However, the large decrease in the translocation ATPase activity suggests that the amino-terminal ATP-binding site, compared with other ATP-binding sites, is more closely connected with the translocation reaction.

Although SecA D133N was defective in the overall protein translocation reaction, SecA D133N retained partial functionality and was able to interact with the membrane components of the translocation machinery as follows. (i) Conformational changes of the mutant SecA caused by interactions with ATP, preproteins, or everted membrane vesicles were the same as that of the wild type, as revealed by the V8 protease digestion pattern (Fig. 4). (ii) The mutant SecA retained the ability to bind ATP significantly and performed the initial stage of translocation so as to carry preproteins to a site accessible to signal peptidase (Fig. 6, also see Ref. 49), whereas translocated species were not observed (Fig. 6B). (iii) The purified SecA D133N blocked in vitro translocation by occupying SecY/E/G (Figs. 5 and 7) with low ATP concentrations. Taking the results together, we conclude that SecA D133N, which has a very low translocation ATPase, is able to perform the initial step of the
translocation until the signal peptide region reaches a site accessible to signal peptidase. This idea is consistent with that proposed by Wickner (3), who studied with nonhydrolyzable ATP analogues.

When the ATP concentration was lower (10–100 μM), wild-type SecA-driven translocation was inhibited by SecA D133N (Fig. 7). This suggests that with low concentrations of ATP, SecA D133N can mediate the translocation pathway to the signal peptide processing step. However, under the standard conditions (ATP concentration, 1 mM) even an excess amount of SecA D133N did not interfere with the wild-type SecA driven proOmpA translocation. The latter situation probably occurs in vivo, because the SecA D133N protein was expressed in vivo without CK4706 cell growth being affected (data not shown). At this stage of our studies, the reason why the translocation inhibition only takes place with a low concentration of ATP is not clear. SecA D133N may be somehow displaced by wild-type SecA efficiently at the SecY/E/G site when there is a high concentration of ATP.

In conclusion, the conserved Asp residue in E. coli SecA (Glu in SecA from some species) is involved in the catalytic function of ATP hydrolysis and translocation activity. The marked decrease in ATPase activity by the replacement of the acidic aspartate residue with the cognate amine, asparagine, at position 133 is consistent with our idea that this carboxyl group activates a water molecule which then attacks the γ-phosphate of ATP in SecA and other nucleotide-binding proteins, such as the RecA protein and F1-ATPase β subunit.

Acknowledgments—We wish to thank Dr. C. A. Kumamoto of Tufts University for strain CK4706, Dr. Mitsuo Tagaya of Tokyo University of Pharmacy and Life Science for the valuable discussion, and Wakana Shimizu for her excellent secretarial support.

REFERENCES

1. Matsuyama, S., and Mizushima, S. (1995) in Advances in Cell and Molecular Biology of Membranes and Organelles, Vol. 4, pp. 61–84, JAI Press, Greenwich, CT.
2. Wickner, W., Driessen, A. J. M., and Hartl, F. U. (1991) Annu. Rev. Biochem. 60, 101–124.
3. Wickner, W. T. (1994) Science 266, 1197–1198.
4. Bicker, K. L., Phillips, G. J., and Silhavy, T. J. (1990) J. Bioenerg. Biomembr. 22, 291–310.
5. Cunningham, K., Lill, R., Crooke, E., Rice, M., Moore, K., Wickner, W., and Oliver, D. (1989) EMBO J. 8, 955–959.
6. Akita, M., Sasaki, S., Matsuyama, S., and Mizushima, S. (1990) J. Biol. Chem. 265, 8164–8168.
7. Matsuyama, S.-I., Kimura, E., and Mizushima, S. (1990) J. Biol. Chem. 265, 8760–8765.
8. Lill, R., Dowhan, W., and Wickner, W. (1990) Cell 60, 271–280.
9. Shinkai, A., Mei, L. H., Tokuda, H., and Mizushima, S. (1991) J. Biol. Chem. 266, 5827–5833.
10. Pandi, J. P., Cabelli, R., Oliver, D., and Tai, P. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8953–8957.
11. Walker, J. E., Driessen, A. J. M., Tokuda, H., and Mizushima, S. (1991) J. Biol. Chem. 266, 5827–5833.
12. Sasaki, M., Sibald, P. R., and Wittinghofer, A. (1990) Trends Biochem. Sci. 15, 430–434.
13. Story, R. M., and Steitz, T. A. (1992) Nature 355, 374–376.