Stepwise Movement of Preproteins in the Process of Translocation across the Cytoplasmic Membrane of Escherichia coli*

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Derivatives of proOmpA possessing the second cysteine residue at position +302 and the first one at different positions were constructed at the DNA level. They were oxidized to form disulfide-bridged loops of different sizes at different positions. In the presence of a proton motive force, proOmpAs possessing a smaller loop could be translocated across the membrane in vitro, whereas ones possessing loops comprising more than 16 amino acid residues were hard to translocate. The sizes of polypeptide chains that had been translocated and had become protease-resistant were determined in both the presence and absence of the proton motive force. The size was the same for all proOmpAs possessing the first cysteine residue between +244 (proOmpA L59) and +274 (proOmpA L29). When the first cysteine residue was moved further away from the N terminus, a sudden increase in size, of approximately 30 amino acid residues, was observed, the size being the same for proOmpA possessing the first cysteine residue between +278 (proOmpA L25) and +293 (proOmpA L10). The shift in size between proOmpA L29 and proOmpA L25 was observed with different proteases exhibiting different substrate specificities. Treatment with these proteases resulted in complete digestion of SecA on everted membrane vesicles, whereas Sec proteins integrated into membranes were considerably resistant to the treatment. These results can be best interpreted as that the translocation of preproteins through the secretory machinery takes place in every 30 amino acid residues and that SecA is responsible for the stepwise movement.

The molecular mechanisms underlying the translocation of secretory proteins across the bacterial cytoplasmic membrane or eukaryotic endoplasmic reticulum membrane have been the subject of extensive studies (1–3). In both cases proteins comprising the translocation machinery have been purified, and reconstitution of the translocation activity from these proteins or a complex of proteins has been successfully performed (4–7). In E. coli cells, a set of Sec proteins, SecA, SecB, SecD, SecE, SecF, SecG, and SecY, have been characterized, and the roles of individual Sec proteins in the translocation reaction have been studied in some detail. SecA, translocation ATPase, is a peripheral membrane protein that plays the central role, especially in the initial stage of the translocation reaction (8, 9). SecY, SecE, and SecG are integral membrane proteins (10–12) and are assumed to play roles together with SecA in the transmembrane movement of preproteins (1, 13, 14). SecD, another membrane-spanning protein (15), is involved in the final stage of the reaction (16), while SecF is in charge of the assembly of SecD and SecY (17). The involvement of SecD and SecF in the proton electrochemical gradient stimulation of preprotein translocation was also indicated (18). Thus, the functions of individual Sec proteins have become much clearer. It is still unclear, however, how preproteins move across the membrane in relation to these Sec proteins.

The translocation reaction is peculiar in that it exhibits no specificity as to the length of the polypeptide chain to be translocated. In E. coli cells, the shortest polypeptide chains so far reported to be translocation-competent have mature domains comprising around 45 amino acid residues (19, 20), the longest one possessing as many as 1000 amino acid residues. Provided that these preproteins are translocated in an unfolded state, the following three possibilities can be considered as to the mode of their movement across the membrane. 1) The machinery translocates polypeptide chains by recognizing peptide bonds one by one. 2) The machinery recognizes a polypeptide of a certain length as a unit, and, therefore, translocation proceeds stepwisely. 3) The translocation of the mature domain takes place continuously in a one-step mode once it has been initiated. The first possibility is unlikely since polypeptide chains possessing an internal non-peptide domain are translocation-competent (21).

Several lines of evidence suggest that the transmembrane movement may take place in a stepwise manner. Tani et al. (22, 23) observed that the requirement of proton motive force $\Delta \mu_9 +$ and ATP for translocation differed with the portion of a polypeptide chain, and Schiebel et al. (24) and Driessen (25) demonstrated that $\Delta \mu_9 -$ and ATP function at different steps of the catalytic cycle of translocation. The translocation of a limited portion of a polypeptide chain (~20 amino acid residues) under certain conditions has also been observed (24). Furthermore, ATP-driven cycles of membrane insertion and deinsertion of SecA molecules during the translocation reaction were observed very recently (26).

In the present work, we provide further evidence supporting the stepwise movement of preproteins across the cytoplasmic membrane of E. coli.

EXPERIMENTAL PROCEDURES

Materials—The reagents for oligonucleotide synthesis were obtained from Applied Biosystems. The oligonucleotide-directed in vitro mutagenesis system was a product of Amersham Corp. SP6 RNA polymerase and restriction enzymes were obtained from Takara Shuzo Co. TranSlabel, a mixture of 70$\%$[35S]methionine and 20$\%$[35S]cysteine,

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1 The abbreviations used are: $\Delta \mu_9 -$ , proton motive force; AMS, 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonate; 2Na; kb, kilobase pair(s); bp, base pair(s); ATP, adenosine 5’-O-(thiotriphosphate); AMP-PNP, adenosine 5’-(\(\gamma\)-imino)triphosphate.
### Table 1

| Plasmids constructed | Oligonucleotides synthesized (5' → 3') | New restriction site formed | Amino acids changed in OmpA | Position of Cys residues in OmpA |
|----------------------|---------------------------------------|----------------------------|-----------------------------|--------------------------------|
| pOA13                | Wild ompA gene                        |                            | 290/302                     |                                |
| pOA290G              | GGT TTC ACG GGT GCT GCA             | Cfr10I                     | C³⁰⁰ → G                    | 302                            |
| pOA10                | AGC ACG CGG TCT GCA GTC GCA GGT G   | PstI                       | V²⁹⁰K → CR                  | 293/302                       |
| pOA16                | GTC ACC GGT GTC ACA GGT ACG GGT     | NspI                       | G²⁸⁷ → C                    | 287/302                       |
| pOA18                | GGT GTT GCC AGT GAC CGG GGT GTC TCA | ApaI                       | V³⁸⁵ → C                    | 285/302                       |
| pOA21                | AGT AAC CGG GCA GTA CTC GCC CAT ACC A | ApaLI                      | S²⁸⁰N → CT                  | 282/302                       |
| pOA25                | GCC CAT ACA ACG TGC GGA             | PmaCI²                     | G²⁹⁷ → C                    | 278/302                       |
| pOA29                | CC CAT ACC ACG TGC GGT GCA CTG TCG CG | ApaLI                      | L²⁷⁸S → CT                  | 274/302                       |
| pOA35                | TGC GAT GTA GAG TTA GGA GAT         | EcoT22I                     | G³⁶⁸ → C                    | 268/302                       |
| pOA39                | GG GAT ATC TTA GCA GGT GTA ATC AAC AA | PstI                       | L²⁹⁰I → CS                  | 264/302                       |
| pOA43                | CAG GTA ATC AAC ACA GAT            | NspI                       | SV³⁶⁰ → TC                  | 260/302                       |
| pOA48                | GA CGG ACA GCG GTA TGC GGC GAG ACC CT | NspI                       | ER³⁵⁵ → AC                  | 255/302                       |
| pOA52                | GCG TCA GAA GCA ACA TGT GTA AGC GT  | NspI                       | OG³⁵¹ → TC                  | 253/302                       |
| pOA59                | G GGT GTA AGC GTC AGA ACA TGT GGT G | NspI                       | IG³⁴⁴ → TC                  | 244/302                       |

*•*, mismatching base.

| a | Restriction site lost on gene manipulation.

1000 Ci/mmol, was purchased from ICN Radiochemicals. Proteinase K was obtained from Merck, and pepsin, trypsin, and Pronase E (protease Type XIV) from Sigma. The SH reagent, 4-acetamido-4-benzenedisulfonate 2Na (AMS), was a product of Molecular Probes Inc. Molecular marker proteins were obtained from Life Technologies, Inc. The SecA protein was purified as described (9). Anti-SecA antibody was raised in rabbits against purified SecA.

Table S1. Oligonucleotides synthesized to introduce a new cysteine codon into the ompA gene.

| Plasmids constructed | Oligonucleotides synthesized (5' → 3') | New restriction site formed | Amino acids changed in OmpA | Position of Cys residues in OmpA |
|----------------------|---------------------------------------|----------------------------|-----------------------------|--------------------------------|
| Wild ompA gene       | Wild ompA gene                        |                            | 290/302                     |                                |
| pOA290G              | GGT TTC ACG GGT GCT GCA             | Cfr10I                     | C³⁰⁰ → G                    | 302                            |
| pOA10                | AGC ACG CGG TCT GCA GTC GCA GGT G   | PstI                       | V²⁹⁰K → CR                  | 293/302                       |
| pOA16                | GTC ACC GGT GTC ACA GGT ACG GGT     | NspI                       | G²⁸⁷ → C                    | 287/302                       |
| pOA18                | GGT GTT GCC AGT GAC CGG GGT GTC TCA | ApaI                       | V³⁸⁵ → C                    | 285/302                       |
| pOA21                | AGT AAC CGG GCA GTA CTC GCC CAT ACC A | ApaLI                      | S²⁸⁰N → CT                  | 282/302                       |
| pOA25                | GCC CAT ACA ACG TGC GGA             | PmaCI²                     | G²⁹⁷ → C                    | 278/302                       |
| pOA29                | CC CAT ACC ACG TGC GGT GCA CTG TCG CG | ApaLI                      | L²⁷⁸S → CT                  | 274/302                       |
| pOA35                | TGC GAT GTA GAG TTA GGA GAT         | EcoT22I                     | G³⁶⁸ → C                    | 268/302                       |
| pOA39                | GG GAT ATC TTA GCA GGT GTA ATC AAC AA | PstI                       | L²⁹⁰I → CS                  | 264/302                       |
| pOA43                | CAG GTA ATC AAC ACA GAT            | NspI                       | SV³⁶⁰ → TC                  | 260/302                       |
| pOA48                | GA CGG ACA GCG GTA TGC GGC GAG ACC CT | NspI                       | ER³⁵⁵ → AC                  | 255/302                       |
| pOA52                | GCG TCA GAA GCA ACA TGT GTA AGC GT  | NspI                       | OG³⁵¹ → TC                  | 253/302                       |
| pOA59                | G GGT GTA AGC GTC AGA ACA TGT GGT G | NspI                       | IG³⁴⁴ → TC                  | 244/302                       |

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successively treated with either anti-SecA or anti-SecD (32) antibodies and anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad), and then developed with a chemiluminescence (ECL) reagent (Amer- sham) on an x-ray film (Fuji).

RESULTS

Construction of Genes That Encode ProOmpAs Possessing Two Cysteine Residues at Different Positions in the Mature Domain—ProOmpA possesses two cysteine residues at positions +290 and +302 of its mature domain. ProOmpA was engineered at the DNA level so as to carry the first cysteine residue at different positions and, therefore, able to form intramolecular disulfide-bridged loops of different sizes at different positions. The primary structures of the proOmpAs encoded are summarized in Fig. 1. These structures have been confirmed on the DNA level.

Mutant ProOmpAs as Well as the Wild-type One Quantitatively Form Disulfide-looped Structures under Oxidative Conditions—35S-Labeled proOmpAs, synthesized and purified, were subjected to oxidation in the presence of ferricyanide. Conversion of sulfhydryl groups to a disulfide bridge was monitored by use of AMS, a sulfhydryl reagent. The results for some of the proOmpAs are shown in Fig. 2. All proOmpAs showed an almost quantitative upward change in position on an SDS gel when they were treated with AMS in the presence of dithiothreitol. No such change was observed in the absence of dithiothreitol. These results indicate that the shift of the band position was due to chemical binding of AMS to sulfhydryl groups of proOmpAs, and that the ferricyanide treatment resulted in the quantitative conversion of proOmpAs to the disulfide-bridged (looped) form.

In Vitro Translocation of Loop proOmpA Derivatives—In vitro translocation of a looped wild-type proOmpA terminates at the loop in the absence of ΔμiH⁻ and proceeds beyond the loop to completion when ΔμiH⁺ is imposed (22, 23). Essentially the same experiment was carried out with a series of looped mutant proOmpAs (Fig. 3). In the absence of ΔμiH⁺, translocation resulted in the appearance of protease K-resistant bands, of which the molecular sizes were smaller than those of the mature OmpAs. The result for L10, of which the loop is smaller than that of wild-type proOmpA, was similar. These results indicate that translocation of all these proOmpAs proceeded to the looped region in the absence of ΔμiH⁺, as in the case of wild-type proOmpA. The completion of translocation was observed in the presence of ΔμiH⁺ when the loop size was smaller than 18 amino acid residues, whereas it was not observed when the size was 20 amino acid residues or more (Figs. 3 and 4). The completion was partial in the cases of L16 and L18. These results indicate that there is a limited loop size as to translocation even in the presence of ΔμiH⁺.

Discontinuous Change in Size of the Translocation-competent Domain—Based on the results of the previous studies (22, 23), we expected that the size of protease K-resistant fragments should become smaller as the loop size becomes larger (see Fig. 1). As judged from the band positions on the gel shown in Fig. 5, however, the sizes of protease K-resistant fragments were essentially the same with proOmpAs with loop sizes of 10–25 (L10–L25). The positions of protease K-resistant bands suddenly shifted downward when the loop size became larger, and the positions remained almost the same from L29 to L59. The downward shift in the band positions on the gel was estimated to be equivalent to about 3 kDa, i.e. about 30 amino acid residues (Figs. 5 and 6A).

The sizes of protease K-resistant fragments in the presence of ΔμiH⁺ were also determined from the data shown in Fig. 3. A shift in the band positions was also observed between L25 and L29, the difference in size being estimated to be about 2.6 kDa (Fig. 6). As described earlier, no intermediate bands were observed with L10 and L13. These results suggest that the translocation mechanism was essentially the same in the presence and absence of ΔμiH⁺, except that the machinery tolerates larger loops in the former. The reason for the slight difference in the shift size in the band positions between +ΔμiH⁺ and −ΔμiH⁺ is unknown.

The Discontinuous Shift Is Independent of the Protease Species—Although the stepwise profiles shown in Figs. 5 and 6 suggest possible stepwise translocation, there was the possibility that such profiles could be due to the substrate specificity of protease K. Therefore, we carried out otherwise the same experiment using different proteases (Fig. 7). The positions of protease-resistant bands varied with the protease species used. This is most probably due to differences in their substrate specificities as discussed later. Nevertheless, the shifts in the positions of major protease-resistant bands took place in all cases between L25 and L29, and the positions of the major bands were essentially the same for L10–L25 and L29–L59, respectively. These results strongly support the view that the stepwise profile is not due to the specificities of the proteases.
used but to the stepwise translocational movement of proOmpA through the secretory machinery. When Pronase E was used, much shorter protease-resistant bands were observed for L29–L59, especially for L52 and L59. L59 also gave a shorter band upon the trypsin treatment. The reason for this appearance was not clear, although it may represent another possible translocation intermediate.

**Characterization of Protease-resistant (Translocated) Polypeptide Fragments—**

The sizes of protease-resistant peptide fragments of OmpA were estimated on gels, and the results are shown in Table II together with the positions of the C-terminal ends, which were estimated from the sizes. In Fig. 8, the results are summarized together with the positions of the first cysteine residues and all preferable sites these proteases may attack. The peptide fragment obtained on pepsin treatment has its C-terminal end around 1299, which is considerably far from those of ones obtained with other proteases (around 1265). This may be due to the fact that there is no cleavage site for pepsin between 1265 and 1299. Based on these data together, the domain in L10–L25 which is still accessible to these proteases after translocation (Δ₁) is

**Fig. 3. Translocation of 35S-labeled proOmpA with or without a disulfide-bridged loop through everted membrane vesicles in the presence or absence of Δ₁.** Five-µl aliquots of disulfide-bridged 35S-proOmpAs (L10-L59 and 290G) in 8 M urea were diluted with 45 µl of 50 mM potassium phosphate, pH 7.5, and divided into five 9-µl portions. They were treated with 1 µl of 100 mM dithiothreitol (lanes 2, 4, and 5) or distilled water and then incubated at 37°C for 10 min and subjected to the translocation assay. The assay mixture comprised 5 µg of everted membrane vesicles, 0.4 µg of SecA, 4 mM MgSO₄, and 5 mM ATP in 50 mM potassium phosphate, pH 7.5 (total volume, 25 µl). NADH was added to lanes 3–5 at the concentration of 5 mM. After a 10-min incubation at 37°C, the samples in lanes 1–4 were treated with 5 µl of protease K (5 µg/µl) for 20 min on ice. OmpAs were recovered by trichloroacetic acid precipitation, washed with acetone and ether, and then analyzed by SDS-polyacrylamide gel electrophoresis. The positions of precursor, mature, and translocation intermediates of OmpA are indicated by P, M, and I, respectively. DTT, dithiothreitol; PK, protease K.

**Fig. 4.** proOmpAs possessing a disulfide-bridged loop were efficiently translocated across membrane vesicles under fully energized conditions when the loop size was smaller than 18 amino acid residues. The extent of translocation was estimated by densitometric analysis of the data given in Fig. 3 (lane 3). The ratio of the density of the mature intermediate band to that of the mature plus intermediate bands is plotted against the loop size represented by the number of amino acid residues involved.

**Fig. 5. Alignment of translocation intermediate bands that arose upon protease K treatment in the process of translocation of the series of looped proOmpAs.** 35S-Labeled proOmpA derivatives indicated were prepared by the small scale method, oxidized with ferri cyanide, and then subjected to the translocation assay in the absence of NADH (Δ₁). The conditions for digestion by protease K were the same as those given in the legend to Fig. 3. The positions of the mature and translocation intermediates of OmpA are indicated by M and I, respectively. The positions of molecular mass markers are shown to the left in kDa. DTT, dithiothreitol.

**Polypeptide Fragments—** The sizes of protease-resistant peptide fragments of OmpAs were estimated on gels, and the results are shown in Table II together with the positions of the C-terminal ends, which were estimated from the sizes. In Fig. 8, these results are summarized together with the positions of the first cysteine residues and all preferable sites these proteases may attack. Around the C-terminal positions thus estimated for L10–L25, possible sites of hydrolysis by the respective proteases exist. Therefore, the domain in L10–L25 which is still accessible to these proteases after translocation (Δ₁) is
The protease-resistant fragments for these proOmpAs were thus estimated to be +233–240, where the possible sites of hydrolysis by the respective proteases are located. For these proOmpA derivatives, therefore, the domain from around +235 to the C terminus is likely accessible to externally added proteases. It should be noted again that position +235 is considerably more amino-proximal than the loop position in many of these proOmpA derivatives. The position of the trypsin-resistant band given by L48 is different from those by others. This is most likely due to the change of a probable site for trypsin of Arg-Arg (−255–256) to Cys-Arg in this mutant proOmpA.

Hydrolysis of Sec Proteins in the Secretory Machinery by Proteases—The everted membrane vesicles used for the translocation assay were treated with proteases, and the Sec proteins that survived were analyzed by immunoblotting (Fig. 9). In one series of experiments (series a), everted membrane vesicles were directly treated with proteases on ice under the conditions used for the analysis of translocated proteins, and protease-resistant fragments were analyzed by immunoblotting against anti-SecA and anti-SecD antibodies. No intact SecA was detected after the treatment with any of these proteases, whereas SecD, a protein mostly localized on the periplasmic side of the membrane (15, 16), was rather resistant to the treatment. Since SecD has several membrane-spanning domains (15), the result indicates that the core region of the membrane is resistant to the treatment. In the other series of experiments, everted membrane vesicles were first incubated with proOmpA, ATP, and succinate to initiate translocation, and then subjected to the protease treatment on ice (Fig. 9, series b). The results were essentially the same. A similar experiment was carried out for SecY with anti-SecY antibodies. A preliminary result indicated that some cytoplasmic domain of SecY was protease-sensitive, whereas its core region as well as its periplasmic domain was resistant (data not shown).

### Table II

| Protease         | ProOmpA derivatives | Protease-resistant fragment |
|------------------|---------------------|-----------------------------|
| Proteinase K     | L10–L25             | L29–L59                     |
|                  | 28.7                | +264                        |
|                  | L29–L59             | 25.8                        |
|                  | +238                |                             |
| Trypsin          | L10–L25             | L29–L59                     |
|                  | 28.8                | +265                        |
|                  | L29–L59             | 26.0                        |
|                  | +240                |                             |
| Pepsin           | L10–L25             | L29–L59                     |
|                  | 32.0                | +296                        |
|                  | L29–L59             | 25.7                        |
|                  | +237                |                             |
| Pronase E        | L10–L25             | L29–L59                     |
|                  | 29.4                | +271                        |
|                  | L29–L59             | 25.3                        |
|                  | +233                |                             |

*Data were taken from Figs. 5 and 7. Only major bands were considered. The molecular mass of each amino acid residue from the N terminus of OmpA was added until the figure reached the size estimated on the gel. The numbers of amino acid residues thus obtained are given.

![Figure 6](http://example.com/fig6.png)

**Fig. 6.** Alignment of translocation intermediate bands that arose upon digestion with pepsin, trypsin, or Pronase E. The methods for the preparation of the series of looped proOmpAs indicated and translocation experiments were the same as those for the experiments described in Fig. 5. Treatment with trypsin and Pronase E was performed in 50 mM potassium phosphate, pH 7.5. For pepsin treatment, the pH was adjusted to 2 with 1 M HCl. Digestion was performed with 25 μg of protease/30 μl on ice for 20 min. The positions of the mature and translocation intermediates of OmpA are indicated by M and I, respectively. The positions of molecular mass markers are shown to the left in kDa. DTT, dithiotreitol.

![Figure 7](http://example.com/fig7.png)

**Fig. 7.** Alignment of translocation intermediate bands that arose upon digestion with pepsin, trypsin, or Pronase E. The methods for the preparation of the series of looped proOmpAs indicated and translocation experiments were the same as those for the experiments described in Fig. 5. Treatment with trypsin and Pronase E was performed in 50 mM potassium phosphate, pH 7.5. For pepsin treatment, the pH was adjusted to 2 with 1 M HCl. Digestion was performed with 25 μg of protease/30 μl on ice for 20 min. The positions of the mature and translocation intermediates of OmpA are indicated by M and I, respectively. The positions of molecular mass markers are shown to the left in kDa. DTT, dithiotreitol.
Taken together, these results suggest that only the cytoplasmic portion of the secretory machinery, including SecA, is susceptible to proteases, whereas the portion of the machinery that is embedded in the membrane or mostly exposed to the periplasmic surface is resistant to the translocation treatment. In other words, the portions of preproteins that are resistant to these proteases have already been inserted into at least the membrane-embedded portion of the secretory machinery.

**DISCUSSION**

In previous works (22, 23), we showed that proOmpA possessing a disulfide-bridged polypeptide loop comprising 13 amino acid residues could be translocated across everted membrane vesicles of the cytoplasmic membrane of E. coli, when the membrane vesicles were fully energized. In the present work, we demonstrated that the size limit of disulfide-bridged loops that the translocation machinery tolerates is 18 amino acid residues (Figs. 3 and 4). The machinery was also reported to tolerate preproteins possessing a side-arm comprising 20 amino acid residues (24), and ones possessing an internal nonpeptide domain in the mature region (21). It is likely, therefore, that the machinery translocates substrates with rather low specificity through a channel that is considerably wide in the fully energized state. It is likely that the channel allowing the passage of polypeptides with such a large loop is not a closed one that fits expanded polypeptide chains, but one that is open toward the environmental lipid bilayer, as discussed previously (33). Evidence for a protein-carrying channel that is laterally open to the lipid bilayer was reported for the endoplasmic reticulum membrane very recently (34).

Evidence has been accumulating that the translocation of preproteins across the membrane may take place in a stepwise manner (22–26). The results presented in the present paper support this view. We previously showed for oxidized proOmpA, which has a disulfide bridge between Cys-290 and Cys-302, thus forming a polypeptide loop of 13 amino acid residues, that translocation in the absence of Δψm ceased when the loop reached the membrane (22, 23). In the present work, we shifted the position of one of the Cys residues (Cys-290) toward the N terminus so as to obtain proOmpA derivatives capable of forming a larger loop closer to the N terminus. If translocation ceases at the loop, the length of polypeptides that can be translocated would become shorter as the position of the first Cys residue becomes closer to the N terminus. However, the size remained almost the same for L10-L25 and L29-L59, respectively, with a considerable change, about 3 kDa, between L25 and L29. The change is not due to protease specificity since four proteases having different substrate specificities gave the same profile as to the position change, suggesting that the profile must reflect a discontinuous event during the translocation. The most probable explanation for the profile may be that depicted in Fig. 10A. We assume that the loop cannot get into the machinery, most likely remaining on the outer surface of the SecA molecule, since the C termini of the protease-resistant domain of OmpAs were always considerably more amino proximal than the loop positions (see Fig. 8). The machinery has a "room" to accommodate a polypeptide chain of a certain length, and translocation through the portion of the translocation machinery that is embedded in the membrane takes place only when the room is filled with a polypeptide.

Providing this is the case, translocation of preproteins in general can be depicted as shown in Fig. 10B, namely the translocation reaction, which is triggered by a signal peptide, takes place stepwisely as the room is fully occupied by a portion of the polypeptide chain under translocation. Since SecA is the only Sec protein that is fully digested on the protease treatment, the room for polypeptide accommodation should reside in the SecA molecule. In this respect, it is very interesting that SecA molecules can be exposed to the periplasmic surface of the cytoplasmic membrane (35, 36) and undergo an ATP-driven cycle of membrane insertion and deinsertion (26). This conformational change of SecA would result in stepwise movement of preproteins. Conformational changes of SecA upon interaction with ATP, prepeptides, phospholipids, and membrane vesicles have been observed (37). SecA exists as dimers (38–40). It may also be possible, therefore, that the stepwise movement is coupled with each half rotation of the SecA dimer. The size difference between before and after the shift in band position on a gel is about 3 kDa, suggesting that the stepwise translocation takes place every ~30 amino acid residues. The movement of a polypeptide chain by about 20 amino acid residues during translocation has been demonstrated upon the addition of ATP analogs ATPγS or AMP-PNP (24).

In order to strengthen the idea of stepwise translocation, we have constructed genes encoding proOmpA derivatives possessing the first cysteine residue much closer to the N terminus. However, due to the difficulty in quantitative conversion...
to the disulfide-bridged structure, translocation experiments could not be performed with them. It should be noted that the appearance of a shorter translocation intermediate are suggested in some of the results with L59.

In the proOmpAs used in the present work, the disulfide-bridged loops were located near the C terminus. One may wonder, therefore, how such good synchronization in the stepwise translocation can be achieved even near the C-terminal end. We recently demonstrated that SecA recognizes not only the positively charged region (9), but also the hydrophobic stretch in signal peptides. If SecA is also able to recognize such stretches in the mature domains of secretory proteins, such stretches could function to enhance the synchronization of the stepwise process. Although the mature domains of secretory proteins are hydrophilic in general (41), they usually possess a few hydrophobic stretches. The OmpA molecule has such a stretch (232–240) right before the region we manipulated in the present work. Possible interaction of hydrophobic stretches in the mature domain and SecA in relation to the mode of translocation is under investigation in this laboratory.

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