Short Hydrophobic Segments in the Mature Domain of ProOmpA Determine Its Stepwise Movement during Translocation across the Cytoplasmic Membrane of Escherichia coli*

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Based on the finding that a series of engineered proOmpAs containing disulfide-bridged loops of different sizes at different positions exhibits a discontinuous mode of polypeptide transit across the cytoplasmic membrane of Escherichia coli, we suggested previously that the translocation of preproteins takes place at every 30 amino acid residues. In the present study, we investigated the molecular mechanism underlying this stepwise translocation. Deletion or relocation of hydrophobic segments of the mature domain of proOmpA (H1, residues 233–237; H2, residues 261–265) significantly altered the pattern of the stepwise translocation. The stepwise mode of polypeptide insertion was also observed with reconstituted proteoliposomes comprising purified SecA, SecY, and SecE. Cross-linking experiments involving a photoactivatable cross-linker revealed that SecY and SecA are the components which interact with the hydrophobic segment of proOmpA. The present results indicate that the hydrophobic segments of the mature domains of preproteins interact with membrane embedded translocase during polypeptide transit across the membrane, which causes a discontinuous mode of polypeptide movement.

The translocation of preproteins across the cytoplasmic membrane of Escherichia coli is mediated through interactions of molecules, including a set of Sec proteins (1–5). The driving force for translocation is provided by the proton motive force and the energy formed through SecA-mediated hydrolysis of ATP (6–9). SecY, SecE, and SecG, a heterotrimeric membrane-embedded translocase, provide an intramembrane pathway for preprotein transit (10–12). Biochemical studies, such as purification of the components of the translocase and their reconstitution into proteoliposomes of specified compositions in E. coli systems, have been performed (13, 14). These studies revealed the minimum components required for translocation and their modes of action in substantial detail. However, the details of the mechanism by which preproteins move through the cytoplasmic membrane remains to be elucidated.

In previous studies, we showed that a proOmpA derivative containing a disulfide-bridged loop between Cys-290 and Cys-302 can be translocated across everted membrane vesicles of the cytoplasmic membrane of E. coli. In the absence of the proton motive force, on the other hand, translocation ceased when the loop reached the membrane (15, 16). Taking advantage of this phenomenon, we analyzed how preproteins are translocated across the membrane. We changed the position of one of the cysteine residues (cysteine-290) toward the N terminus so as to obtain proOmpA derivatives with disulfide-bridged loops of different sizes at different positions (17). We expected that the lengths of the translocated polypeptides would become shorter as the first cysteine residue becomes closer to the N terminus, if translocation ceases at the position of the loop. In contrast to our expectation, however, we found that the size of the translocated polypeptides remained almost the same for OmpA derivatives containing loops of 10–25 and 29–59 amino acid residues, respectively, with a change of about 3 kDa. This suggested that the in vitro translocation of proOmpA through the secretory machinery takes place in every 30 amino acid residue segments. Under certain conditions, the movement of a polypeptide chain by about 20 amino acid residues during translocation was also demonstrated upon the addition of a nonhydrolyzable ATP analog, ATPyS or AMP-PNP (7). SecA may be involved in this discontinuous polypeptide transition. The binding of ATP to SecA results in the membrane insertion of a 30-kDa fragment of the SecA protein (18), which may be accompanied by the insertion of segments of preproteins into the membrane.

Although the insertion-deinsertion cycle of SecA may partly explain the stepwise translocation of preproteins, the details of the mechanism remained unclear. It is quite difficult to imagine that SecA strictly recognizes every 30 amino acid residue segments, which have a wide variety of physicochemical properties. We therefore assume that the mature domains of preproteins have some elements that control their stepwise movement.

In the present study, we examined whether or not hydrophobic segments of the mature domain of proOmpA determine the stepwise movement during translocation across everted membrane vesicles of E. coli. We focused on the fact that many outer membrane proteins in addition to OmpA periodically contain hydrophobic segments in their mature domains, although they are hydrophilic in general (19). We relocated or replaced hydrophobic segment(s) with an artificial hydrophobic sequence(s), and compared the patterns of stepwise movement.

1 The abbreviations used are: ATP-γ-S, adenosine 5'-O-(thiotriphosphate); AMP-PNP, adenosine 5'-β,γ-imino)triphosphate; APDP, N-[4-(p-azidosalicylamido)butyl]-3'-2'-pyridyldithio)propionamide; PAGE, polyacrylamide gel electrophoresis.
EXPERIMENTAL PROCEDURES

Materials—Everted membrane vesicles were isolated from E. coli K003 (Lpp·, ΔuncB-C-Tn10) as described previously (20). SecA was purified as described previously (21). Mutant proOmpAs were purified as described by Crooke et al. (22), and SecB was purified as described by Weiss et al. (23). All proOmpA derivatives used for the in vitro translocation reactions were synthesized in vitro in the presence of EXPRESS®S10 protein labeling mix (DuPont NEN) (17). SecE and SecY were purified as described by Akimaru et al. (14). Na125I (100 mCi/ml) was purchased from ICN. IODO-GEN, 5,5'-dithiobis(2-nitrobenzoic acid), and N-[4-p-azidobenzylamido]butyl-3'-2'-pyridyldithio)propionamide (APDP) were from Pierce. Irradiation of samples was performed with a UV lightbox from Funakoshi (Funa-UV-linker FS-1500).

METHOD—DNA was isolated from the cell pellet by the method of Uchida et al. (13). One-third of the 35S-labeled proOmpA dissolved in 6 M urea and 100 mM potassium phosphate (pH 7.5) was incubated with 60 units of proteinase K for 15 min at 37°C. The sample was dialyzed in the presence of 10 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl2, and 1 mM dithiothreitol at 4°C for 1 h. The sample was then applied to a Sephadex G-50 column to remove Na125I. To prepare 125I-SEC-bound membrane vesicles, 4 nM 125I-SecA and 200 μg/ml urea-washed membrane vesicles were incubated for 15 min on ice in a 100-μl reaction buffer consisting of 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl2, and 0.2 mg/ml bovine serum albumin. The membrane vesicles were isolated and then resuspended in the same buffer. Five microliters of 50 μM APDP, 5 μM phosphocreatine, creatine kinase (100 μg/ml), and 20 μg/ml proteinase K were added to the sample, and the mixture was incubated in reaction buffer for 15 min at 37°C, followed by proteinase K treatment (final, 0.1 mg/ml) for 15 min on ice. The proteins were precipitated with trichloroacetic acid and then washed with acetone twice, then the washed precipitate was rehydrated in 6 M urea and 100 mM potassium phosphate (pH 7.5), and 50 μl of diluted APDP was added. After 1 h at room temperature, the proteinase K was washed with Tris-buffered saline, and then washed with acetone twice, then the washed precipitate was dissolved in 6 M urea and 50 mM potassium phosphate (pH 7.5).

RESULTS

Construction of proOmpA Derivatives—purification of proOmpA derivatives for the in vitro translocation reaction was performed according to the method of Uchida et al. (13). The resultant plasmids are referred to as pTDL16-L43. The pTDL plasmid DNA was isolated from the E. coli CJ236, and uracil-containing single-stranded plasmid DNA was isolated. Oligonucleotide-directed mutagenesis was performed according to the method of Funk (26). The following oligonucleotide primers were used: proOmpAL16–43H1, 5'-GTCGCGTGAACCTGCGCACACAACG-3' (spil); proOmpAL16–29H2, 5'-GCCGGGATTCTTGGAGAGCTCTTTATCACGACATGT-3' (spil); proOmpAL16–29ΔH1, 5'-GGGATTCTTGGAGAGCTCTTTATCACGACATG-3' (spil); proOmpAL43H2, 5'-GCCGGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL43H2, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL43H2-DH1, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL85H2-DH1, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL43H2-DH1, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL85H2-DH1, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL43H2-DH1, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI); proOmpAL85H2-DH1, 5'-GGGATTCTTGGAGAGCTCTTTATCAGAAGAGTATCAGAC-3' (SacI). The restriction sites created are shown in parentheses. The cysteine residues (Cys-290 and Cys-302) were introduced at Cys-260 was replaced with a glutamin residue, and the positions of the cysteine residues used for the formation of a disulfide bridge with those of the two hydrophobic regions. We constructed, by the oligonucleotide-directed mutagenesis method, genes encoding derivatives of proOmpA in which either or both of the hydrophobic segments were replaced with artificial hydrophilic acidic amino acid sequences (Fig. 1D). As judged from the hydrophobic profile, the replacement was rendered both hydrophobic segments completely hydrophilic (Fig. 1C).

The Effects of Hydrophobic Segments in the Mature Domain of proOmpA—These proOmpA derivatives were then subjected to in vitro translation into everted membrane vesicles of E. coli in the absence of the proton motive force (Fig. 2). As shown in Fig. 2A, the sizes of the proteinase K-resistant fragments (band A) were essentially the same for proOmpAs containing loops of 16 and 21 amino acid residues (L16 and L21). When the size of the loop increased (L35), a sudden decrease in the size of the fragment, by about 5 kDa, occurred and several fragments were observed (collectively referred to as band B). The sizes of the fragments were the same for L35 and L43. L29 exhibited an intermediate profile between those of L21 and L35. These results are essentially the same as those of Uchida et al. (17). This stepwise profile was not due to the substrate specificity of proteinase K, because similar profiles were observed when other proteases with different substrate specificities were used (17).

As shown in Fig. 2B, when the H1 segment was replaced with a hydrophilic sequence, the sizes of the protease K-resistant fragments were the same for all proOmpA derivatives (L16, L21, L29, L35, and L43). Similar results were obtained when trypsin was used instead of proteinase K (data not shown), suggesting that the observed profile was not due to the specificity of the protease. When the H2 segment was replaced with...
a hydrophilic one, no fragment was observed at the position of band A for L16 or L21. Instead, bands C* and C were observed for L16 and L21, respectively. It should be noted that a slight decrease in size occurred between L16 and L21 (Fig. 2C), suggesting that translocation occurred continuously. When both the H1 and H2 segments were replaced, the size of the proteinase K-resistant fragments continuously decreased as the loop size increased, except for L35 and L43 (Fig. 2D). Discontinuous translocation between L35 and L43 can be explained by the fact that appropriate proteinase K cleavage sites do not exist from around residues +260 to +252, where the translocation of L43 is predicted to cease at the position of its loop. These results suggest that the hydrophobic segments are involved in the stepwise movement. Similar profiles were observed when translocation was performed in the presence of the proton motive force (data not shown), suggesting that the mechanism of SecA-dependent translocation is principally the same in the absence and presence of the proton motive force, as we discussed previously (17). Furthermore, when the translocation reaction was conducted in the presence of everted membrane vesicles derived from a strain harboring the mutation in the prlA666, which was shown to be a particularly strong suppressor for a variety of maltose-binding protein export defects (27), the patterns of the translocation of looped proOmpA derivatives were the same as that obtained in the presence of the wild-type membranes (data not shown).

To confirm that the hydrophobicity is important for the stepwise movement, we inserted another H1 segment just before the original H1 segment to increase the hydrophobicity (proOmpA2xH1). As shown in Fig. 2E, the hydrophobic segment 2xH1 showed substantial stop-translocation activity. The size of proteinase K resistant fragment, 26 kDa, was the same as that of the proOmpA containing loops of 43 amino acid residues (L43), implying that the translocation of proOmpA2xH1 was interrupted at the H1 region. These results suggest that the hydrophobicity itself is important for the translocation stall.

If hydrophobic segments really determine the discontinuous transition of proOmpA through the membrane, it was expected that the relocation of hydrophobic segments would lead to a change in the stepwise translocation pattern. We therefore constructed a series of proOmpAs, in which hydrophobic segment H1 was shifted about 14 residues closer to the N terminus, and then subjected them to in vitro translocation (Fig. 3A). Proteinase K digestion of the translocated mutant proOmpAs with the relocated H1 region (proOmpAH0+1) revealed a change in the stepwise profile (Fig. 3B). In the case of wild-type proOmpA derivatives, a shift of band A to B occurred around a loop size of 29 (Fig. 2A), whereas proOmpA derivatives with the relocated H1 region showed a shift at a loop size of 35. This result strongly supports the idea that hydrophobic segments are directly involved in the discontinuous transition of a polypeptide across membrane vesicles.

**H1 and H2 Segments of ProOmpA Are Responsible for the Formation of Translocation Intermediates**—When the in vitro translocation reaction was performed with a low concentration of ATP (10 μM), translocation intermediates of proOmpA were observed (7, 9). We examined whether or not the proteinase K-resistant fragment that was formed on partial translocation of proOmpA with a disulfide-bridged loop was identical with

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**Fig. 1.** A, hydropathy profile of proOmpA. Hydropathy was calculated according to Kyte and Doolittle with a span of seven residues. Hydropathic stretches H1 and H2 are indicated by bold bars. B, structures of proOmpA derivatives. The amino acid residues of the signal peptide (−21 to −1) and the mature domain (+1 to +325) are indicated with the positions of cysteine residues. The amino acid residues replaced by a cysteine residue are shown in parentheses. The name of each proOmpA derivatives, loop size, and hydrophobic stretches H1 and H2 are also shown. WT, wild-type. C, amino acid sequences of the H1 and H2 regions. Each segment was replaced by an artificial amino acid sequence, which is shown with the amino acid residue positions. The hydropathy patterns of these mutated proOmpA derivatives are also shown. D, structures of mutated proOmpA derivatives. The name of each proOmpA with removed hydrophobic segment(s) is indicated.
translocation intermediates accumulated with a low concentration of ATP. A low concentration of ATP permitted a slow translocation reaction and thereby resulted in protease-resistant intermediates, termed I29 and I16 (Fig. 4, lane 1). Translocation intermediate I29 showed the same mobility as band B on SDS-polyacrylamide gel electrophoresis, indicating that translocation was interrupted at the same site (Fig. 4, lanes 1 and 2). On the other hand, limited translocation of the proOmpA devoid of the H1 and H2 segments gave several bands, which gave a different pattern from I29 (Fig. 4, lane 3). These results suggest that hydrophobic segments in the mature domain of proOmpA are responsible for the accumulation of translocation intermediates with a low concentration of ATP.

Minimum Components Required for Stepwise Translocation—Reconstitution studies revealed that SecA, SecE, and SecY are the minimum components mediating preprotein translocation (13, 14). To determine whether or not these components are enough for the stepwise movement of proOmpA derivatives, an in vitro preprotein translocation reaction with reconstituted proteoliposomes comprising purified SecA, SecE, and SecY was carried out with a series of looped proOmpAs. As shown in Fig. 5, proOmpAs with disulfide-bridged loops exhibited a discontinuous mode of translocation, which was similar to that observed with everted membrane vesicles. This implies that the stepwise movement of proOmpA derivatives is achieved only through SecA, SecE, SecY, and phospholipids.

Cross-linking with the H2 Region and Translocase—Using a photoactivable and reducible cross-linker, APDP, Joly and Wickner (26) showed that OmpA in a translocation intermediate is cross-linked to SecA and SecY, suggesting that translocation occurs through a proteinaceous channel. To determine whether or not the H2 segment interacts with SecA and SecY during translocation, we introduced a cysteine residue (Cys-260) in the H2 segment and replaced Cys-302 with a glutamine residue and then APDP was attached to the cysteine residue of the radiolabeled proOmpA. The translocation reaction was conducted with a low concentration of ATP (10 μM) to produce translocation intermediates (Fig. 6A, lane 2). After membrane vesicles had been isolated by centrifugation to remove free 35S-ProOmpA derivatives, they were irradiated with UV light. This photolysis allowed a reactive group attached to Cys-260 to form a covalent bond with its nearest neighbor. To determine whether or not SecA and SecY are cross-linked, immunopre-
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The results of our previous study involving proOmpAs with disulfide-bridged loops of different sizes at different positions suggested that the in vitro translocation of proOmpA through the secretory machinery takes place in every 30 amino acid residues (17). How can such exact synchronization of the stepwise movement be achieved from the N terminus to the C terminus of proOmpA? Although the secretory machinery including SecA, which repeats the insertion-deinsertion cycle during translocation (18, 29), may be responsible for the stepwise movement of preproteins, preproteins themselves may also have elements that control such movement. In the present work, we carried out systematic analysis of the stepwise movement of proOmpAs with different distributions of hydrophobic segments in the mature domain of proOmpA. Our results strongly indicate that hydrophobic segments are determinants for the stepwise movement of proOmpA. We also demonstrated that the formation of translocation intermediates with a low concentration of ATP (7, 9) is due to the hydrophobic segments in the mature domain of preproteins. Hydrophobic portions in the mature domain may transiently be arrested in the Sec machinery in the process of translocation.

The observation that proOmpA translocation takes place in about every 30 amino acid residue segments (17) can be explained by the fact that the distance between the H1 and H2 segments is about 30 residues. We assume that the loop and hydrophobic segments cooperatively act to arrest the transit of a polypeptide chain across the membrane. When the loop size is small, as in the cases of L16 and L21, the H1 segment passes through the membrane, and the H2 segment acts as an arrest signal (Fig. 1A). The H2 segment may be a stronger arrest signal than the H1 segment because the arrest of proOmpA transit occurred at the H2 segment, but not at the H1 segment, when the concentration of ATP was low (Fig. 4). When the loop size is larger, the H1 segment arrests the transit of a polypeptide, probably due to steric hindrance or another reason. It should be noted that the distance between the two hydrophobic segments is about 30 amino acid residues. This coincides with the difference in size between bands A and B. When the H1 segment is replaced with a hydrophilic sequence, the H2 segment arrests the transit irrespective of the loop size. When the H2 segment is replaced, translocation continuously occurs after the H1 segment has passed through the membrane (Fig. 2C). It was expected that the replacement of both segments results in continuous translocation. However, as shown in Fig. 2D, L35

The indicated 35S-proOmpA derivatives were preincubated with ATP and proOmpA containing a disulfide-bridged loop to form membrane-inserted SecA. Proteolysis of the membrane-inserted SecA gave a 30-kDa fragment, as observed for the membrane-inserted SecA, which had been formed with wild-type proOmpA (Fig. 7). Removal of the H1 and H2 segments from the looped proOmpA did not alter the efficiency of membrane insertion of 125I-SecA, suggesting that the hydrophobic segments play no role in the insertion of SecA into the membrane.

**Discussion**

We next examined whether or not the presence of hydrophobic segments in the mature domain of proOmpA affect the insertion of SecA into the membrane. 125I-SecA was incubated with ATP and proOmpA containing a disulfide-bridged loop to form membrane-inserted SecA. Proteolysis of the membrane-inserted SecA gave a 30-kDa fragment, as observed for the membrane-inserted SecA, which had been formed with wild-type proOmpA (Fig. 7). Removal of the H1 and H2 segments from the looped proOmpA did not alter the efficiency of membrane insertion of 125I-SecA, suggesting that the hydrophobic segments play no role in the insertion of SecA into the membrane.

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A limited translocation and nearest neighbor analysis. Translocation intermediates were prepared under the conditions given in the legend to Fig. 4 with APDP-conjugated proOmpAL43C302Q prepared as described under “Experimental Procedures.” APDP-proOmpAL43C302Q in urea was diluted in the reaction mixture (25 μl) described in the legend to Fig. 2, without ATP, followed by incubation at 37 °C for 2.5 min. After the 10-min incubation with 10 μM ATP (lanes 2, 4, and 6) or with 5 mM AMP-PNP (lanes 3 and 5) at 37 °C, membrane vesicles were isolated by centrifugation and then resuspended in buffer comprising 50 mM potassium phosphate (pH 7.5), 5 mM MgSO4, and 50 mM KCl, 5 mM ATP, 5 mM phosphocreatine and creatine kinase (10 μg/ml), as described under “Experimental Procedures.” The samples were incubated at 37 °C for 15 min and then digested with proteinase K (5 mg/ml) for 15 min on ice. After trichloroacetic acid precipitation, the samples were analyzed by SDS-PAGE and fluorography. The arrowheads indicate the protease-inaccessible 30-kDa bands. in the mature domain.

Hydrophobic segments, which cause the stepwise translocation, consist of four hydrophobic amino acid residues. Such sequences seem to be appropriate for transfer through the membrane, i.e. the hydrophobicities of such hydrophobic segments are below the threshold of the hydrophobicity required for the stop of transfer. It is possible that proOmpA translocation pauses not only at H1 and H2, but also at other short hydrophobic segments in the mature domain.

Similar discontinuous translocation may occur in other outer membrane proteins. Outer membrane proteins generally form cross-β-structures (30, 31), and their most hydrophobic regions are significantly less hydrophobic than the membrane-spanning sequences of inner membrane proteins (19). The lack of long hydrophobic regions in outer membrane proteins is quite reasonable, because such regions hinder the transfer of preproteins through the Sec machinery. On the other hand, inner membrane proteins that span the bilayer one time have hydrophobic regions of 20–25 amino acid residues. The lengths of these regions in the α-helical conformation are long enough to span the membrane (32).

A polypeptide crosses the membrane through a tunnel or pore comprising SecY, SecE, and SecG (26). On the other hand, the peripheral cytoplasmic factor, SecA, plays roles in both initiation and the elongation step of the entire process of the polypeptide transit (7, 9). In the process of translocation, a SecA arm accompanies the polypeptide chain into the membrane-embedded translocase, so that a portion of SecA is transiently located in the membrane, as revealed by the occurrence of a 30-kDa SecA fragment that is resistant to proteolytic cleavage (18). We showed here that the hydrophobic segments in the mature domain of proOmpA derivatives had no effect on the formation of the 30-kDa proteolytic fragment of SecA. This may suggest that SecA does not participate in the specific recognition of hydrophobic segments and that other proteins such as SecY may play this role. Further study is required to clarify this possibility.

In conclusion, our present observations clearly showed that short segments comprising four hydrophobic amino acid residues are determinants for the stepwise translocation. Since such segments are ubiquitous in secretory proteins (19), stepwise movement may be common for protein translocation across the E. coli cytoplasmic membrane.
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