**In Vitro Analysis of the Stop-transfer Process During Translocation across the Cytoplasmic Membrane of *Escherichia coli***

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Ken Sato‡§¶,** Hiroyuki Mori§¶,** Masasuke Yoshida§¶,** Mitsuo Tagaya‡, 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.

In this study, using a derivative of proOmpA containing an artificial stop-transfer sequence (proOmpA2xH1), we analyzed the process of stop-transfer during translocation across the cytoplasmic membrane of *Escherichia coli*. ProOmpA2xH1 did not interfere with the transit of wild-type proOmpA. When proOmpA2xH1 was anchored in the membrane, membrane-inserted SecA was deinserted with the reversion of the inverted topology of SecG. Cross-linking experiments revealed that the anchored proOmpA2xH1 that does not interact with either SecY or SecA. These results, taken together, suggest that proOmpA2xH1 leaves the translocation pathway by means of a specific interaction between the stop-transfer sequence and the translocational channel.

In *Escherichia coli*, the translocation of proteins across the cytoplasmic membrane is mediated through protein-protein interactions involving a set of Sec proteins (1–4). Secretory proteins are synthesized with an NH2-terminal signal sequence that targets them for export. The amino-terminal positive charges and central hydrophobic region of the signal sequence are important for the interaction of preproteins with SecA (5), a translocation ATPase (6). Upon the binding of ATP and a preprotein, SecA changes its conformation (7), and a portion of SecA is inserted deep into the cytoplasmic membrane (8, 9), which can be detected as the appearance of a protease-resistant ~30-kDa fragment (8). The hydrolysis of ATP results in deinsertion of the SecA (10). Coupled with this insertion-deinsertion cycle, the membrane topology of SecG becomes inverted (11). SecG, SecE, and SecY are components of the membrane-embedded translocase (3) that provides a proteinaceous channel for the transit of preproteins (12).

The transit of preproteins across the membrane is stopped by particular amino acid sequences termed “stop-transfer sequence” (13). Although the hydrophobicity of the stop-transfer sequence is important for its function, the details of the mechanism of the stop-transfer process remains unclear. One possibility is that the stop-transfer sequence specifically interacts with the translocase via its hydrophobic region, and induces a conformational change of the translocase (14). Another possibility is that the stop-transfer sequence simply leaves the translocase as a consequence of the interaction with the hydrophobic core of the lipid bilayer (15).

Previously, we showed that the short hydrophobic segments in the mature region of proOmpA interact with the membrane translocase during polypeptide transit across the cytoplasmic membrane of *E. coli* (16), which causes a discontinuous mode of polypeptide translocation (17). These segments are less hydrophobic than the threshold required for the cessation of polypeptide transit. However, when such a short hydrophobic segment was duplicated, the resultant segment showed substantial stop-translocation activity (16). In this study, using a derivative of proOmpA containing the stop-transfer segment, we analyzed the process of stop-transfer. Based on the results obtained, we propose that the hydrophobic stop-transfer segment leaves the translocation pathway by means of a specific interaction between the hydrophobic segment and the translocational channel, which triggers the stop-transfer.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains*—*E. coli* strains JM109 (recA1, endA1, gyrA96, thi-1, hsdR17, relA1, supE44, lac-proAB, (F, truD36, proAB, lacIq, lacYq, lacZΔm15) (18); CJ236 (lacIq, ung1, thi-1, relA1, pCH105 (F cam*)) (19); K003 (Lpp", *puncB*-C-Tn10) (20); MM66 (F, orad139, dargF-lacU169, rpsL150, relA1, fliB5301, deoC1, ptsF25, geneXwm, supF") (21); K1297 (chtid-33::Tn10, secY24, orad139, rpsE, dargF-lacU169, rpsL150, relA1, fliB5301, deoC1, ptsF25, rbsR1/F (lacP lacPLS, lacZ", lacY", lacA") (22); pST30 (cat, plac-syd) (23); and RK4788 (F, dargF-lacU169, orad139, recA1, rpsL150, β5-301, deoC1, thi, secY219, non, metE70, AbutB, ompA, zcb-Tn10) (24) were used.

*Materials*—Na231 (100 mM cMCl) was purchased from ICN. IODOGEN was from Pierce. ATP, AMP-PNP,1 creatine kinase, and creatine phosphate were obtained from Boehringer Mannheim. Proteinase K was purchased from Merck. Restriction enzymes were from Takara Shuzo Co. Sephadex G-50 (medium) and protein A-Sepharose CL-4B was purchased from Pharmacia Biotech Inc. N9(9-NTA)-agarose was from QIA GEN. A polyclonal anti-SecG antibody was kindly provided by Drs. K.-i. Nishiyama and H. Tokuda. A horseradish peroxidase-conjugated anti-rabbit antibody was obtained from Bio-Rad.

Everted membrane vesicles for *in vitro* translocation were isolated from *E. coli* K003 (Lpp", *puncB*-C-Tn10) as described previously (25). SecC was purified as described previously (5). The wild-type and mutant proOmpAs were purified as described by Crooke et al. (26) from *E. coli* RK4788 harboring the plasmids encoding them, and the disulfide bridge of proOmpA43 was formed according to the method of Uchida et al. (17). SecB was purified as described by Weiss et al. (27). All proOmpA derivatives used for the *in vitro* translocation reactions were synthesized in *vitro* in the presence of EXPRE350585 protein labeling mix (NEN Life Science Products), and partially purified as described previously (17).

Construction of ProOmpA Derivatives—Plasmids pOA43, pTDL43, and pTDL43C302 were prepared as described previously (16). To add a His6 tag to the carboxyl terminus of proOmpA2xH1, oligonucleotide-
directed mutagenesis was performed according to the method of Kunkel (28). For the detection of mutation, an A/II site was also deleted. Plasmid pTD-2xH1 (16) was transformed into E. coli CJ236, and then the uracil-containing single-stranded phagemid DNA was isolated. Using uracil-containing trichloroacetic acid, the mutagenic oligonucleotide complementary DNA strand was synthesized, and the resulting double-stranded DNA was transformed into JM109. Plasmid pTD-2xH1-His6 was isolated on the basis of the A/II site. Plasmids pKS-ompA and pKS-2xH1 were prepared by the insertion of a 1.3-kilobase pair EcoRI-HindIII fragment of pOA13 and pTD-2xH1, respectively, into the corresponding site of pSTV28.

Cell Fractionation—Cells of RK4788 harboring pKS-ompA or pKS-2xH1 were grown in LB medium, and at A600 = 0.6 the cells were harvested. Half of the cells were precipitated with trichloroacetic acid to give a "whole cell" sample. The other half were pelleted, and then inner membrane vesicles were isolated according to the method of Yamada et al. (25).

Purification of His6-tagged ProOmpA2xH1—Kl207 cells harboring pST30 and pTD-2xH1-His6 were cultured until the mid-log phase in LB medium supplemented with 50 μg/ml ampicillin, 10 μg/ml chloramphenicol, and 0.4% glucose. Isopropyl-1-thio-

RESULTS

Membrane Insertion of 125I-Labeled SecA—Purified SecA was iodinated according to the method of Economou and Wickner (8) with a minor modification, as described below. Na125I and SecA were incubated on ice for 20 min in a tube that had been coated with IODOGEN. The reaction mixture was transferred to another tube containing 2 mM diethythreitol to stop iiodination, and then applied to a Sephadex G-50 column to remove Na125I. To prepare 125I-SeqA-bound membrane vesicles, 4 nm 125I-SeqA and 200 μg/ml urea-washed membrane vesicles were incubated for 15 min on ice in 100 μl of a reaction buffer (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. 125I-SeqA-bound membrane vesicles, 50 μg/ml SecB, 5 mM phosphocreatine, creatine kinase (10 μg/ml), and 20 μg/ml proOmpA43 or proOmpA2xH1 were preincubated in the reaction buffer for 2 min at 37 °C (800 μl each). One hundred μl was removed as a control at time 0. The reaction mixture of SecA was incubated with ATP, 100-μl aliquots were removed as indicated. The aliquots were digested with proteinase K (final, 0.1 mg/ml) for 20 min on ice. The proteins were precipitated with trichloroacetic acid, and then analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography and quantification.

Inversion of the Membrane Topology of SecG—Membrane topology analysis of SecG was performed according to the method of Nishiyama et al. (11) with minor modifications. The translocation mixture (total, 120 μl) comprised 0.2 mg/ml membrane vesicles, 60 μg/ml SecA, 50 μg/ml SecB, 25 μg/ml proOmpA derivative, 1 mM ATP, 1 mM MgSO4, 10 mM creatine phosphate, and 100 μg/ml creatine kinase in 50 mM potassium phosphate, pH 7.5. Translocation mixtures containing proOmpA43 or proOmpA2xH1 were incubated in 20 mM NaHCO3 buffer at 37 °C for the indicated times, and then 10 mM AMP-PNP and 10 mM MgSO4 were added. After 5 min of incubation at 37 °C, the reaction mixtures were chilled on ice for 2 min, and then aliquots (20 μl) were treated with 20 μl of proteinase K at the indicated concentrations on ice for 30 min. The samples were precipitated with trichloroacetic acid, washed with acetone, and then analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography.

In Vivo Translocation of ProOmpA Derivatives—The translated 35S-labeled proOmpA derivatives were subjected to in vivo translocation essentially according to the method of Uchida et al. (17). The translocation mixture (25 μl) comprised a 35S-labeled proOmpA derivative, membrane vesicles (5 μg of protein), 2 mM ATP (or 10 μM ATP) or 10 mM AMP-PNP, 5 mM MgSO4, 50 mM potassium phosphate (pH 7.5), 15 μg/ml SecB, and 40 μg/ml SecA. A cold proOmpA derivative was added to a final concentration of 5 μg/ml, if necessary. The ATP concentration was maintained through regeneration with 10 mM creatine phosphate and 10 μM/ml creatine kinase. After a 10-min incubation at 37 °C, the mixture was treated with proteinase K (200 μg/ml) for 20 min at 0 °C, subsequently solubilized with trichloroacetic acid, and then analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography.
RK4788 cells (ΔompA) harboring a low copy plasmid encoding proOmpA2xH1 or wild-type proOmpA without the induction of the proteins. As shown in Fig. 1, most proOmpA2xH1 was partitioned in the inner membrane fraction (84%) (lane 6), while little, if any, wild-type proOmpA was associated with the inner membrane (lane 4). These results suggest that 2xH1 acts as a membrane anchor in vivo as well as in vitro.

Stop-transfer Process Induces the Deinsertion of SecA—To investigate the process of membrane anchoring in more detail, we examined how the presence of a stop-transfer sequence affects the insertion-deinsertion cycle of SecA. SecA changes its conformation in the presence of ATP and a preprotein (7). This conformational change causes SecA to become inserted deep into the membrane, and the inserted portion of a 30-kDa fragment is resistant to protease treatment. Upon ATP hydrolysis, SecA changes its conformation again and thereby becomes deinserted from the membrane (8). As shown in Fig. 3, in the presence of looped proOmpAL43, whose translocation is interrupted by the intramolecular disulfide-bridged loop comprising 43 amino acid residues (17), the 30-kDa fragment was formed in an ATP-dependent manner, and its amount increased with time, reaching steady state after 15 min. This time course is similar to that of wild-type proOmpA (10). When the membrane insertion was conducted in the presence of proOmpA2xH1, accumulation of the membrane-inserted form of SecA was completed within 5–6 min, and subsequently deinsertion was observed. SecA-proOmpA2xH1 interaction is maintained through 20 min, as revealed by the sensitivity of SecA to protease V8 (7), indicating that the result is not for the aggregation of proOmpA2xH1 (data not shown).

ATP Hydrolysis by SecA during Translocation—The ATPase activity of SecA is remarkably enhanced by the membrane translocase, acidic lipids (30), and preproteins (31). This enhanced ATPase activity is referred to as translocation ATPase activity. We next examined the effect of proOmpA2xH1 on the ATPase activity of SecA. ProOmpAL43 enhanced the ATPase activity of SecA to a similar extent to that observed for wild-type proOmpA (Fig. 4). This may imply that enhanced ATP hydrolysis continues even when polypeptide transition is blocked by an intramolecular disulfide-bridged loop. Similar results were observed when translocation was blocked by a derivative of proOmpA carrying a side chain of 20 amino acid residues via a cysteine residue (32). Although proOmpA2xH1 also enhanced the ATPase activity of SecA, the enhancement lasted for only approximately 100 s. The lack of enhancement after 100 s is not due to the inactivation of SecA because the addition of wild-type proOmpA caused reactivation of ATP hydrolysis.

Topology Inversion of SecG—We next examined how the presence of a stop-transfer sequence influences the topology inversion of SecG (11). SecG possesses two transmembrane derivatives.
regions, and its amino- and carboxyl-terminal regions are located in the periplasm. In everted membrane vesicles, proteinase K cleaves sites close to the NH₂ terminus of 11.4-kDa SecG, producing a 9-kDa fragment. This fragment is immunodetected by anti-SecG, which recognizes the carboxyl-terminal 16 amino acid residues of SecG, because the COOH-terminal region is located inside the vesicles. In the presence of ATP and proOmpA, on the other hand, no fragment is detected by the antibody because the COOH-terminal region is cleavable by proteinase K as a consequence of the inversion of the membrane topology of SecG (11). Consistent with the previous results (11), the 9-kDa protease-resistant fragment was formed when SecA was incubated with everted membrane vesicles in the absence of a preprotein (Fig. 5A). When translocation was conducted in the presence proOmpAL43 and blocked by the addition of AMP-PNP at 3 min, the 9-kDa fragment was not formed (Fig. 5B). Although the previous study showed that SecG is almost completely cleaved by proteinase K under the translocation conditions (11), we found that only a small amount of SecG was cleaved in the presence of proOmpAL43. A similar result was obtained when wild-type proOmpA was used (data not shown). Perhaps the COOH-terminal region of SecG is present on the outside of membrane vesicles, but is not completely exposed and therefore is inaccessible to proteinase K. However, it is obvious that SecG changes its conformation in both preprotein- and ATP-dependent manners, as revealed by the changes in the proteolytic patterns. This conformational change was maintained for at least 15 min. When translocation was conducted in the presence of proOmpA2xH1 and terminated at 3 min, the proteolytic pattern was essentially the same as that observed in the presence of proOmpAL43 (Fig. 5C). In contrast, when translocation was stopped at 15 min, the proteolytic pattern was remarkably different from that observed in the absence of proOmpAL43, and rather similar to that observed in the absence of the preprotein. These results, taken together, suggest that proOmpA2xH1 containing a stop-transfer sequence is translocated through the normal translocation pathway at an early stage, but leaves the pathway at a later stage of translocation.

**Nearest Neighbor of the Anchored 2xH1 Segment**—To directly demonstrate that proOmpA2xH1 does not interact with the translocation machinery at a later stage of translocation, we performed a cross-linking experiment using a photoactivatable and reducible cross-linker, APDP. We previously used this reagent and demonstrated that a translocation intermediate of a looped proOmpA interacts with SecY and partly with SecA (16). We replaced a serine residue (Ser-233) in the middle of the 2xH1 segment with a cysteine residue (proOmpA2xH1C) (Fig. 6A), and then APDP was attached to this cysteine residue. After photolysis, SecA and SecY were immunoprecipitated with anti-SecA and anti-SecY, respectively, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis in the presence of dithiothreitol to cleave the cross-linked proteins. As shown in Fig. 6B, a significant amount of cross-linked 35S-proOmpA2xH1 was not immunoprecipitated by either anti-SecA (lane 4) or anti-SecY (lane 3). This is not due to the inability to photo-cross-link the derivative of proOmpA2xH1 with a Cys residue in the 2xH1 region with APDP to the Sec machinery. When the deinsertion of SecA was blocked by the addition of AMP-PNP, as described in Fig. 7B, prior to photolysis, a significant amount of cross-linked 35S-proOmpA2xH1 was immunoprecipitated by either anti-SecA (lane 2) or anti-SecY (lane 3). These results suggest that the 2xH1 segment transiently interacts with the Sec machinery. Once anchored, the 2xH1 region could not interact with the Sec machinery.

**Alkali Extraction of the Membrane-anchored ProOmpA2xH1**—To assess the state of proOmpA2xH1 anchored in the membrane, membrane proteins were extracted with 0.2 M Na₂CO₃ (pH 11) after completion of the translocation reaction. This alkali treatment extracts all peripheral membrane proteins and uninserted polytopic membrane proteins in mammalian cells (33, 34) and in bacterial cells (22, 35). As shown in Fig. 7A, 60% of the anchored proOmpA2xH1 was partitioned into the alkali soluble fraction, whereas 17% of the translocation intermediate of the looped proOmpA was observed in the same fraction (Fig. 7A). When AMP-PNP was added to inhibit the deinsertion of SecA from the membrane prior to alkali...
In this study we examined the stop-transfer process of a proOmpA derivative containing a stop-transfer sequence (proOmpA2xH1). The use of an in vitro translocation system enabled us to analyze the translocation process at defined stages of polypeptide transfer. The major conclusion of this study is that when the transfer of a polypeptide is halted by a stop-transfer sequence, the preprotein leaves the functional translocation site, which allows the passage of the following preprotein molecule. Cross-linking analysis confirmed that the membrane-anchored hydrophobic segment is located outside the translocation channel. Our data also showed that the cessation of translocation causes deinsertion of SecA and the return the inverted topology of SecG to the original one. Alkali extraction experiments revealed that the state of proOmpA2xH1 anchored in the membrane is different from that of the translocation intermediate formed as a consequence of the presence of an intramolecular loop. When deinsertion of SecA was interrupted by AMP-PNP, proOmpA2xH1, as well as the looped proOmpA, was not extracted on alkali treatment.

It has been proposed that secretory proteins are translocated across the cytoplasmic membrane through a proteinaceous channel (12). When a segment with moderate hydrophobicity enters the translocation channel, the passage of the polypeptide is interrupted and the preprotein is excluded from the channel. There are two possible explanations for this mechanism. First, translocation arrest may be triggered by an interaction between the hydrophobic stop-transfer segment and the hydrocarbon of the phospholipid, and then the hydrophobic segment spontaneously leaves the translocase. Second, the translocation apparatus itself may recognize the hydrophobic segment, and decide to continue its transfer or to integrate it into the membrane. In the former case, the stop-transfer process is dependent on the affinity of the hydrophobic segment with the hydrophobic core of the lipid bilayer. If this is the case, it is reasonable to assume that the anchored segment would be spontaneously integrated into the lipid bilayer regardless of whether SecA is deinserted or not. However, alkali extraction experiments in the presence of AMP-PNP revealed that the anchored segment remains in the translocase unless SecA is deinserted, suggesting that the polypeptide is not spontaneously integrated into the membrane. Therefore, the second of the above two alternatives seems to be more likely.

We recently demonstrated that the short hydrophobic segments within the mature region of a preprotein promote translocation stalling. This event seems to be at least partly due to an interaction between the hydrophobic stretch and the translocase (16). Here, we propose that the hydrophobic segment itself functions as a kind of “integration signal” for Sec-dependent insertion of membrane proteins, such as type I membrane protein (having only a single transmembrane segment and its carboxyl terminus located on the cytoplasmic side of the membrane). The entrance of a stop-transfer sequence of a preprotein into the translocation channel may trigger the release of SecA from the translocase and thereby allows the preprotein to leave the active translocation site. This indicates that the discrimination between secretory proteins and membrane proteins is conducted by the translocase that can estimate the hydrophobicity of the polypeptide chain of preproteins. The results described in this study are consistent with this proposal.

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