Translocation of Conjugated Presecretory Proteins Possessing an Internal Non-peptide Domain into Everted Membrane Vesicles in Escherichia coli*

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Polypeptides comprising 20 amino acid residues (Y2) were covalently bound to the carboxyl terminus of a truncated proOmpA (proOmpA-D72C) through N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (X). The length of the inverted linker domain was 2.8 nm. proOmpA-D72C-X-Y2 thus synthesized was subjected to in vitro translocation into everted membrane vesicles of Escherichia coli. The conjugated protein was translocation-competent in terms of both polypeptide bonds, when a proton motive force was imposed. The translocation was ATP-dependent. The polypeptide kinase treatment resulted in the digestion of SecA, SecE, and SecY in the membrane, suggesting that the polypeptide kinase resistance of the Y2 domain was not due to its interaction with these Sec proteins in the secretory machinery. In the absence of Δμm, the translocation ceased at the linker domain. Upon the imposition of Δμm, the linker-Y2 domain underwent translocation, which did not require ATP hydrolysis as in the case of the translocation of the latter portion of usual secretory proteins. The translocation was prevented by anti-Y2 IgG even when Δμm was imposed. Another conjugated protein, which possesses a polypeptide comprising 61 amino acid residues after the linker (proOmpA-D72C-X-Lpp'), was synthesized. This compound was also translocated into everted membrane vesicles with cleavage of the signal peptide. These results suggest that substances to be translocated through the secretory machinery need not necessarily be solely held together by polypeptide bonds.

The translocation of secretory proteins across the prokaryotic cytoplasmic membrane or the eukaryotic endoplasmic reticulum membrane is mediated by a specific translocation machinery. In Escherichia coli, which is prokaryotic, the machinery is composed of a group of Sec proteins including SecA (1-3), SecE, SecY (4-6), and probably SecD (7, 8). Although genetic evidence supports the participation of SecF in the secretion process (7), no biochemical evidence has been available as to this matter. Since the machinery is believed to be for proteins and since proteins are generally translocated as unfolded linear polypeptide chains (9), it was thought highly likely that the machinery recognizes peptide bonds along polypeptide chains.

A series of recent findings suggests, however, that the machinery may permit the translocation of a molecule possessing non-peptide bonds when the molecule is equipped with the signal peptide. proOmpA, the precursor of an outer membrane protein of E. coli, has Cys residues at +290 and +302 of its mature domain. proOmpA with a disulfide bridge between them was translocated in vitro without cleavage of the bridge when the cytoplasmic membrane was fully energized (10). When the 2 Cys residues were irreversibly cross-linked with a chemical reagent, translocation also took place (11). Although the translocation of such artificial substrates may take place along the looped polypeptide chain, it is alternatively possible that the machinery translocates the substrates across the non-peptide linker domain.

To examine the latter possibility, in the present work, we chemically cross-linked a signal peptide-possessing truncated proOmpA to a polypeptide chain via N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (MPHP). This artificial substrate was translocation-competent in vitro in terms of both polypeptide K resistance and signal peptide cleavage. These results suggest that substances to be translocated through the secretory machinery need not necessarily be solely held together by peptide bonds.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli K003 (Lpp'-uncB-C-Tn10), K002 (Lpp'-uncB-C'-Tn10), and JM103/ppp' (lacF') were used (12). Plasmid pSI053 (13) carries the ompA gene that encodes proOmpA. Plasmid pK125 (14) carries the ompF-lpp gene that encodes proOmpF-Lpp. Plasmid pTTQ18 (15), an expression vector carrying the tac promoter-lac operator, was purchased from Amerham International.

Materials—A polypeptide corresponding to a hydrophilic region of SecY (Ala146-Arg180), with Cys at the amino terminus, was used (16) (Fig. 1B). Hereafter, it will be called peptide Y2. Leader peptidease was prepared as described (17). Polyonal anti-peptide Y2 antibody was prepared as described (16).

Preparation of Everted Membrane Vesicles—Everted membrane vesicles were prepared from E. coli K003 (3586-3593, 1993). The amount of membrane vesicles was expressed as that of protein, which was determined by the method of Lowry et al. (18).

Construction of a Plasmid Possessing a Gene Encoding a Truncated proOmpA Derivative with an Additional Cysteine Residue at Its Carboxyl Terminus—A polynucleotide linker, d(5'-GATCCG-TGCTAAATCTAGATTAGCAGC-3'), synthesized with an Applied Biosystems DNA synthesizer 380B, was self-annealed. Plasmid pSI053 carrying the SP6 promoter-controlled ompA gene was used as the

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The abbreviations used are: MPHP, N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine; AMP-PNP, adenosine 5'- (β,γ-imino)triphosphate.
starting plasmid (13). Plasmid pS1053 possesses a unique BamHI site in the region corresponding to the carboxy-terminal portion of OmpA. After digestion of pS1053 with BamHI, the resultant fragment was isolated and ligated with the polynucleotide linker described above to construct pOAD 72C. This plasmid carries a gene that encodes proOmpA-D72C, a truncated proOmpA (Met1-Pro70) with an extra Cys residue at its C-terminal end. Lpp' preparation was about 50%. The deduced chemical structure of Lpp' was grown on L broth (100 ml) at 37 °C. When reached 1.0, the signal peptide of proOmpF-Lpp, was digested with EcoRI and HindIII fragments, between the Cys residues, with MPHP should, therefore, produce a conjugated compound, the chemical structure of which is shown in Fig. 1A. Afterward this conjugated compound will be called proOmpA-D72C-X-Y2. The distance between the ϕ-carbons of the two Cys residues is 2.8 nm (Fig. 1C), which corresponds to the length of 7.8 polypeptide units.

The cross-linking of 35S-labeled proOmpA-D72C synthesized in vitro (Fig. 2A, lane 1) with peptide Y2 via MPHP resulted in a larger compound with a molecular mass of 31 kDa (Fig. 2A, lane 2), which is approximately the same as that of proOmpA-D72-X-Y2. The material, which was purified on a polyacrylamide gel, was immunoprecipitated with both anti-OmpA and anti-Y2 antisera (Fig. 2A, lanes 3–6). We conclude, therefore, that this band material is proOmpA-D72C-X-Y2.

Translocation of proOmpA-D72C-X-Y2 into Everted Membrane Vesicles in the Presence of ATP and Δψ^+—Upon mixing with everted membrane vesicles, proOmpA-D72C-X-Y2 was converted to a smaller form. The difference was about 2 kDa, which is the same as the molecular mass of the signal peptide, and the conversion was accompanied by the acquisition of protease K resistance (Fig. 2B). The results suggest that the entire proOmpA-D72C-X-Y2 molecule is completely translocated across the membrane, in spite of the fact that it possesses an internal non-peptide domain. The translocation required ATP, as in the case of usual protein translocation.

The translocation of presecretory proteins is usually enhanced when Δψ^+ is imposed (12, 22, 23). Furthermore, the imposition of Δψ^+ permits the translocation of some nonlin-

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**Preparation of Phospolipid-Linked proOmpA-D72C**—In vitro transfection of the gene encoding proOmpA-D72C was performed with SP6 RNA polymerase, as described (19). The translation reaction was carried out at 37 °C for 30 min in the presence of Tran32-labeled (1.2 nCi/ml) according to the method described previously (13). After the translation, trichloroacetic acid was added to the mixture to a final concentration of 10%. The resultant precipitate was successively washed with 10% trichloroacetic acid, acetone, and then diethyl ether, dried, and then dissolved in 8 μl urea, 50 mM potassium phosphate (pH 7.5) as to make the radioactivity level about 1.0 × 10^6 dpm/μl.

**Preparation and Purification of Phospolipid-Linked proOmpA-D72C**—Precipitate was washed with acetone and then diethyl ether, dried, and then dissolved in 8 μl urea, 50 mM potassium phosphate (pH 7.5) containing 35S-labeled proOmpA-D72C (5 × 10^6 dpm) was mixed with 50 μl of 1 μM peptide Y2. 375 μl of 2 M urea, 50 mM potassium phosphate (pH 7.5) and then disrupted by sonication for 2 h. 100 μl of 1% SDS, 5% β-mercaptoethanol, 10% glycerol, and then boiled for 5 min. This solution was then applied to an SDS-polyacrylamide gel for electrophoresis. The region containing the cross-linked precursor was cut out from the gel, and elution was carried out as described (20). A 9-fold volume of acetone was then added to the eluate, and the resultant precipitate was washed with 90% acetone three times, dried, and then dissolved in 8 μl urea, 50 mM potassium phosphate (pH 7.5) so as to make the radioactivity level about 1.0 × 10^6 dpm/μl. The total radioactivity recovered was about 1.0 × 10^6 dpm/μl.

**Construction of a Plasmid Carrying a Gene Encoding a Lipoprotein Derivative (Lpp')—Polynucleotide linkers, d(5'AATTCTAG-GAGGTTTAAATTTATGTGCGC-3') and d(5'GCACATAAATT- TAAACCTCCTAG-3'), were synthesized and annealed. Plasmid pK125 (14) was used as the source of the ompF-lpp gene. The EcoRI-HindIII fragment, carrying the ompF-lpp gene, was isolated and placed under the control of the tac promoter-operator of pTTQ18 (15) to construct pTac-OmpF-Lpp. This plasmid, which possesses a unique SacII site at the position corresponding to the cleavage site of the signal peptide of proOmpF-Lpp, was digested with EcoRI and SacII, and the larger fragment was isolated. The fragment was ligated with the synthetic linker described above to construct pTac-Lpp'.
Translocation of Proteins Containing a Non-peptide Domain

**Fig. 1. Structure of proOmpA-D72C-X-Y2 possessing an internal non-polypeptide domain.** A, schematic representation of proOmpA and its derivatives. Cysteine residues are indicated. The closed box denotes the linker domain. The first amino acid residue of the mature domain is numbered +1, and the last amino acid residue of the signal peptide is numbered -1. B, the amino acid sequence of peptide Y2 is shown using one-letter symbols. The sequence derived from SecY is enclosed by an open box. C, the structure of the linker domain. The two terminal cysteine residues cross-linked with the linker, and the distance between the cysteine residues is also shown.

**Fig. 2.** Translocation of proOmpA-D72C-X-Y2 possessing an unusual chemical structure in its polypeptide backbone in the presence of Δμo⁺ in vitro. A, the preparation of proOmpA-D72C-X-Y2. In vitro synthesized ³⁵S-labeled proOmpA-D72C (lane 1) was cross-linked with peptide Y2 via MPHP (lane 2), and then the cross-linked product was purified by means of gel electrophoresis (lane 3). Lanes 4–6, immunoprecipitates of the purified sample with control serum (lane 4), anti-OmpA antiserum (lane 5), and anti-Y2 antiserum (lane 6). All samples were analyzed on a polyacrylamide gel, followed by fluorography. The positions of proOmpA-D72C-X-Y2 and proOmpA-D72C are indicated by closed and open arrowheads, respectively. B, in vitro translocation of purified ³⁵S-labeled proOmpA-D72C-X-Y2 (25 μl) was carried out for 5 min with or without 5 μg of membrane vesicles (MV) prepared from *E. coli* K002 (unc⁻) in the presence or absence of 1 mM ATP, as indicated (note that Δμo⁺ can be formed from ATP through F₁,F₂-ATPase coded for by the unc genes). The samples were then treated with proteinase K (PK) (lanes 2–4). The positions of the precursor (P) and the mature protein (M) are indicated. C, in vitro translocation reactions (25 μl) were carried out with or without 5 μg of membrane vesicles (MV) prepared from *E. coli* K003 (Δunc) in the presence or absence of 1 mM ATP or 5 mM NADH, as indicated. The samples were then treated with proteinase K (lanes 2–4). The positions of the precursor, the mature protein, and bands P1 and I-2 are indicated. The positions of molecular weight standards are also indicated.
ear polypeptide chains, such as ones possessing a peptide loop formed as the result of disulphide bridge formation (10) or intramolecular chemical cross-linking (11). proOmpA-D72C-X-Y2 also required ΔµH+ for its complete translocation (Fig. 2C). In the absence of ΔµH+, partial translocation took place, resulting in the appearance of a protease K-resistant band of a smaller molecular mass, I-2, together with a minor band, I-1. Since the position of I-2 on the gel was essentially the same as that of the formic acid-treated OmpA, which comprises Ala'→Asp228 of OmpA and hence almost the same in size of a smaller molecular mass, 1-2, together with a minor band, X-Y2 also required precipitation with 10% trichloroacetic acid, dissolved in 8 M urea, 50 mM potassium phosphate (pH 7.5). Samples were then subjected to formic acid treatment and 6 were then subjected to formic acid treatment.

Chemical Characterization of the I-1 and I-2 Band Materials—The I-2 band material did not react with the anti-Y2 antibody (Fig. 3), supporting the view that I-2 represents a translocation intermediate which was stuck to the membrane at the linker domain. In contrast to I-2, the I-1 band material seemingly reacted with the anti-Y2 antibody, suggesting that I-1 may represent an intermediate, of which the MPHP domain stuck to a far internal locus of the translocation machinery in the membrane, and this made a portion of the Y2 domain protease K-resistant. Since the I-1 band was faint, further experiments are required.

We then determined whether the incompletely translocated OmpA molecule detected as bands I-2 and I-1 possesses the signal peptide or not. The translocation of 35S-labeled proOmpA-D72C-X-Y2 was performed in the absence of ΔµH+, followed by digestion with protease K. The samples were precipitated with 10% trichloroacetic acid, dissolved in 8 M urea, and then digested with 24 volumes of 50 mM potassium phosphate (pH 7.5). Samples were then treated with leader peptidase in the presence of Triton X-100 and then analyzed on an SDS gel. Leader peptidase treatment did not result in a shift of band I-2 (Fig. 4A, lanes 3 and 4). Band I-2 in lane 4 of Fig. 4A was considerably thinner than that in lane 3. This may be, at least partly, due to the broadening of the band in lane 4. Densitometric determination revealed that band I-2 in lane 4 accounted for about 50% of that in lane 3 in terms of the density on fluorogram. Furthermore, no processed band was observed right beneath band 1-2 in lane 4. In contrast to the case of band I-2, the processing of proOmpA-D72C-X-Y2 to its mature form was clearly observed under the same conditions (Fig. 4A, lanes 1 and 2). These results indicate that the band I-2 material did not possess the signal peptide. Although the data were less clear, it is likely that the band I-1 material possesses no signal peptide either.

The absence of the signal peptide was also supported, although not proved, by the results of another experiment (Fig. 4B). OmpA contains the Asp228-Pro230 sequence, which can be specifically cleaved off in the presence of formic acid (10). Upon formic acid treatment, proOmpA-D72C-X-Y2 (lanes 1 and 2) and mature OmpA-D72C-X-Y2 (lanes 3 and 6) were converted to polypeptides with molecular masses of about 28 and 26 kDa, respectively. These values were consistent with those expected from their molecular masses. After formic acid treatment, the sample containing the band I-1 and I-2 materials gave a band at the position corresponding to 26 kDa (lanes 3 and 4). This supports the view that the band I-1 and I-2 materials do not possess the signal peptide. It does not prove it, however, since the band position after formic acid treatment was almost the same as that of I-2, and hence it was unclear whether or not the band I-2 material possessed the Asp228-Pro230 sequence.

The Protease K-resistant nature of the Y2 Domain Was Not Due to Its Interaction with SecA, SecE, or SecY—Both SecA originally existing in the everted membrane vesicles and that externally added to the translocation mixture were quite sensitive to protease K treatment, irrespective of their localization (Fig. 5A). It was previously shown that SecA became more sensitive to a protease, V8, in the presence of presecretory proteins (24), suggesting that SecA interacting with presecretory proteins is also susceptible to protease digestion. It is likely, therefore, that SecA had nothing to do with the protection of the incompletely translocated proOmpA-D72C-X-Y2 against protease K, which in turn suggests that the SecA molecule is not the site where the passage of the linker domain is prevented. SecE and SecY in the everted membrane vesicles were also susceptible to protease K under the assay conditions for translocation (Fig. 5B), excluding the possibility that the protease K-resistant nature of the Y2 domain was due to the protection by SecE or SecY of this domain.
Inhibition of the Translocation of proOmpA-D72C-X-Y2 by Anti-Y2 IgG—Although anti-Y2 IgG specifically interacts with peptide Y2 or the Y2 region of the SecY protein, it does not inhibit in vitro protein translocation (16, 25). We used this IgG to examine the process of translocation of ProOmpA-D72C-X-Y2 (Fig. 6). The translocation of the 35S-labeled substrate was performed in the absence of ΔμHᵢ⁺ (Fig. 6, lane 2), followed by treatment with anti-Y2 IgG. This treatment resulted in the appearance of two new bands (I-3 and I-4) at lower positions on the gel (Fig. 6, lane 3). This indicates that 1) the Y2 domain of proOmpA-D72C-X-Y2, which has been partially translocated in the absence of ΔμHᵢ⁺, was still accessible to the externally added anti-Y2 IgG, and 2) the translocation intermediate may move backward as the result of the interaction of the Y2 domain with anti-Y2 IgG. Such a retrograde movement in the absence of ΔμHᵢ⁺ has been suggested with proOmpA (26).

Upon the imposition of ΔμHᵢ⁺, the anti-Y2 IgG-free substrate underwent complete translocation, whereas the anti-Y2 IgG-treated one did not (Fig. 6, lanes 4 and 5). When the entire translocation experiment was performed in the presence of peptide Y2, no inhibition of translocation by anti-Y2 IgG was observed (data not shown).

The Intermediates Detected as Bands I-1 and I-2 Undergo Complete Translocation Even in the Presence of AMP-PNP When ΔμHᵢ⁺ Is Imposed—AMP-PNP completely inhibits the initiation of translocation but not its later stage (22, 26, 27). We examined whether or not the translocation of the Y2 domain is inhibited by AMP-PNP (Fig. 7). The translocation of proOmpA-D72C-X-Y2 was started in the presence of ATP, but not NADH, to accumulate the translocation intermediates. AMP-PNP was then added to stop further initiation of the translocation reaction, followed by the addition of NADH to impose ΔμHᵢ⁺. The addition of NADH resulted in the shift of the intermediate bands to the mature position, even in the presence of AMP-PNP (Fig. 7, lane 5). Since the initiation of a new translocation reaction did not take place at all in the presence of AMP-PNP (Fig. 7, lane 6), it is strongly indicated that the translocation of the linker-Y2 domain of proOmpA-D72C-X-Y2 can take place, as the translocation of the latter part of the intact proOmpA can do so (26, 27).

proOmpA-D72C-X-Lpp' Possessing a Larger Carboxyl-terminal Peptide after the Linker Can Also Be Translocated—proOmpA-D72C-X-Y2 possesses only 20 amino acid residues after the linker. To make more certain that the region after the linker can indeed be translocated across the membrane, a larger polypeptide chain was placed after the linker molecule. A lipoprotein derivative (Lpp') possessing Met-Cys-Ala-Glu in place of Cys at the amino terminus (Lpp' ) was constructed,
on a DNA level, and cross-linked to $^{35}S$-labeled proOmpA-
D72C via MPHP (proOmpA-D72C-X-Lpp') (Fig. 8, A and B). This artificial substrate possesses 61 amino acid residues
after the linker. Met of this domain exists as a side arm due
to the cross-linking at Cys. The structure of proOmpA-D72C-
X-Lpp' was confirmed by its molecular mass, and its reactivity
with both anti-OmpA and anti-lipoprotein antisera (Fig. 8C). The band materials that appeared above proOmpA-
D72C-X-Lpp' (Fig. 8C, lane 4) was most likely proOmpA-
D72C-X-Lpp' that had been cross-linked with proteins contami-
ning in the Lpp' preparation.

Upon mixing with everted membrane vesicles and ATP,
proOmpA-D72C-X-Lpp', of which the proOmpA region was
radiolabeled, was converted to a smaller form, which was
proteinase K-resistant (Fig. 9, lanes 1–4). The band that
appeared was immunoprecipitated with anti-lipoprotein anti-
serum (Fig. 9, lanes 5 and 6), suggesting that the band
represents OmpA-D72C-X-Lpp'. Taken together, the results
suggest that the entire substrate was translocated. A protein-
ase K-resistant band, that appeared right below proOmpA-
D72C, was most likely the translocation product of the latter,
which was contaminating in the sample. Since the transloca-
tion of proOmpA-D72C-X-Lpp' was not as efficient as that
of proOmpA-D72C-X-Y2, further precise analyses were not

**DISCUSSION**

In the present work, we provided evidence suggesting that
substrates to be translocated through the protein secretory
machinery in the cytoplasmic membrane of *E. coli* need
not necessarily be solely held together by peptide bonds. The
translocation substrates we found to be competent possess an
internal non-polypeptide domain, the length of which is 2.8
nm. The length is about the same as that of an octapeptide.
Furthermore, it accounts for about one-third of the thickness
of the cytoplasmic membrane. The maximum length and
chemical nature of such a non-polypeptide domain acceptable
to the secretory machinery remain to be determined. It should
be noted in this respect that MPHP possesses one peptide
linkage with the same orientation (–CO–NH–) as that in the
protein domain and one with the opposite orientation
(–NH–CO–), whereas other portions are entirely different
from the polypeptide structure.

One may argue that the results observed in the present
work do not represent the complete translocation beyond the
non-peptide region, *i.e.*, the carboxyl-terminal domain after

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**Fig. 8. Structure of proOmpA-
D72C-X-Lpp'.** A, schematic represen-
tation of proOmpA-D72C-X-Lpp'. Cys-
teine residues are indicated. The closed
box denotes the linker region. The first
amino acid residue of the mature domain
of OmpA and that of Lpp' are both num-
bered +1, and the last amino acid residue
of the signal peptide is numbered −1. B,
the amino acid sequence of Lpp' is
shown using one-letter symbols. C, *in*
*vitro* synthesized $^{35}S$-labeled proOmpA-
D72C was cross-linked with Lpp' via
MPHP (lane 1). After cross-linking, the
reaction mixture was immunoprecipita-
ted with control serum (lane 2), anti-
lipoprotein antiserum (lane 3), and anti-
OmpA antiserum (lane 4). All samples
were analyzed on a polyacrylamide gel,
followed by fluorography. The positions
of proOmpA-D72C-X-Lpp' and pro-
OmpA-D72C are indicated.
the linker domain being protected from protease digestion by interaction with the secretory machinery or the lipid bilayer. Although we cannot entirely exclude this possibility, it is unlikely because of the following reasons.

1. The Y2 domain is extremely hydrophilic, 9 out of the 20 residues being charged ones. The Lpp' domain is highly hydrophilic as well. They, therefore, hardly stay in the lipid bilayer. The total length of the Lpp' domain is 21.6 nm. It is possible that the translocation pauses at certain stages or certain points of the substrate. The transient accumulation of an intermediate during the proOmpA translocation in the absence of ΔμH⁺ (27) supports this view. Intermediate I-19 detected in the process of proOmpA translocation may also support this view (26).

Although ΔμH⁺ is not essential for the translocation of some secretory proteins, it considerably enhances the rate of translocation of all secretory proteins so far examined (12, 13, 22, 23, 28). Furthermore, ΔμH⁺ permits the translocation of substrates with anomalous structures, such as ones possessing a polypeptide loop (10, 11) or polypeptide side chains. It is likely that ΔμH⁺ somehow physically or physiologically widens the channel through which substrates are translocated. We further demonstrated in the present work that substrates which possess a non-polypeptide domain in their otherwise peptide chains can also be most likely translocated when ΔμH⁺ is imposed. This suggests that the machinery does not translocate polypeptide chains by recognizing peptide bonds one by one, but does it in a less specific manner through a channel which is widened upon the imposition of ΔμH⁺. On the contrary, the initiation of the translocation reaction is a highly specific process. It requires a signal peptide, which is characterized by amino-terminal positive charges and a central hydrophobic domain (29-32). It is probable that the initial process, which is highly specific, induces ΔμH⁺-dependent widening of a channel, which in turn allows the faster translocation of the following polypeptide chain of the passage of a domain, which can be heterogeneous in size and molecular structure.

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