**In Vitro Kinetic Analysis of the Role of the Positive Charge at the Amino-terminal Region of Signal Peptides in Translocation of Secretory Protein across the Cytoplasmic Membrane in Escherichia coli**

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By using an *in vitro* system for the translocation of secretory proteins in *Escherichia coli*, detailed and quantitative studies were performed as to the function of the positively charged amino acid residues at the amino terminus of the signal peptide. Uncleavable OmpF-Lpp, a model secretory protein carrying an uncleavable signal peptide, and mutant proteins derived from it were used as translocation substrates. When the positive charge was altered by introducing different numbers of Lys or Arg residues into the amino terminus, the rate of translocation was roughly proportional to this number, irrespective of whether the charged amino acid residues were Lys or Arg. When the amino-terminal LysArg was replaced by His residues, translocation took place more efficiently at pH 5.5 than pH 8.0, whereas that of the wild-type was about the same as the two pH values. We conclude that the signal peptide requires a positive charge at its amino-terminal region to function in the translocation reaction and that the rate of translocation is roughly proportional to the number of the positively charged group, irrespective of whether the charged amino acid species that donates the charge. Evidence suggesting that the positive charge is involved in the binding of precursor proteins to the membrane surface to initiate translocation is also presented.

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Secretory proteins generally possess an amino-terminal extension as the signal peptide. Although signal peptides show little primary sequence homology, they share unique features in their overall structures. Typically, signal peptides possess 1 or more positively charged amino acid residues at the amino terminus, which are followed by a stretch of hydrophobic residues (1). All prokaryotic signal peptides so far reported have a positive charge, suggesting its essentiality in protein secretion, whereas some eukaryotic ones, although only a small number, have no positive charge (2, 3) or are even negatively charged (2). For example, rat PC12, a neuron-derived cell line, expresses amino-terminal charge on the rate of translocation of secretory proteins in vivo in prokaryotic *Escherichia coli* cells by means of gene manipulation (4–7): the replacement of the positively charged amino acid residues with negatively charged ones resulted in the accumulation of signal peptide-containing precursor proteins. It should be mentioned, however, that the same studies also showed that alteration of the positive charge to a neutral one did not result in precursor accumulation (4, 5, 7). One experiment with a heterologous system, composed of *E. coli* membrane vesicles and a Staphylococcal secretory protein, exhibited partial suppression of translocation upon neutralization of the positive charge (6). It is unclear, therefore, whether or not the positive charge is directly required for protein translocation across the cytoplasmic membrane. One of the problems with these *in vitro* experimental systems is that detailed kinetic studies on the effect of the amino-terminal charge on the rate of translocation of secretory proteins are difficult. It is also difficult to determine *in vivo* the stage of the secretion process the charge affects.

The development of an *in vitro* system in *E. coli* for the translocation of secretory proteins across the cytoplasmic membrane made biochemical studies possible. The system comprises inverted membrane vesicles and signal peptide-containing precursor proteins. We recently established the conditions for preparing membrane vesicles exhibiting efficient translocation of secretory proteins (8). We also recently showed that a model secretory protein, in which the site of signal peptide cleavage has been converted to an uncleavable one, exhibits much faster and more quantitative translocation into membrane vesicles than the wild-type one with a cleavable signal peptide (9). The enhanced translocation is most likely due to a lack of premature cleavage of the signal peptide before the translocation. Using membrane vesicles thus prepared and secretory proteins including this uncleavable one, the mechanism underlying protein translocation has been studied in detail (10–13).

In the present work, the effect of the positive charge at the amino-terminal region of the signal peptide on the rate of *in vitro* translocation was studied in detail. The number of the positive charge was altered by deleting basic amino acid residues from the amino-terminal region of the signal peptide or by replacing them with different numbers of Lys, Arg, or His. The signal peptide was found to require a positive charge at its amino-terminal region to function in the translocation reaction, and the rate of translocation was roughly proportional to the number of the positively charged group, irrespective of the amino acid species that donates the charge. Evidence suggesting that the positive charge is involved in the binding of precursor proteins to inverted membrane vesicles is also presented.
**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases, DNA-modifying enzymes, and SP6 RNA polymerase were from Takara Shuzo Co. Proteinase K was from Merck, TranS-Label, a mixture of 70% [32P]methionine (1000 Ci/mmol, 1 Ci = 37 GBq) and 20% [35S]cysteine, was obtained from ICN Radiochemicals. Un cleavable Om fpLpp (9), a model secretory protein possessing an uncleavable signal peptide, and mutant proteins derived from it by gene manipulation were used as substrates for in vitro translocation. The structures of their signal peptide regions are shown in Fig. 1.

**Bacterial Strains**

The E. coli strains used were Q13lp for preparation of cytosolic fraction S150-2, K003 (Lpp ΔuncB C-T1n10) for preparation of membrane vesicles (14), and JM83 (15) for DNA manipulation.

**In Vitro Transcription, Translation, and Translocation Reactions**

In vitro transcription of genes coding for uncleavable Om fpLpp and its mutant forms was performed with SP6 RNA polymerase as described (16). The translation reaction was carried out in the presence of TranS-Label as described previously (9). The translation reaction was carried out using purified inverted membrane vesicles at 37 °C after the addition of 5 mM ATP and 5 mM succinate (6). The translation mixture was then treated with proteinase K (1 mg/ml) on ice for 10 min. After cooling on ice, the mixture was centrifuged at 100,000 g for 30 min at 4 °C. The pellet obtained was washed twice with 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, and 0.1 M sodium chloride. The pellet was then suspended in 50 mM potassium phosphate (pH 7.5), 5 mM MgSO4, and then analyzed by polyacrylamide gel electrophoresis and fluorography. The amount of radioactivity in the OmpF-Lpp band on a gel was determined densitometrically with a Shimadzu CS-930 chromatogram-scanner. The efficiency of translocation was expressed as the ratio of the intensities of protein bands on fluorograms before and after the proteinase K treatment.

**Removal of Small Molecules from the Translation Mixture**

By gel filtration. The removal of small molecules from the translation mixture was carried out as described previously (9) with minor modifications: the posttranslational supernatant was passed through a column of Sephadex G-75 superfine equilibrated with 50 mM potassium phosphate (pH 7.5).

**Binding Assay**

45 μl of 50 mM potassium phosphate (pH 7.5), 5 mM MgSO4 containing 30 μg of membrane vesicles and 15 μl of 50 mM potassium phosphate (pH 7.5) containing the gel-filtered uncleavable Om fpLpp were preincubated separately at 37 °C for 3 min and then mixed to initiate binding at 37 °C for 10 min. After cooling on ice, the mixture was centrifuged at 100,000 g for 30 min at 4 °C. The pellet fraction was suspended in 50 mM potassium phosphate (pH 7.5), 5 mM MgSO4, and then analyzed by polyacrylamide gel electrophoresis and fluorography. The amount of radioactivity in the Om fpLpp band on a fluorogram was determined densitometrically.

**Plasmid Construction**

pSS205—Plasmid pINIK10 (14) was digested with EcoRI-SalI. The EcoRI-SalI fragment containing the omfpLpp gene was isolated and ligated with the EcoRI-SalI large fragment of pUC18 to construct p1HL. p1K1 was then subjected to site-directed mutagenesis (17) with d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS602); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS603); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS604); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS605); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS606); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS607); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS608); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS609); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS610); d(5' -AAATCTAGGAGGTTTAAATTTATGATGAAAACAC-3') and d(3'-GATCCATCATCATCATCCAAATTTAAATACTACTTTTTTTTTTGGT5') (pSS611).

**RESULTS**

**Translocation of Uncleavable OmpfpLpp Was Inhibited by Replacement of the Positively Charged Amino Acid Residues at the Amino Terminal of the Signal Peptide with Neutral or Negatively Charged Amino Acids—Uncleavable OmpfpLpp was subjected to mutagenesis, so that the positive charge at the amino-terminal region, that is +2 (LysArg) for the wild-type, was altered to 0 (AsnGly), or -2 (GluASP) (see Fig. 1), and then the posttranslational translocation into inverted membrane vesicles was examined (Fig. 2). After the translocation reaction, 90% of the wild-type precursor became proteinase K-resistant. As the charge was altered from +2 to -1, the translocation efficiency decreased drastically (5%). Essentially no translocation was observed when the charge at the relevant position was -1 or -2.

**Kinetic Analysis of Translocation of Precursor Proteins Possessing Different Numbers of Lysine Residues—Most prokaryotic signal peptides possess a +2 net charge at the amino-terminal region. They are mostly composed of Lys and Arg, with a higher number of the former than of the latter (19). The number of the positive charge at the amino-terminal region of the signal peptide of uncleavable OmpfpLpp was altered by introducing tetrapeptides composed of different numbers of Lys and Asn, and the rates of translocation of the individual mutant proteins were determined (Fig. 3). The

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The amino acid sequences of the amino-terminal region of the signal peptide domain are shown. The amino acid residues of the wild-type precursor in boldface type were replaced by the residues indicated for individual mutant precursors. The carboxyl-terminal amino acid residue of signal peptides is numbered −1.

**FIG. 1.** Wild-type and mutant precursors (uncleavable OmpF-Lpp). The amino acid sequences of the amino-terminal region of the signal peptide domain are shown. The amino acid residues of the wild-type precursor in boldface type were replaced by the residues indicated for individual mutant precursors. The carboxyl-terminal amino acid residue of signal peptides is numbered −1.

| Plasmid          | Net Charge at Amino-terminus | Signal Sequence                  |
|------------------|------------------------------|----------------------------------|
| pK127 (Wild Type)| +2                           | MetMetLysArgAsn                   |
| pSS208           | 0                            | AsnGlyAsn                        |
| pSS210           | −1                           | AsnAspAsn                        |
| pSS211           | −2                           | GluAspAsn                        |
| pSS500           | 0                            | AsnArgAsnArgAsn                  |
| pSS502           | +2                           | LysLysLysAsn                     |
| pSS503           | +3                           | LysLysLysLys                     |
| pSS504           | +4                           | LysLysLysLysLys                  |
| pSS602           | +2                           | ArgArgAsnAsn                     |
| pSS604           | +4                           | ArgArgArgArg                     |
| pKRN130          | +4                           | LysArgArgArg                     |
| pSS700           | 0                            | AsnAsnAsnAsn                     |
| pSS701           | +1                           | HisAsnAsnAsn                     |
| pSS702           | +2                           | HisHisAsnAsn                     |
| pSS703           | +3                           | HisHisHisAsn                     |

**FIG. 2.** Alteration of the positively charged amino acid residues at the amino terminus of the signal peptide to neutral or negatively charged residues inhibited the translocation of uncleavable OmpF-Lpp into membrane vesicles. The LysArg sequence at the amino-terminal region of the signal peptide of uncleavable OmpF-Lpp was changed so as to make the region neutral (AsnGly) or negatively charged (AsnAsp or GlnAsp). The plasmids used were pK127 (lanes 1–3), pSS208 (lanes 4–6), pSS210 (lanes 7–9), and pSS211 (lanes 10–12). The net charge at the amino-terminal region of the signal peptide is shown in parentheses. mRNA transcribed with the SP6 system was added to the translation mixture to synthesize the precursor protein. The translation mixture was incubated at 37 °C for 40 min and then the reaction mixture was centrifuged at 10,000 × g for 3 min to remove aggregates. The supernatant (15 μl) was added to the translocation mixture (final volume, 60 μl) in the presence of membrane vesicles (15 μg of protein), 5 mM ATP and 5 mM succinate, and then the translocation was carried out at 37 °C for 15 min. The sample was then chilled in ice water and treated with proteinase K and then analyzed by polyacrylamide gel electrophoresis and fluorography. X-ray films of the fluorograms were subjected to densitometric scanning to determine the degree of translocation. 100% translocation means that all of the precursor protein became resistant to proteinase K.

**FIG. 3.** Time courses of the translocation of uncleavable OmpF-Lpp possessing different numbers of lysine residues. The number of the positive charge at the amino-terminal region of the signal peptide was altered by introducing tetrapeptides composed of different numbers of Lys and Asn, as indicated in Fig. 1. The plasmids used were pK127 (A), pSS500 (O), pSS502 (D), pSS503 (O), and pSS504 (Δ). The net positive charge at the amino-terminal region of the signal peptide is indicated in parentheses. The translocation reaction (200 μl) was carried out with the translation mixture and membrane vesicles (50 μg of protein) at 37 °C in the presence of 5 mM ATP and 5 mM succinate. At the indicated times, aliquots (20 μl) were withdrawn and the reaction was terminated on ice. The aliquots were then treated with proteinase K and then analyzed by polyacrylamide gel electrophoresis and fluorography. X-ray films of the fluorograms were subjected to densitometric scanning to determine the degree of translocation. 100% translocation means that all of the precursor protein became resistant to proteinase K.

Translocation kinetics to the wild-type. The rate of translocation became faster as the number of Lys was further increased. It should be noted that a more than 3-fold increase, compared to the wild-type, in the initial rate of translocation was achieved with the (Lys)_2 mutant. These results suggest that the rate of translocation is proportional to the number of the positive charge and that a +2 charge is by no means the best in terms of the rate of translocation.

**Arginine Residues Were as Active as Lysine Residues at the Amino Terminus in Translocation—**Experiments similar to those illustrated in Fig. 3 were carried out, different numbers of Arg being introduced in place of Lys (Fig. 4, A and B). The translocation efficiency again was found to depend on the number of the positive charge at the amino-terminal region, irrespective of whether the residues responsible were of Lys or Arg.

**Histidine Residues Were Also Active as Donors of the Amino-terminal Positive Charge at a Low pH**—The pK values of Lys and Arg are 10.8 and 12.5, respectively. The pK values of His, another basic amino acid, on the other hand, is 6.0–6.5, indicating that only a very small fraction of this residue is positively charged in the translocation mixture, the pH of which is 7.8. Indeed, the rate of translocation of mutant precursors possessing different numbers of His at the amino-terminal region of the signal peptide was slow at pH 8.0. At pH 6.5, however, the rate was much faster (Fig. 5). The rate of translocation of the wild-type precursor was about the same at the two pH values (Fig. 5A). It should be noted that the reason we chose Asn as the counterpart for Lys is that it is neither charged, hydrophobic, nor in possession of a large side-chain. No significant translocation was observed with the (Asn)_2 mutant. The (Lys)_2(Asn)_2 mutant exhibited similar
rate of translocation again was found to depend on the number of His at the two pH values (Fig. 5, B-E). The rate of translocation of the (His)₄ mutant at pH 6.5 was about two-thirds of that of the wild-type.
increases, and 3) the enhancement is independent of the species of amino acids that donate the charge, but dependent on the total net positive charge. It should be mentioned in this respect that the rate of translocation of the precursor protein possessing (Lys), or (Arg), was severalfold faster than that of the wild-type one possessing two positively charged amino acid residues at the relevant position (Figs. 3 and 4). Almost all prokaryotic signal peptides possess a +2 or +1 charge at this position (19). These facts indicate that naturally existing signal sequences are not necessarily ideal in terms of the rate of translocation. The reason for this inefficient arrangement of amino acid residues remains to be determined.

A difference between the present in vitro work and previous in vivo studies (4–7) is the result to the role of the positive charge. In previous in vivo studies, although the replacement of the positively charged residues with negatively charged ones resulted in strong suppression of the translocation, that with neutral residues had no detectable effect on translocation (4, 5). Contrary to this, one in vivo experiment with a foreign secretory protein demonstrated partial suppression upon neutralization of the positively charged region (6). In the present in vitro work, removal of the positive charge resulted in almost complete suppression of the translocation (Figs. 2 and 3). A decrease in in vitro translocation upon neutralization of the positively charged domain of the signal peptide was also observed with mutant proPhoE, the in vivo translocation of which was rather normal (7).

The difference may be due to the fact that the in vivo data so far presented have not included ones on the kinetics of the translocation reaction. Furthermore, when the translocation reaction is not a rate-limiting one in the in vivo expression of a gene for a secretory protein, defects at the level of translocation are not necessarily reflected by the accumulation of precursors. The partial suppression upon neutralization observed in vivo with a foreign secretory protein (6) may have been due to its inefficient translocation. It should also be discussed in this respect whether or not the amino-terminal amino group plays a role as the positive charge. Precursor proteins synthesized in vitro most likely retain the initiator fMet at the amino terminus. It is unclear, on the other hand, whether or not precursors synthesized in vivo retain the fMet. It seems likely that the initiator fMet is cleaved off in vivo when followed by Ala (21), a positive charge thus being created. Some of the precursors used in the present in vivo studies possessed Ala at this position, whereas others did not. The cleavage of fMet, therefore, may not be relevant as to the discrepancy.

Another interesting observation in the present study was that the translocation efficiency primarily depends on the net positive charge, irrespective of the species of amino acids that donate the charge. The positive charge-donating groups in Lys, Arg, and His are amino, guanidyl, and imidazolyl groups, respectively. The present work also showed that signal sequences with longer polylsine or polyarginine residues exhibited higher translocation efficiencies. Assuming that these positively charged amino acid residues are involved in the interaction with the membrane surface as suggested (Table I), the responsible surface component(s) would be one(s) that is/have highly negatively charged. Phosphoryl groups of phospholipids in the membrane bilayer are certainly a candidate for this component, as originally suggested by Inouye (20) in his loop model. It should be mentioned in this respect that negatively charged phospholipids are required for the spontaneous insertion of proapolipoprotein, a secretory protein, into liposomes or cytoplasmic membrane vesicles (22). The involvement of phosphatidylglycerol, a negatively charged phospholipid, in protein translocation has also been reported (23). The importance of negatively charged phospholipids has also been reported as to the import of proteins into mitochondria, for which positively charged amino acid residues at the amino-terminal region of precursor proteins are also important (24).

Another interesting candidate that recognizes the positive charge is SecA, a protein component essentially required for the translocation of secretory proteins across the cytoplasmic membrane (11, 25–27). SecA is a peripheral membrane protein that is associated with the inner surface of the cytoplasmic membrane (26). It possibly exists as a homooligomer, with a subunit molecular mass of 102 kDa (28). The subunit is rich in acidic amino acids, the sum of Glu plus Asp exceeding that of Lys plus Arg by 29 residues. We recently found that the signal peptide domain of secretory proteins interacts with SecA and that the interaction is enhanced when the number of Arg or Lys at the amino terminus of the signal peptide is increased. The essentiality of SecA in the functional interaction of proOmpA, the precursor form of a secretory protein, with inverted membrane vesicles has also been demonstrated (29). It is highly probable, therefore, that the positively charged domain of the signal peptide is recognized by SecA on the membrane surface to initiate translocation. The involvement of phospholipids as a primary, but rather nonspecific, site for the interaction with signal peptides cannot be excluded, however.

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