Effects of Total Hydrophobicity and Length of the Hydrophobic Domain of a Signal Peptide on in Vitro Translocation Efficiency*

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The hydrophobic domain of the signal peptide of OmpF-Lpp, a model secretory protein, was systematically engineered so as to be composed of different lengths of polyleucine residues or polymers with alternate leucine and alanine residues, and the effects of the length and nature of the hydrophobic stretch on the rate of in vitro translocation were studied using everted membrane vesicles of Escherichia coli. The translocation reaction exhibited high substrate specificity as to the number of hydrophobic residues. The results suggest that the hydrophobic domain is recognized specifically by a component(s) of the secretory machinery rather than nonspecifically by the hydrophobic region of the membrane. The in vitro translocation thus demonstrated required SecA and ATP and was markedly enhanced upon imposition of the proton motive force, as in the case of secretory proteins possessing a natural signal peptide. The highest translocation rate was obtained with the octamer in the case of polyleucine-containing signal peptides, whereas it was the decamer in the case of ones containing both leucine and alanine. These results suggest that the total hydrophobicity of the hydrophobic region of the signal peptides is an important determinant of the substrate specificity.

Secretory proteins are generally synthesized as a precursor form possessing a signal peptide at its amino terminus. Although no amino acid sequence homology is found among signal peptides, they share some common features. For example, in Escherichia coli, they possess 1) one or more positively charged amino acid residues at their amino terminus and 2) a cluster of 10-15 hydrophobic amino acid residues in their central region (1). A large number of signal peptide mutants have been obtained. Replacement of the amino-terminal positively charged amino acid residues with negatively charged ones resulted in the in vivo accumulation of signal peptide-containing presecretory proteins, suggesting the importance of the positive charge for protein secretion (2-5). Later, the importance of the positive charge for the transmembrane translocation reaction was quantitatively demonstrated using an in vitro translocation system (6).

The importance of the central hydrophobic region for protein secretion has also been demonstrated in vivo. Deletion of this hydrophobic region or insertion of a charged amino acid residue into the region resulted in severe inhibition of protein secretion (7, 8). Furthermore, one of the deletion mutations thus obtained was suppressed upon insertion of a leucine residue, which resulted in an artificial hydrophobic stretch (8, 9).

In spite of the accumulation of a large amount of such evidence, the basic principles of the hydrophobic region, which is essentially required for protein translocation, remain unclear. This is mostly due to the fact that this region consists of a variety of amino acid residues, some of which are even hydrophilic. To overcome this problem, the hydrophobic region was systematically engineered on a DNA level so as to be composed of one species of hydrophobic amino acid residue. Kendall et al. (10-12) constructed, in E. coli, genes encoding alkaline phosphatases possessing various lengths of either polyleucine, polyvaline, polyalanine or polyisoleucine residues as the hydrophobic stretch. Yamamoto et al. (13) studied the secretion from yeast cells of human lysozyme possessing an artificial polyleucine-containing signal peptide. Both groups showed in vivo that the artificial signal peptides thus constructed were active as to secretion when the length of the hydrophobic stretch was appropriate. A longer hydrophobic stretch was required when hydrophobicity/residue was lower, suggesting the importance of the total hydrophobicity (11). They also showed that the secretion took place over a rather wide range as to the length of the hydrophobic stretch. This was more prominent in the secretion of alkaline phosphatase from E. coli cells (11). Although in vivo studies are important in many respects to get an insight into functions of signal peptides, in vitro studies are advantageous in other respects such as direct demonstration of the translocation process. In the present work the effect of the length of the hydrophobic stretch, composed of leucine alone or leucine and alanine, on the rate of in vitro translocation was studied. The in vitro translocation exhibited high substrate specificity as to the number of hydrophobic residues, the maximum being observed for the octamer in the case of leucine polymers and the decamer in the case of polymers comprising leucine and alanine. The results suggest that the hydrophobic domain is specifically recognized by a factor(s) involved in the secretory machinery rather than nonspecifically by the hydrophobic region of the membrane, and that the total hydrophobicity is an important factor for the substrate specificity.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, DNA-modifying enzymes, and SP6 RNA polymerase were purchased from Takara Shuzo Co. Protease K was from Merck. Trans35S-label, a mixture of 70% [35S]methionine (1000 Ci/mmol, 1 Ci = 37 GBq) and 20% [35S]cysteine, was obtained from ICN Radiochemicals. Both uncleavable and cleav-
able OmpF-Lpps, model secretory proteins possessing uncleavable and cleavable signal peptides, respectively (14, 15), and mutant proteins derived from them through gene manipulation were used as substrates for in vitro translocation.

**Bacterial Strains**—The E. coli strains used were K003 (LppΔinsertion-Tn10) for preparations of everted membrane vesicles and S100 (15), MM66 (F−, ΔlacI, araP139, rpsL, recA, thi, secA, Tn10, sus3, trpC) (16) for preparation of SecA-depleted S150, BL21(DE3)/pJW25 (17) for preparation of SecB overproduced S150, and MH1160recA (F−, Δlac169, araD139, rpsL, recA, thi, secAB, Tn19, SlSOO) (15), “66 (F−, Alac169, araP139, rpsL, recA, thi, SecAB”, Tn19, pCHPL. This plasmid encodes a mutant uncleavable OmpF-Lpp, PL, of the signal peptide region of uncleavable OmpF-Lpp was isolated from pK127 and ligated with M13 mp19 replicative form DNA, and then the pCH7AL-pCH12AL-series OmpF-Lpps. Amino acid residues in the signal peptide are numbered -1 to -22; the amino terminus of the mature domain being +1. B, in vitro transcription and translation of wild-type uncleavable OmpF-Lpp and PL OmpF-Lpp (C). Portions of the fluorograms are also shown. OmpF-Lpps added to the individual reaction mixtures are at the left. The translocation assay was also carried out in the absence of everted membrane vesicles (●) in the presence of 10 mM AMP-PNP (Δ).

**Analysis of the Extent of Aggregation of OmpF-Lpps by Centrifugation**—The translocation mixture was subjected to either sonication in 6 M urea or centrifugation for 30 min at 4°C, and the pelleted membranes were suspended in 50 mM potassium phosphate, pH 7.6 (22). More than 80% of the SecA was removed from the membranes after treatment (without sonication in 6 M urea). Aliquots (1 ml) were then centrifuged at 100,000 × g for 1 h in a Beckman TL-100 ultracentrifuge (rotor 1003), and the supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography (6). The amount of protein in the OmpF-Lpp band on a gel was determined densitometrically with a Shimadzu CS-930 chromato-scanner. The amount of protein bands on fluorograms before and after the proteinase K treatment.

**Removal of SecA from Everted Membrane Vesicles with 6 M Urea**—Everted membrane vesicles prepared from K003 (21) were suspended in 50 mM potassium phosphate, pH 7.6, mixed with an equal volume of 12 M urea and then placed in ice water for 1 h. The mixture was centrifuged at 156,000 × g for 30 min at 4°C, and the pelleted membranes were suspended in 50 mM potassium phosphate, pH 7.6 (22). More than 80% of the SecA was removed from the membranes through this treatment (23).

**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 (6) with minor modifications. The post-translational supernatant was passed through a column of Sephacry G-75 superfine equilibrated with 50 mM potassium phosphate, pH 7.6.

**Plasmid Construction**—pCHPL, an EcoRI-SalI fragment carrying the 59-terminal region of the ompf-lpp gene encoding the signal peptide, is under the control of the SP6 promoter (19). pK127 was partially digested with SspI and then with SalI site were located right before the region encoding the hydrophobic domain.

**Urea and Translocation**—The translation reaction was performed as described (6).

**Sonication of Translation Products in 6 M Urea and Translocation Reaction**—To the mixture after translation, an equal volume of 12 M urea dissolved in 50 mM potassium phosphate, pH 7.6, 2 mM dithiothreitol was added. The mixture was then subjected to 30-s sonication four times, with 30-s intervals, in a bath type sonicator (W-l03T, Watanabe Co., Ltd.). The translocation reaction was started by adding this mixture to 9 volumes of the translocation mixture (50 mM potassium phosphate, pH 7.6, 2 mM ATP, 5 mM succinate, 100 pg/ml creatine kinase, 30 mM creatine phosphate, 20 mg/ml partially purified SecA (20), and 0.3 pg/ml everted membrane vesicles), which had been preincubated at either 37 or 25°C. The translocation mixture was then treated with 1 mg/ml proteinase K at 25°C for 10 min and the translocates were detected by SDS-polyacrylamide gel electrophoresis followed by fluorography (6). The amount of protein bands on fluorograms before and after the proteinase K treatment.

**Analysis of the Extent of Aggregation of OmpF-Lpps by Centrifugation**—The translocation mixture was subjected to either sonication in 6 M urea followed by 10-fold dilution as described above or the control treatment (without sonication in 6 M urea). Aliquots (1 ml) were then centrifuged at 100,000 × g for 1 h in a Beckman TL-100 ultracentrifuge (rotor 1003), and the supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography as to OmpF-Lpp contents.

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cleavable carboxyl-terminal region of the signal peptide and ones encoding various lengths of the polymers of leucine or leucine and alanine were prepared, as listed in Table I. The codons for leucine and alanine used are those used for the ompF lp gene (24). The synthetic DNA fragments were then ligated with the Sphl-SacI large fragment of pX127 to construct L- and AL-series plasmids (pCH7AL-pCH12AL, pCH7AL-pCH12AL). The DNA sequences of all the mutant ompF-lpp genes thus constructed were confirmed as described (25).

Le-series Plasmids (pCH7Le-pCH9Le) and AL-series Plasmids (pCH8ALc-pCH11ALc)—These plasmids carry ompF-lpp genes encoding cleavable OmpF-Lpps possessing different lengths of a central hydrophobic stretch comprising leucine or both leucine and alanine residues. An outline of the construction and the structures of these cleavable OmpF-Lpps are shown in Fig. 7. The method was essentially the same as that for the L-series and AL-series plasmids, except that synthetic DNA fragments encoding the cleavable carboxyl-terminal region of the signal peptide was used (see Table I).

RESULTS

Substitution of Leucine for Proline in the Signal Peptide Does Not Affect the Efficiency of in Vitro Translocation—Some presecretory proteins including proOmpF possess a proline residue, an a-helix breaker, in their hydrophobic region. Since the a-helical conformation has been discussed in relation to the functions of signal peptides (26-28), the effect of the presence of a proline residue on the rate of protein translocation was studied (Fig. 1). PL, an OmpF-Lpp mutant in which the proline residue at -11 has been replaced with a leucine residue, was translocated even faster than the wild-type OmpF-Lpp, suggesting that this proline residue is not essential for translocation. This is consistent with the results of an in vivo secretion study (10). The translocation of PL was SecA dependent (data not shown) and completely inhibited in the presence of AMP-PNP, like that of the wild-type (29).

Most of the L-Series OmpF-Lpps Are Proteinase K-resistant But Can Be Converted to Be Proteinase K-sensitive by Sonication in the Presence of 6 M Urea—The hydrophobic region of PL is composed of 12 amino acid residues. We replaced them with a polyleucine stretch of various lengths and tried to determine its function in in vitro translocation. However, most of the L-series OmpF-Lpps, except 0L and 5L, were more or less resistant to proteinase K and were not translocated into membrane vesicles. It was highly likely that these mutant OmpF-Lpps tended to form aggregates, which are proteinase K resistant and at the same time incompetent as to in vitro translocation. SecB has been reported to help in maintaining the translocation competent conformation of some secretory proteins (30). The addition of a cytosolic translocation was studied (Fig. 1). PL, an OmpF-Lpp mutant in which the proline residue at −11 has been replaced with a leucine residue, was translocated even faster than the wild-type OmpF-Lpp, suggesting that this proline residue is not essential for translocation. This is consistent with the results of an in vivo secretion study (10). The translocation of PL was SecA dependent (data not shown) and completely inhibited in the presence of AMP-PNP, like that of the wild-type (29).

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No Table I

| Sequence | Hydrophobic region |
|----------|-------------------|
| 5'-CTGTTCATCGCTTCTG-3' 3'-GACAATGACGAA-5' | 5L |
| 5'-CTGTTCATCGCTTCTG-3' 3'-GACGAAGACATGACGAA-5' | 7L |
| 5'-CTGTTCATCGCTTCTG-3' 3'-GACGAAGACATGACGAA-5' | 8L |
| 5'-CTGTTCATCGCTTCTG-3' 3'-GACGAAGACATGACGAA-5' | 9L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 10L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 12L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 13L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 14L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 16L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 18L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 20L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 22L |
| 5'-TTACTGTTCTGCTTCTG-3' 3'-AATGACGAA-5' | 24L |

For cleavable OmpF-Lpps

* For the construction of OL, the synthetic oligonucleotide lacking 3'-terminal GAC was used.
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fraction, S150, prepared from SecB-overproducing cells did not solve this problem, however.

We found that sonication in the presence of 6 M urea followed by 10-fold dilution made these mutant OmpF-Lpps sensitive to proteinase K. This proteinase-sensitive state lasted for at least 3 h after the 10-fold dilution. Neither sonication in the absence of urea nor the urea treatment alone had such an effect. It should be noted, however, that mutant OmpF-Lpps possessing polyleucine stretches longer than 14 residues were still slightly resistant to proteinase K even after the sonication in 6 M urea.

The effect of sonication in 6 M urea on the state of L-series OmpF-Lpps was also examined by ultracentrifugation. The wild-type OmpF-Lpp, 7L, 8L, and 10L were centrifuged at 100,000 x g for 1 h as described under “Experimental Procedures,” and amounts of these OmpF-Lpps in the supernatant and pellet fractions were determined by polyacrylamide gel electrophoresis, followed by fluorography (Table II). Although the sonication in 6 M urea resulted in the increase, to some extent, in the amount of OmpF-Lpps in the supernatant fraction, the increase was not significant. Furthermore, the wild-type OmpF-Lpp, which is highly active for the in vitro translocation, was recovered by 40% in the pellet fraction. These results suggest that the translocation incompetency of the nontreated OmpF-Lpps is not largely due to their states of aggregation. It may rather be due to the conformation of individual molecules.

The Effect of the Number of Leucine Residues in the Hydrophobic Region of the Signal Peptide on the Rate of Translocation—Precise kinetic studies as to in vitro translocation were then performed on L-series OmpF-Lpps subjected to sonication in 6 M urea (Figs. 3 and 4). No translocation was observed with 0L, indicating that a hydrophobic stretch is essential. The translocation was also not observed with 5L or those possessing a polyleucine stretch of 12 residues or more (Fig. 4). Significant translocation was only observed with 7L-9L, the maximum rate being observed for 8L (Fig. 3A). The rate of translocation of 8L was comparable to that of PL. Since the rate of translocation of 8L at 37 °C was too fast to correctly measure the initial rate, translocation experiments were also carried out at 25 °C (Fig. 3B).

The initial rates of in vitro translocation thus determined are summarized in Fig. 4. The profile showed a high substrate specificity in terms of the number of leucine residues comprising the hydrophobic region. The initial rate of transloca-

| Gene product | Sonication in 6 M urea | Control treatment | | | |
|--------------|------------------------|-------------------|----------------|----------------|----------------|----------------|
| (ProOmpF-Lpp derivative) | Sup | Pellet | Sup | Pellet | |
| Wild type proOmpF-Lpp | 89.3 | 10.7 | 58.2 | 41.8 | |
| L-series | | | | | |
| 7L | 67.6 | 32.4 | 43.6 | 56.4 | |
| 8L | 65.6 | 34.4 | 42.9 | 57.1 | |
| 10L | 65.5 | 34.5 | 53.5 | 46.5 | |
| AL-series | | | | | |
| 9AL | 92.8 | 7.2 | 70.5 | 29.5 | |
| 10AL | 91.0 | 9.0 | 64.6 | 35.4 | |
| 12AL | 88.0 | 12.0 | 51.6 | 48.4 | |

FIG. 3. Translocation kinetics in the cases of L-series OmpF-Lpps possessing different numbers of leucine residues in the hydrophobic region. The L-series OmpF-Lpps indicated were sonicated in 6 M urea, 10-fold diluted with the translocation mixture, and then subjected to the translocation assay at 37 °C (A) or 25 °C (B) (O). At the indicated times, portions (60 μl) were withdrawn, and the reaction was terminated on ice, followed by treatment with 15 μl of 1 mg/ml proteinase K for 10 min at 25 °C. The translocated proteins, which were proteinase K-resistant, were then detected on an SDS-polyacrylamide gel by means of fluorography, followed by densitometric scanning. In B, fluorograms are also shown with translocation times (min). OmpF-Lpps added to the individual reaction mixtures are at the left. The assay was also carried out in the presence of 10 mM AMP-PNP (Δ) or in the absence of everted membrane vesicles (●).

FIG. 4. Effect of the number of leucine residues on the initial rate of in vitro translocation of L-series OmpF-Lpps. The translocation in the first 1 min assayed at 37 °C (●) or 25 °C (○) was expressed as a percentage of the total input OmpF-Lpps.

The Translocation of L-Series OmpF-Lpps Requires SecA, ATP, and ΔH1—it—The translocation of some secretory proteins has been reported to be SecA-independent in vivo, even though they possess signal peptides (31, 32). The SecA requirement for the in vitro translocation of L-series OmpF-Lpps was examined using urea-treated membrane vesicles, which are SecA-deficient. The translocation of these mutant proteins was SecA-dependent, as in the case of the wild-type proteins. Results with 8L are presented in Fig. 5A. The translocation was also dependent on ATP and was enhanced in the presence of ΔH1, which was generated by the addition
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...concentrations of SecA, ATP, and succinate were 20 ng/µl, 1 and 5 mM, respectively. The translocation reaction was carried out in the presence or absence of SecA as described (22). The reaction was carried out using everted membrane vesicles from which SecA had been removed by urea treatment as described (22). The concentrations of SecA, ATP, and succinate were 20 ng/µl, 1 and 5 mM, respectively. The translocation reaction was carried out in the absence of both SecA and membrane vesicles (A). B, [35S]OmpF-Lpps translated in the normal S-100 fraction were gel filtered, sonicated in 6 M urea, and then subjected to in vitro translocation at 37 °C in the presence of 1 mM ATP and 5 mM succinate (O), 1 mM ATP (△), 5 mM succinate (●), or none (■) as indicated. For both A and B, the samples were then treated as described in the legend to Fig. 3.

It was shown that the mode of translocation of mutant proteins possessing a polyleucine stretch as the hydrophobic domain of the signal peptide is essentially the same as that of naturally occurring secretory proteins.

**Translocation of Mutant OmpF-Lpps Possessing Different Numbers of Alanine and Leucine Residues in the Hydrophobic Region of the Signal Peptide—Leucine is one of the most hydrophobic amino acids. We showed in Figs. 3 and 4 that the rate of translocation was specific as to the number of leucine residues comprising the signal hydrophobic domain. In order to determine whether the apparent specificity is determined by the number of hydrophobic amino acid residues, i.e., the length of the hydrophobic polypeptide chain, similar experiments were carried out using AL-series OmpF-Lpps, in which the hydrophobic region is composed of alternate alanine and leucine residues. The hydrophobicity of the former amino acid residue is much weaker than that of the latter. The chemical structures of these OmpF-Lpps are summarized in Fig. 2.

All the AL-series OmpF-Lpps examined were highly sensitive to proteinase K digestion. Kinetic profiles of the translocation of these OmpF-Lpps with or without sonication in 6 M urea were essentially the same, although the rate of translocation after the urea treatment was slightly slower (data not shown). The following experiments were, therefore, carried out without sonication in 6 M urea prior to the translocation assay. The AL-series OmpF-Lpps were also subjected to the ultracentrifugation analysis (Table II). They were more soluble than the L-series ones and the solubility was further increased upon the urea sonic treatment. However, a significant portion of them was recovered in the pellet fraction in the absence of the urea sonic treatment (Table II), and the treatment did not result in the increase in their translocation competency as mentioned above. Taken together with the results in the case of the L-series OmpF-Lpps, it is likely that the solubility as revealed by ultracentrifugation is correlated to some extent to the translocation competency, it does not directly represent the translocation competent conformation.

The results of kinetic analysis of the translocation of these mutant OmpF-Lpps are presented in Fig. 6, A and B, and the initial rates of their translocation are compared in Fig. 6C. As in the case of L-series mutants, a sharp response of the rate of translocation to the length of the hydrophobic domain was observed. It should be noted that the number of hydrophobic amino acid residues for the maximum translocation activity was larger than that in the case of L-series OmpF-Lpps. 10AL exhibited the maximal translocation rate, whereas 10L was almost completely inactive. These results suggest that the total hydrophobicity is an important determinant of the translocation specificity.

**Translocation Experiments with Mutant OmpF-Lpps Possessing Cleavable Signal Peptides**—The cleavage of the signal...
peptide is an important and useful criterion of protein translocation across the membrane. To confirm that the translocation data as revealed by protease K resistance in the preceding experiments really represent translocation across the membrane, we have constructed, at a DNA level, cleavable OmpF-Lpps corresponding to some of the uncleavable OmpF-Lpps (Fig. 7) and examined for in vitro translocation.

Translocation of these cleavable OmpF-Lpps was accompanied by the signal peptide cleavage (Fig. 7B). Furthermore, translocation profiles as a function of the numbers of hydrophobic residues were essentially the same irrespective of whether the signal peptide is cleavable or uncleavable in both L-series and AL-series OmpF-Lpps (Fig. 8A). Thus, the difference in the number of hydrophobic amino acid residues for the maximum translocation activity between L-series and AL-series OmpF-Lpps was again demonstrated.

The translocation of some of the cleavable OmpF-Lpps was accompanied by accumulation of a small amount of precursor forms, which were proteinase K-resistant and hence should have been translocated (Fig. 8B). Topological relation between signal peptidase and the signal cleavage site may be inappropriate in some mutant OmpF-Lpps.

**DISCUSSION**

Requirements for the hydrophobic region of the signal peptide for translocation across the cytoplasmic membrane were studied. This was performed by using gene-engineered signal peptides possessing hydrophobic regions with simple compositions. In order to focus solely on the translocation process, for which the signal peptide is essential, an *E. coli in vitro* system involving everted membrane vesicles was employed. We further employed, in the first part of the present work, uncleavable OmpF-Lpp as the starting presecretory protein for the derivation of mutant proteins as to the hydrophobic region, the reasons being as follows. 1) This protein possesses a small hydrophilic mature domain, and the influence of the conformation of the mature domain on the translocation kinetics is assumed to be insignificant. 2) This protein can be translocated highly efficiently and has been extensively used for precise *in vitro* kinetic studies (13, 33). 3) This protein possesses an uncleavable signal peptide and hence the translocation process can be focused on (14). The unprocessing of the signal peptide does not interfere with the *in vitro* translocation reaction. Obviously the use of uncleavable OmpF-Lpps was disadvantageous in another respect; the signal peptide cleavage as a criterion of translocation being unable to observe. To overcome the disadvantage, OmpF-Lpps with a cleavable signal peptide were used in the later part of this work.

The translocation of synthetic signal peptide-possessing OmpF-Lpps observed as protease K-resistant was ATP-, Δ*μ*+- and SecA-dependent. Furthermore, the translocation was accompanied by the signal peptide cleavage when OmpF-Lpps possessing a cleavable signal peptide with the same hydrophobic composition were used. These results indicate that the observed protease K resistance represents translocation across rather than insertion into the membrane, irrespective of whether OmpF-Lpps are of L-series or AL-series.

One interesting observation in the present work was that secretory proteins of which the hydrophobic region of the signal peptide had a simple composition exhibited a sharp response in the rate of translocation to the number of hydrophobic amino acid residues, although the lengths of the hydrophobic regions of naturally existing signal peptides are quite diverse. This was the case for both the L-series and AL-series signal peptides. As to the *in vivo* secretion of *E. coli* alkaline phosphatase possessing polyoleucine-containing signal peptides, Kendall et al. (11) observed the secretion maximum ranging from 10 to 15 leucine residues. Their results were, however, not necessarily inconsistent with ours, since their *in vivo* assay was only carried out with signal peptides containing 5, 10, 15, and 20 leucine residues. Yamamoto et al. (13), using human lysozyme preceeded by a polyoleucine-containing signal peptide of various lengths, found the length optimum for secretion from yeast cells to be 8 leucine residues, which is the same as that found in the present work. The specificity as to the length of the stretch of leucine residues was, however, still lower than that observed in the present work. Since Yamamoto et al. (12) performed an *in vitro* study on eukaryotic cells over a 5-day time course, it is difficult to compare their results with those of our *in vitro* study with a 1-min time course. We should also discuss the possibility that
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The sharp decrease in the rate of translocation with the increase in the number of hydrophobic amino acid residues is due to aggregation, which makes OmpF-Lpps translation-incompetent. Although this possibility cannot be excluded completely, the following evidence suggests that it is unlikely: 1) the sharp decrease was also observed with AL-series OmpF-Lpps, which did not show any indication of aggregation, and 2) no difference was observed between 8L and 10L as to their state of conformation in the reaction mixture as revealed by proteinase K resistance, and 3) no clear cut relationship was observed between the extent of aggregation and translocation competency. It should also be noted in this respect that signal peptides possessing a long polyleucine stretch are not always artificial. Natural signal peptides possessing a polyleucine stretch comprising 8 or 7 leucine residues have been reported (34-36).

Although the optimal number of amino acid residues of the hydrophobic region differed between L-series and AL-series OmpF-Lpps, the high specificity of translocation as to the number of the residues of the hydrophobic region suggests that this region is recognized in a specific manner by the secretory machinery, rather than nonspecifically by the hydrophobic region of the membrane, such as the lipid bilayer. The recognition of the signal peptide by SecA has been suggested (37, 38). Although the importance of the positive charge at the amino terminus of the signal peptide in the interaction with SecA has been demonstrated (38), the role of the hydrophobic region remained unknown. We examined, by means of chemical cross-linking, the effect of the length of the hydrophobic stretch on the interaction with SecA. No significant translocation was observed for lOL, whereas lOAL exhibited the maximal translocation activity. These results (data not shown). The roles of other components of the secretory machinery in the recognition of the hydrophobic region are unknown. In an eukaryotic system, a methionine-secretory machinery in the recognition of the hydrophobic region are unknown. In an eukaryotic system, a methionine-

Acknowledgments—We thank Dr. P. J. Bassford Jr. for the SecE-overproducing strain, M. Kato for computer search of the protein database, and I. Sugihara for excellent secretarial support.

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