Lipoprotein Sorting Signals Evaluated as the LolA-dependent Release of Lipoproteins from the Cytoplasmic Membrane of *Escherichia coli*

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Various bacteria possess lipoproteins, which have a lipid-modified cysteine at the N terminus. In *Escherichia coli*, lipoproteins are anchored to the periplasmic side of either the inner or outer membrane through fatty acyl chains covalently attached to an N-terminal cysteine. Aspartate at position 2 functions to retain lipoproteins in the inner membrane, although the retention is perturbed depending on the residue at position 3. We previously revealed that LolCDE and LolA play critical roles in this lipoprotein sorting. To clarify the sorting signals, the LolA-dependent release of lipoprotein derivatives having various residues at positions 2 and 3 was examined in spheroplasts. When the residue at position 3 was serine, only aspartate at position 2 caused the retention of lipoproteins in spheroplasts. We then examined the release of derivatives having aspartate at position 2 and various residues at position 3. Strong inner membrane retention occurred with a limited number of species of residues at position 3. These residues were present at position 3 of native lipoproteins having aspartate at position 2, whereas residues that inhibited the retention were not. It was also found that a strong inner membrane retention signal having residues other than aspartate at position 2 could be formed through the combination of the residues at positions 2 and 3. These results indicate that the inner membrane localization of native lipoproteins is ensured by the use of a limited number of strong inner membrane retention signals.

 Various bacteria possess lipoproteins, which have a lipid-modified cysteine at the N terminus. In *Escherichia coli*, lipoproteins are anchored to the periplasmic side of either the inner or outer membrane through N-terminal fatty acyl chains (1, 2). Lipoproteins are synthesized as precursors in the cytoplasm and then are translocated across the inner membrane, followed by sequential modification reactions leading to the formation of mature lipoproteins on the periplasmic side of the inner membrane (1, 2). Five Lol proteins are involved in the sorting and outer membrane localization of lipoproteins (3–5). LolCDE in the inner membrane releases outer membrane-directed lipoproteins from the inner membrane in an ATP-dependent manner (5, 6), leading to the formation of a complex between the lipoprotein and LolA (3), a periplasmic chaperone. The LolA-lipoprotein complex crosses the periplasm and then interacts with outer membrane receptor LolB, which mediates the anchoring of lipoproteins to the outer membrane (4). The lipoprotein-sorting signal is recognized at the release step, and the inner membrane-specific lipoproteins are not released (3–5).

Inouye and collaborators (7) first revealed the importance of aspartate at position 2 for the inner membrane localization of *E. coli* lipoproteins. They showed that replacement of serine at position 2 of an outer membrane-specific lipoprotein by aspartate caused the protein to remain in the inner membrane. Furthermore, replacement of aspartate at position 2 of an inner membrane-specific lipoprotein by another residue caused outer membrane localization of the protein. Taken together, these results indicate that aspartate at position 2 functions as an inner membrane retention signal for lipoproteins. However, they also found that when histidine or lysine was present at position 3, some lipoproteins were localized in the outer membrane even though aspartate was present at position 2 (8).

Seidel et al. (9) recently reported a sensitive method for examining the inner membrane localization of lipoprotein derivatives. They constructed a maltose-binding protein (MalE) derivative having a lipid-modified cysteine at the N terminus (lipoMalE) and then expressed it in a chromosomal *malE* deletion mutant. When aspartate was at the N-terminal position, lipoMalE was localized in the inner membrane, where it functioned as a maltose-binding protein, thereby enabling the mutant to grow in the presence of maltose (9). In contrast, lipoMalE localized in the outer membrane was not functional and did not support *malE* mutant growth. Systematic substitution of the residue at position 2 of lipoMalE revealed that in addition to aspartate, five other residues (phenylalanine, tryptophan, tyrosine, glycine, and proline) supported mutant growth (9). Lipid modification and partial inner membrane localization of these lipoMalE derivatives were confirmed, indicating that aspartate at position 2 is not the sole inner membrane retention signal. Because the residue at position 3 of lipoMalE was fixed at asparagine, it seemed possible that substitution of the residue at position 3 with other residues might cause different results. As noted by the authors, limited degradation of lipoMalE releases a small amount of a periplasmic form that can function in maltose transport. Therefore, the Mal" growth assay seems to be too sensitive for examining the membrane localization of lipoproteins.

So far as we examined, lipoproteins released as a complex with LolA were quantitatively localized in the outer membrane in a LolB-dependent manner (10), indicating that a lipoprotein-sorting signal can be assessed as LolA-dependent release. Here we systematically examined the influence of the residues at positions 2 and 3 on the LolA-dependent release of lipoproteins from the inner membrane.


### EXPERIMENTAL PROCEDURES

**Growth of Bacteria and Materials**—*E. coli* DLP79-36 (Hfr C pneum lpp) was obtained from Dr. Masaaki Wachi. LB broth was used as the standard medium. The growth of *E. coli* cells was followed by monitoring the optical density at 660 nm. Labeling experiments were carried out in M63 (NaCl)-maltose minimal medium (11) supplemented with 20 μg/ml thiamine, 20 μg/ml thymine, and 40 μg/ml each of all amino acids except methionine and cysteine. When required, chloramphenicol was added at the concentration of 25 μg/ml. Restriction endonucleases, T4 DNA ligase, and a polymerase chain reaction kit were purchased from Takara Shuzo Co. Tran35S-label (a mixture of 70% [35S]methionine and DNA ligase, and a polymerase chain reaction kit were purchased from PerkinElmer Life Sciences, as described (3). The amounts of L10P lipoproteins was terminated by chilling the reaction mixture in ice.

**spheroplasts** as a complex with LolA. The membrane incorporation of L10P derivatives having vari-

**gene for L10P under the control of P BAD was mutagenized using a pJYL10P (6) carrying L10P derivatives having different residues at positions 2 and 3, raised in rabbits (12). LolA was purified according to the reported method (3).

**Construction of Plasmids Encoding L10P Derivatives**—To construct L10P derivatives having different residues at positions 2 and 3, a pYJL10P (6) carrying araC, the chloramphenicol resistance gene, and a gene for L10P under the control of PBAD was mutagenized using a QuikChange site-directed mutagenesis kit (Stratagene) with a specified pair of oligonucleotides. For the construction of L10P(XS) having various residues at position 2 and serine at position 3, the oligonucleotides shown in Table I were used. The oligonucleotides used to construct L10P(SN), (GN), (FN), and (WN) were 5′-GCCCTACTCTGCTGCGGCCGCGCCGCGC-3′ (6) and 5′-CGATTGATCACGCTAAATTACC-3′ (6) in which X1–X4 and X5–X6 for the respective derivatives are shown below.

**Release of L10P Derivatives from Spheroplasts**—The LolA-dependent release of L10P derivatives from spheroplasts was examined as described previously (3). Briefly, DLP79-36 cells harboring a specified plasmid were grown at 37 °C, induced with 0.2% arabinose for 5 min, and then converted into spheroplasts in the presence of 0.2 m M EDTA and 10 μg/ml lysozyme. A suspension (300 μl) containing 5 × 10^8 spheroplasts was kept on ice for 3 min in the presence and absence of 10 μCi of Tran35S-label was then added for a 2-min labeling at 30 °C. The labeling was chased for 2 min by the addition of non-radioactive methionine and cysteine (each at 12 mM). The release of L10P derivatives was terminated by chilling the reaction mixture into ice water and analyzed after fractionation into spheroplasts and medium by centrifugation at 16,000 × g for 2 min.

**In Vitro Membrane Incorporation of L10P Derivatives**—LolB-dependent incorporation of L10P derivatives into outer membranes was examined as described (4) with [35S]labeled L10P derivatives released from spheroplasts as a complex with LolA. The membrane incorporation of lipoproteins was terminated by chilling the reaction mixture in ice water and analyzed after fractionation into a supernatant and pellet by centrifugation at 100,000 × g for 30 min.

**Immunoprecipitation and SDS-PAGE**—L10P derivatives with an anti-Lpp antibody were immunoprecipitated with anti-Lpp antibodies and analyzed by SDS-PAGE, followed by fluorography with Enlightening (PerkinElmer Life Sciences), as described (3). The amounts of L10P derivatives were determined with an ATTO Densitograph.

### RESULTS

**Only Asp at Position 2 Functions as the Inner Membrane Retention Signal of L10P**—L10P is a derivative of Lpp and possesses Pro in place of Leu at position 10 (Fig. 1A). L10P was shown previously to be suitable for release assays because the derivative remained release-competent for a long time after its synthesis (6). Furthermore, L10P mislocalized in the inner membrane forms a covalent linkage between C-terminal Lys and peptidoglycan, causing growth inhibition (11). Therefore, the inner membrane accumulation of L10P can be qualitatively examined as growth inhibition. We replaced Ser at position 2 with other residues, and the LolA-dependent release of 20 L10P(XS) derivatives, in which X denotes 1 of 20 amino acid residues at position 2, was examined in spheroplasts. Only L10P(DS) having Asp at position 2 remained in spheroplasts in the presence of 2 μg/ml LolA. The other 19 amino acid residues at position 2 caused efficient release of derivatives like L10P(SS) (Fig. 1B). Although the results are not shown, these 19 L10P derivatives released in the presence of LolA were quantitatively incorporated into the outer membrane in a LolB-dependent manner. When expressed in cells, only L10P(DS) inhibited growth (see Fig. 2A). These results were consistent with the “+2 rule” for lipoprotein-sorting signals, i.e., Asp at position 2 is the inner membrane retention signal (7). However, we also found that a certain proportion of L10P(DS) was released when a higher amount of LolA was added (Fig. 1, B and C). These results suggest that Asp position 2 alone is not sufficient for an absolute inner membrane retention signal, because the *in vivo* concentration of LolA was estimated to be higher than 20 μg/ml (3). It has been suggested that the residue at position 3 affects the potency of Asp at position 2 (8). We therefore further tested the “+2 rule” by constructing 20 L10P(DX) derivatives, which possessed Asp at position 2 and 1 of 20 residues at position 3.

**The Residue at Position 3 Influences the Potency of the Inner Membrane Retention Signal Asp at Position 2**—Each L10P(DX) derivative was induced with 0.2% arabinose, and then the inhibition of growth was examined (Fig. 2). L10P(DS) (Fig. 2A) and 12 other derivatives, possessing Asp, Glu, Gln, Asn, Arg, Gly, Pro, Met, Phe, Tyr, Trp, or Leu (data not shown) at position 3, caused growth inhibition. On the other hand, L10P(DC) (Fig. 2B) and six other derivatives, having Ala, Ile, Thr, Val, Lys, or His (data not shown) at position 3, did not inhibit growth, suggesting that these seven residues negate the potency of the inner membrane retention signal Asp at position 2. It has been reported that His and Lys at position 3 cause the localization of a portion of lipoproteins in the outer membrane even when the residue at position 2 is Asp, whereas Gln and Tyr at position 3 do not affect their inner membrane localization (8).

The LolA-dependent release of L10P(DX) was examined in spheroplasts. When assayed with a subsaturating concentra-

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1 The abbreviations used are: Lpp, the major outer membrane lipoprotein; PAGE, polyacrylamide gel electrophoresis.

### Table I

| Oligonucleotides used for the construction of L10P(XS) derivatives |
|----------------------------------|
| Derivative | X1–X4 | X5–X6 | Derivative | X1–X4 | X5–X6 |
| AS | GCC | GCC | MS | ATG | CAT |
| CS | TGC | GCA | NS | AAC | GCT |
| ES | GAG | CTC | PS | CCC | GGG |
| FS | TTC | GAA | QS | CAG | CTC |
| GS | GGC | GCC | RS | CGG | GGG |
| TS | ATC | CTC | TS | ACC | GGT |
| IS | ATC | GAT | VS | GTC | GAC |
| KS | AAG | CTT | WS | TGG | CCA |
| LS | CTC | GAG | YS | TAC | GTA |

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The sequences of primers A and B are 5′-GGTTTACTCTGCTGCGGCCGCGCCGCGC-3′ and 5′-CGATTGATACGCTAAATTACC-3′, respectively.
tion (2 μg/ml) of LolA (6), no release occurred with 13 L10P(DX) derivatives (Fig. 3). These derivatives were ones having an inhibitory effect on growth (Fig. 2). A small portion of other L10P(DX) derivatives was released in a LolA-dependent manner, whereas the majority of these derivatives remained in spheroplasts. Faster migrating bands were observed with derivatives having Cys, Ala, or Val at position 3. These bands were observed for supernatant fractions irrespective of the presence or absence of LolA, suggesting that they are non-lipidated degradation products.

When examined with a saturating concentration (20 μg/ml) of LolA, LolA-dependent release occurred with the most derivatives (Fig. 4). However, the amounts of the released derivatives varied depending on the species of the residue at position 3. When the residue at position 3 was Thr, Cys, Ala, Ile, Val, Lys, or His, the efficiency of the release was more than 50%. These derivatives were ones having no inhibitory effect on growth. When the residue at position 3 was Asp, Glu, or Gln, no release occurred even with 20 μg/ml LolA, indicating that Asp-Asp, Asp-Glu, and Asp-Gln are strong inner membrane retention signals. Asp-Asn and Asp-Arg also functioned as strong inner membrane retention signals because only a portion of lipoproteins was released. The release assays with 2 and 20

| Derivative | X₁-X₆ | X₇-X₁₂ | Derivative | X₁-X₆ | X₇-X₁₂ |
|-----------|--------|--------|-----------|--------|--------|
| DA        | GACGCA | TGGCTC | DN        | GACAC  | GTGTC  |
| DC        | GACTGC | GCAGTC | DP        | GACCC  | GGGTC  |
| DD        | GACGAC | TCTGTC | DQ        | GACGAG | CTGTC  |
| DF        | GACTTC | GAACTC | DR        | GACCTC | GAGTC  |
| DG        | GACGTC | GCCGTC | DT        | GACACC | GTGTC  |
| DH        | GACCA  | GTGTC  | DV        | GACGTC | GACGTC |
| DI        | GACATC | GTAGTC | DW        | GACTGG | CCAGTC |
| DK        | GACAAA | TTTGTC | DY        | GACTAC | GTAGTC |
| DL        | GACCTC | GAGTTC | ND        | AAAGAC | GTGTC  |
| DM        | GACATG | CATGTC | SD        | TCCGAC | GTGGA  |

**Fig. 1.** Among 20 L10P(DX) derivatives, only L10P(DS) remains in the inner membrane. A, amino acid sequence of L10P. The residues at positions 11–57 are not shown. B, the release of L10P(SS) and L10P(DS) from spheroplasts was examined in the presence of the indicated concentrations of LolA. C, the amounts of L10P derivatives released at the indicated LolA concentrations were determined and expressed as percentages of the total amounts of the derivatives.

**Fig. 2.** Effect of the expression of L10P(DX) on the growth of E. coli cells. E. coli DLP79-36 cells harboring a plasmid encoding 1 of 20 L10P(DX) derivatives were induced (closed circles) or not induced (open circles) with 0.2% arabinose at the times indicated by arrows. Growth was monitored as described under “Experimental Procedures.” The results obtained with L10P(DS) and L10P(DC) are shown in A and B, respectively. Essentially the same results as for L10P(DS) were obtained for derivatives having the following residues at position 3: Asp, Glu, Gln, Asn, Arg, Gly, Pro, Met, Phe, Tyr, Trp, and Leu. Besides L10P(DC), derivatives having the following residues at position 3 exhibited no growth inhibition: Ala, Ile, Thr, Val, Lys, and His.
Asp at position 2 have chosen a residue at position 3 that functions as a very weak inner membrane retention signal. The major fraction of L10P(ND) remained in the outer membrane (9), it seemed highly unlikely that Asp at position 3 causes partial inner membrane localization of AcrE. It has been reported that Asp introduced at position 3 causes partial inner membrane localization of a lipoprotein (8). Because the N-terminal third residue of AcrE is Asp, we analyzed the effects of Asn and Asp on inner membrane retention (Fig. 6). These observations indicate that native lipoproteins with Asp at position 2 have chosen a residue at position 3 that ensures inner membrane retention.

Asn-Asp Is a Combined Inner Membrane Retention Signal—AcrE is a lipoprotein component of the multidrug exporter system functioning in the inner membrane of E. coli (13, 14) and possesses Asn instead of Asp at position 2 (15). Because L10P(ND) was released as efficiently as L10P(NS) was (Fig. 1), and because lipoMalE having Asn at position 2 was exclusively localized in the outer membrane (9), it seemed highly unlikely that Asn at position 2 alone causes the inner membrane retention of AcrE. It has been reported that Asp introduced at position 3 causes partial inner membrane localization of a lipoprotein (8). Because the N-terminal third residue of AcrE is Asp, we analyzed the effects of Asn and Asp on inner membrane retention (Fig. 6). As shown in Fig. 1, L10P(SS) was completely released even with 2 μg/ml LoLA. On the other hand, most L10P(SD) molecules remained in spheroplasts with 2 μg/ml LoLA but were released with 20 μg/ml LoLA, indicating that Asp at position 3 functions as a very weak inner membrane retention signal. The major fraction of L10P(ND) remained in spheroplasts even with 20 μg/ml LoLA, indicating that the combination of Asn at position 2 and Asp at position 3 is a strong inner membrane retention signal. Judging from the LoLA-dependent release, the inner membrane retention potency of L10P(ND) is similar to that of L10P(DR) (Fig. 5).

Consistent with the LoLA-dependent release, expression of L10P(ND) inhibited the growth of cells, whereas that of L10P(SD) had no effect (data not shown).

Phe-Asn and Trp-Asn Are Artificial Inner Membrane Retention Signals—Seydel et al. (9) recently examined lipoprotein-sorting signals with a lipid-modified derivative of MalE (lipoMalE), which has 4 N-terminal residues (CDNS) derived from a lipoprotein, PulA (16), followed by the mature region of MalE (17). They found that when the residue at position 2 was Asp, Phe, Gly, Pro, Trp, or Tyr, a portion of lipoMalE was localized in the inner membrane, where it caused MalE-dependent growth (9). Because the majority of lipoMalE having Gly at position 2 was localized in the outer membrane (9), a small amount of functional lipoMalE enables cells to grow in the presence of maltose. In contrast to the results with lipoMalE, the LoLA-dependent release revealed that, among 20 L10P(XS) derivatives, only L10P(DS) remained in the inner membrane (Fig. 1). Because the residue at position 3 is Asn for lipoMalE and Ser for L10P(XS), we thought that the different residue at position 3 might have caused the different results. We therefore examined the LoLA-dependent release of L10P derivatives having Ser, Gly, Trp, or Phe at position 2 and either Asn or Ser at position 3 (Fig. 7). Consistent with the reported results, the major fractions of L10P(WN) and L10P(FN) remained in spheroplasts even in the presence of a saturating concentration of LoLA, whereas both L10P(SN) and L10P(GN) were efficiently released. In contrast, the four derivatives having Ser at position 3 were efficiently released. Because native lipoproteins possessing Trp-Asn or Phe-Asn as the N-terminal 2nd and 3rd residues are not present in E. coli, they are artificial inner membrane retention signals. These results also indicate that...
Asn at position 2 (Fig. 6) or 3 (Fig. 7) enhances the potency of inner membrane retention signals.

**DISCUSSION**

The results presented here further support the importance of Asp at position 2 for the inner membrane retention of lipoproteins. When the residue at position 3 was fixed as Ser, only Asp at position 2 caused the retention of most lipoprotein molecules in the inner membrane (Fig. 1). When the residue at position 2 was fixed as Asp, significant inner membrane retention occurred with many species of residues at position 3 (Fig. 4). However, as suggested previously (8), our results revealed that the inner membrane retention because of Asp at position 2 was variously affected by the species of residue at position 3, indicating that Asp at position 2 alone has a tendency to cause the retention of lipoproteins in the inner membrane but is not sufficient for a strong inner membrane retention signal. The strongest inner membrane retention signals were found to be Asp-Asp, Asp-Glu, and Asp-Gln. Because Asp-Asn was also a potent inner membrane retention signal, it appeared that an acidic residue or its amide form at position 3 makes Asp at position 2 the strongest inner membrane retention signal. Asp-Arg was also a strong inner membrane signal, whereas Asp-Lys rather functioned as an outer membrane signal. We cannot explain at present why similar basic residues at position 3 caused opposite results. It is also puzzling why native lipoproteins having Asp-Glu are absent in *E. coli*, whereas Asp-Asp and Asp-Gln are utilized for native inner membrane-specific lipoproteins. In any event, it seems clear that only potent inner membrane retention signals are used for native inner membrane-specific lipoproteins. In contrast, ambiguous sorting signals causing less efficient release (Fig. 4) and hence localization in both membranes (8, 9) were not found for native lipoproteins. This is presumably important for most, if not all, lipoproteins involved in various activities in the cell envelope.

Among the 90 native lipoproteins encoded by the chromosome, only AcrE is known to remain in the inner membrane without Asp at position 2 (13, 15). The residues at positions 2 and 3 of this lipoprotein are Asn and Asp, respectively. The LolA-dependent release of L10P(ND) revealed that the strong inner membrane retention of this derivative was dependent on both Asn and Asp (Fig. 6). Thus, Asp at position 2 is not absolutely essential for the inner membrane retention signal, although this is a rare case. The residue at position 4 of L10P is Asn (Fig. 1) and that of AcrE is Lys. It is therefore highly unlikely that the 4th residue also contributes to the inner membrane retention of AcrE or L10P(ND). Moreover, it has been reported that the introduction of Asp at position 4 did not affect the outer membrane localization of lipoproteins, whereas Asp introduced at position 3 functioned as a weak inner membrane retention signal (8). These observations suggest that the 4th N-terminal residue has little influence on the lipoprotein-sorting signal.

L10P(NS) was efficiently released, whereas L10P(DS) was strongly retained in the inner membrane (Fig. 1), indicating that post-translational conversion of Asn into Asp through a deamidation reaction did not take place for L10P(NS). It may be possible that combinations of residues at positions 2 and 3 other than those examined here also function as inner membrane retention signals. However, even if this were the case, the number of combined inner membrane retention signals present in native lipoproteins is likely to be limited, because Ser is most frequently found at both positions 2 and 3 of native lipoproteins. Therefore, as far as native lipoproteins are concerned, we think that the evaluation of sorting signals described here seems to be nearly complete.

Why do lipoproteins having an inner membrane retention signal remain in the inner membrane? It seems clear that most lipoproteins in *E. coli* are outer membrane-specific, and only a handful of lipoproteins remain in the inner membrane. This seems to be related to how Gram-negative bacteria generate outer membrane-associated proteins. Proteins having a cluster of hydrophobic amino acid residues remain in the inner membrane and cannot reach the outer membrane, because a cluster of hydrophobic residues functions as a stop transfer or signal anchor sequence (18). Instead, proteins spanning the outer membrane are known to have an amphipathic β-structure (18) possessing alternating hydrophobic residues, which does not function as a stop transfer or signal anchor sequence. Lipid modification of lipoproteins occurs on the outer surface of the inner membrane (1, 5) and therefore does not inhibit the translocation (5). Thus, both β-structure and lipid modification are characteristic of outer membrane-associated proteins. This seems to be the reason why the Lol system has been developed to transport most lipoproteins to the outer membrane. If the physiological significance of the Lol system is the transport of lipoproteins to the outer membrane, the inner membrane retention signal may be a signal that somehow enables lipoproteins to avoid the action of the Lol system. The detailed molecular mechanism underlying the signal recognition by the Lol system is currently under investigation. The strongest inner membrane retention signals revealed here and non-native inner membrane retention signals found by Seydel et al. (9) seem to be useful for such investigations.

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