Mechanism Underlying the Inner Membrane Retention of Escherichia coli Lipoproteins Caused by Lol Avoidance Signals

Received for publication, July 20, 2003
Published, JBC Papers in Press, August 1, 2003, DOI 10.1074/jbc.M307836200

Takashi Hara, Shin-ichi Matsuyama‡, and Hajime Tokuda§
From the Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

Escherichia coli lipoproteins are localized to either the inner or outer membrane depending on the residue at position 2. The inner membrane retention signal, Asp at position 2 in combination with certain residues at position 3, functions as a Lol avoidance signal, i.e. the signal inhibits the recognition of lipoproteins by LolCDE that releases lipoproteins from the inner membrane. To understand the role of the residue at position 2, outer membrane-specific lipoproteins with Cys at position 2 were subjected to chemical modification followed by the release reaction in reconstituted proteoliposomes. Sulfhydryl-specific introduction of nonprotein molecules or a negative charge to Cys did not inhibit the LolCDE-dependent release. In contrast, oxidation of Cys to cysteic acid resulted in generation of the Lol avoidance signal, indicating that the Lol avoidance signal requires a critical length of negative charge at the second residue. Furthermore, not only modification of the carboxylic acid of Asp at position 2 but also that of the amine of phosphatidylethanolamine abolished the Lol avoidance function. Based on these results, the Lol avoidance mechanism is discussed.

Bacterial lipoproteins are synthesized as precursors in the cytoplasm and then translocated across the inner membrane by the Sec translocation machinery (1, 2). Subsequent processing to mature lipoproteins occurs on the periplasmic side of the inner membrane. The N-terminal Cys of mature lipoproteins is modified by thioether-linked diacylglycerol and amino-linked acyl chain (3). In Escherichia coli, lipoproteins are anchored through N-terminal lipids to the periplasmic leaflet of either the inner or outer membrane. It has been proposed that Asp at position 2 makes lipoproteins specific to the inner membrane, whereas other residues direct lipoproteins to the outer membrane (4). We recently revealed that the inner membrane retention of lipoproteins is determined by Asp at position 2 but also affected by the residue at position 3 (5). Thus, strong inner membrane retention occurs with Asp at position 2 and Aep, Glu, Gln, or Aan at position 3. Other residues at position 3 decrease the inner membrane retention of lipoproteins to various extents depending on the species. Importantly, E. coli native lipoproteins specific to the inner membrane only utilize these strong inner membrane signals (5).

The Lol system, which is composed of five Lol proteins, is involved in the sorting and outer membrane localization of lipoproteins. The LolCDE complex in the inner membrane belongs to the ATP-binding cassette transporter superfamily and comprises two copies of an ATPase subunit, LolD, and one copy each of the integral membrane subunits LolC and LolE. This complex releases outer membrane-directed lipoproteins from the inner membrane in an ATP-dependent manner (6–8), leading to the formation of a water-soluble complex comprising one molecule each of lipoprotein and LolA in the periplasm (9, 10). The LolA-lipoprotein complex then interacts with outer membrane receptor LolB, which catalyzes the anchoring of lipoproteins to the outer membrane (11, 12). The crystal structures of LolA and LolB are very similar to each other despite the fact that their amino acid sequences are dissimilar (13). The inner membrane retention signal functions as a Lol avoidance signal and inhibits the recognition of lipoproteins by LolCDE, thereby causing the retention of lipoproteins in the inner membrane (14). The Lol avoidance is not caused by a difference in the mode of lipid modification between inner membrane-specific and outer membrane-specific lipoproteins (15). A LolC mutant, LolC(A40P), that releases lipoproteins possessing the Lol avoidance signal was recently isolated (16). Because both LolA and LolB could interact with inner membrane-specific lipoproteins released by the mutant LolCDE complex, the inner membrane retention signal was found to only function against LolCDE. However, it remains to be clarified why Asp at position 2 has such a specific function.

To clarify critical properties required for the Lol avoidance signal, the second residue of lipoproteins was chemically modified, and the release of the modified lipoproteins was examined in proteoliposomes reconstituted with E. coli phospholipids and LolCDE. Here we show that the distance between Ca and the negative charge of the second residue is critical for the Lol avoidance signal. Furthermore, phosphatidylethanolamine was found to be essential for the Lol avoidance mechanism.

EXPERIMENTAL PROCEDURES

Materials—Globomycin was a kind gift from Masatoshi Inukai (Sankyo Co.). Sucrose monocaprate and octylglucoside were purchased from Dojindo Laboratories. Avidin-HRP was a kind gift from Fujioji Laboratories. Avidin-HRP was from Amersham Bio-

**This work was supported by grants (to H. T.) from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Life Science, Rikkyo University, 3-34-1, Nishi-ikebukuro, Toshima-ku, Tokyo 171-8501, Japan.
§ To whom correspondence should be addressed. Tel.: 81-3-5841-7830; Fax: 81-3-5841-8464; E-mail: htokuda@iam.u-tokyo.ac.jp.

The abbreviations used are: avidin-HRP, streptavidin-horseradish peroxidase conjugant; PE, phosphatidylethanolamine; CL, cardiolipin; PC, phosphatidyicholine; IPTG, isopropyl-β-D-thiogalactopyranoside; octylglucoside, n-octyl-β-D-glucopyranoside; sucrose monocaprate, β-n-fructopyranosyl-β-D-glucopyranoside monodecanolate; maleimide biotin, (+)-biotinyl-3-maleimidopropionanilidyl-3, 6-dioxoactanediamine; iodoacetyl biotin, (+)-biotinyl-iodoacetamidyl-3, 6-dioxoactanediamine; biotin PEO-amine, (+)-biotinyl-3, 6-dioxoactanediamine; SNA, sulfo-N-hydroxysuccinimide acetate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochloride; AMS, 4-acetamido-4’-maleimidystilbene-2, 2’-disulfonic acid disodium salt; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; SH, sulphydryl; MES, 4-morpholinethanesulfonic acid.
Bacteria and Plasmids—E. coli K12 strain JM83 (F− ara λlac proAB) pRS415 (800 lacZΔM15) (18) was grown on L broth at 30 or 37 °C. When required, the medium was supplemented with 50 μg/ml ampicillin or 25 μg/ml chloramphenicol. Plasmids pTPH21 (15) and pKM301 (14) carry pal-his and lolE, respectively, under the control of tacPO and lacP. pKM402 (14) carries lolC and lod-lhs under the control of fBAD.

Construction of Pal Derivatives—To construct plasmids encoding His-tagged Pal(2D), Pal(2D3D), and Pal(2D3D4C) under the control of tacPO and lacP, pTPH21 was digested with Sall and HindIII, followed by Klenow enzyme to make the ends blunt, and then subjected to self-ligation. The Mg1-SphI fragment of the resultant plasmid encompassing the signal cleavage region of pal was replaced with the specified double-stranded synthetic oligonucleotides listed in Table I.

Construction of Synthetic Lipoproteins—A synthetic lipoprotein precurso was designed so as to have the signal peptide of Pal at the N terminus, a T7 tag and a His tag at the C terminus of the mature lipid, and no carbonyl acid in the mature region except for the C-terminal one. The gene for the designed lipoprotein was inserted into the EcoRI-HindIII site of pTTQ18 (Amersham Biosciences) under the control of tacPO and lacP. However, expression of the initially designed lipoprotein was very poor. To improve the expression, synthetic oligonucleotides containing random codons except those for Asp and Gln were inserted into the mature region. The plasmids thus constructed were transformed into JM83, and the expression of lipoproteins was examined. A plasmid, pTHSCLP(SS), was isolated from the clone that expressed the highest amount of SCLP(SS) and SCLP(DQ) were kept in 0.1 M MES-NaOH (pH 5.0) containing 1% octylglucoside, 50 mM MgSO4, 0.1 M NaCl, and then dialyzed overnight against 500 ml of the dilution buffer. The reconstituted proteoliposomes were collected by centrifugation at 100,000 × g for 2 h and resuspended in 0.1 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM MgSO4 and 0.1 M NaCl and then dialyzed overnight against 500 ml of the dilution buffer. The reconstituted proteoliposomes were collected by centrifugation at 100,000 × g for 2 h and resuspended in 0.1 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM MgSO4, 0.1 M NaCl, 2 mM ATP, and 1 mM TCEP.

Release of Lipoproteins from Reconstituted Proteoliposomes—The release method (14) was slightly modified. E. coli phospholipids (0.8 mg) and LolCDE. was kept at −80 °C in 50 mM potassium phosphate (pH 7.5) containing 2% sucrose monophosphate and 1 mM TCEP at 2 mg/ml. SCLP(SS) and SCLP(DQ) were kept in 0.1 M MES-NaOH (pH 5.0) containing 2% octylglucoside. LolA was purified as reported (9). Anti-Pal (8), anti-OmpA (19), and anti-SecG (20) antibodies were raised in rabbits as described. Anti-T7 tag antibodies were obtained from Novagen.

Release of Lipoproteins from Reconstituted Proteoliposomes—The release method (14) was slightly modified. E. coli phospholipids (0.8 mg) and LolCDE. was kept at −80 °C in 50 mM potassium phosphate (pH 7.5) containing 2% sucrose monophosphate and 1 mM TCEP at 2 mg/ml. SCLP(SS) and SCLP(DQ) were kept in 0.1 M MES-NaOH (pH 5.0) containing 2% octylglucoside. LolA was purified as reported (9). Anti-Pal (8), anti-OmpA (19), and anti-SecG (20) antibodies were raised in rabbits as described. Anti-T7 tag antibodies were obtained from Novagen.

Oxidation of Cys was performed according to the method of Hirs (21). For cross-linking of biotin PE-amine to the carboxylic acid of lipoproteins by means of a cross-linker, EDC, lipoproteins were incubated at 30 °C for 2 h in 100 mM MES-NaOH (pH 5.0) containing 1% octylglucoside, 50 mM EDTA, and 10 mM biotin PEO-amine. The reaction mixture was dialyzed against 200 ml of 100 mM MES-NaOH (pH 5.0) containing 1% octylglucoside for 2 h and then 200 ml of 200 mM HEPES-NaOH (pH 8.0) containing 2% octylglucoside for 6 h.

Oxidation of Cys was performed according to the method of Hirs (21). For cross-linking of biotin PE-amine to the carboxylic acid of lipoproteins by means of a cross-linker, EDC, lipoproteins were incubated at 30 °C for 2 h in 100 mM MES-NaOH (pH 5.0) containing 1% octylglucoside, 50 mM EDTA, and 10 mM biotin PEO-amine. The reaction mixture was dialyzed against 200 ml of 100 mM MES-NaOH (pH 5.0) containing 1% octylglucoside for 2 h and then 200 ml of 200 mM HEPES-NaOH (pH 8.0) containing 2% octylglucoside for 6 h.
Performic acid (200 µl) was added to the lipoprotein suspension, followed by standing at −20 °C for 150 min. After diluting the reaction mixture with ice-cold water, the lipoproteins were precipitated with acetone and then solubilized in 100 mM potassium phosphate (pH 7.5) containing 2% sucrose monocaprate.

E. coli phospholipids (100 mg) in 50 mM HEPES-NaOH (pH 8.0) containing 2% octylglucoside were treated with 25 mM SN at 4 °C and then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol to terminate the reaction.

Other Methods—SDS-PAGE for Pal derivatives and synthetic lipoproteins, SCLP(SS) and SCLP(DQ), was performed according to Laemmli (22) and Hussain et al. (23), respectively. Immunoblotting (24) and subcellular fractionation (11) were performed as described.

RESULTS

SH-specific Modification at Position 2 Does Not Inhibit the LolA- and LolCDE-dependent Release of Lipoproteins—Outer membrane lipoprotein Pal has no Cys residue except for the N-terminal lipid-modified one. Pal(2C), which possesses Cys at position 2 (Table I) and a His6 tag at the C terminus, was constructed and expressed in E. coli. It was previously shown that the His6 tag attachment does not affect lipoprotein sorting (15). Membrane localization of Pal(2C) was then examined in vivo. Consistent with our previous in vitro results (5), Cys at position 2 did not function as the Lol avoidance signal, and Pal(2C) was exclusively localized in the outer membrane (data not shown). Pal(2C) was purified and treated with SH-specific reagents. Modified Pal(2C) was anchored to proteoliposomes reconstituted with LolCDE and then subjected to the release reaction in the presence and absence of LolA (Fig. 1). Surprisingly, introduction of large molecules such as iodoacetyl biotin or AMS did not inhibit the release. Iodoacetyl biotin-treated Pal(2C) released into the supernatant reacted with biotin-specific streptavidin (Fig. 1A, lower panels), confirming the attachment of biotin to the released Pal(2C). Although the results are not shown, the modified Pal(2C) in the supernatant existed as a complex with LolA and was subsequently incorporated into outer membranes in a LolB-dependent manner. Taken to-
Lol Avoidance Signals of E. coli Lipoproteins

Fig. 3. Construction and expression of synthetic lipoproteins. A, the sequences of the precursor forms of synthetic lipoproteins, proSCLP(SS) and proSCLP(DQ), are indicated. B, E. coli JM83 cells harboring pTTQ18 (vector) or pTHSCLP(SS) were grown at 30 °C with or without 5 μg/ml globomycin. At the middle of the exponential phase of growth, 1 mM IPTG was added to induce the expression of SCLP(SS) for 1 h. The cells were analyzed by SDS-PAGE and Western blotting with antibodies against the T7-tag or OmpA. The precursor (p) and mature (m) forms of SCLP(SS) are indicated. C, JM83 cells harboring pTHSCLP(SS) or pTHSCLP(DQ) were induced by the addition of 1 mM IPTG for 1 h and then disrupted by a French pressure cell. Soluble fractions containing cytosol and periplasm (Sol), total membranes (TM), inner membranes (IM), and outer membranes (OM) were fractionated and then analyzed by SDS-PAGE and immunoblotting with antibodies against the T7 tag, SecG and OmpA. Total proteins of induced (+IPTG) or uninduced (−IPTG) cells were also analyzed.

gether, these results indicate that introduction of nonprotein molecules to the second residue of outer membrane-specific lipoproteins has no effect on their interaction with Lol proteins.

Pal(2C) was modified with iodoacetic acid to introduce a negative charge to the second position. However, this also did not inhibit the Pal(2C) release, which was dependent on both LolCDE and LolA (Fig. 1B), whereas Pal(2D) possessing Asp at position 2 was not released from proteoliposomes. It has been reported that Glu at position 2 does not exhibit the Lol avoidance function (5). These results suggest that the introduction of a negative charge alone is not sufficient for conversion of an outer membrane lipoprotein into an inner membrane one.

We next examined whether dimerization through a disulfide bond affects the release of Pal(2C). Pal(2C) was either reduced with TCEP or oxidized with potassium ferricyanide and then reconstituted into proteoliposomes. The Pal(2C) dimer was only detected with potassium ferricyanide-treated Pal(2C) (Fig. 1C). The monomeric forms of Pal(wt) and Pal(2C) were released on the addition of LolA, whereas LolA did not induce the release of dimeric Pal(2C), indicating that only the lipoprotein monomer acts as a substrate for the release reaction.

Oxidation of Cys at Position 2 Inhibits the Release of Lipoproteins—Because Glu at position 2 does not function as the Lol avoidance signal (5), it seems possible that the distance between the negative charge and Cα of the main chain is critical for the Lol avoidance signal. To examine this, Cys of Pal(2C) was oxidized with performic acid to generate cysteic acid, of which the Cα-negative charge distance is similar to that of Asp (for details, see Fig. 7). As shown in Fig. 2, the LolA-dependent release of Pal(wt) was not affected by treatment with performic acid. Pal(2D) remained in proteoliposomes after the treatment with performic acid. Strikingly, the release of Pal(2C) was nearly completely inhibited by this treatment. We also oxidized Pal(3C) and Pal(4C), which possess Cys at positions 3 and 4, respectively. Inhibition of the release reaction by performic acid treatment was dependent on the position of Cys; inhibition of the release was significant with Pal(3C) and almost nothing with Pal(4C). These results are consistent with the observation that Asp at position 2 functions as the Lol avoidance signal, whereas at position 4 does not (25). After the performic acid treatment, none of the Pal derivatives possessing Cys formed a disulfide-bonded dimer upon treatment with potassium ferricyanide, indicating that SH was completely converted to sulfonate.

Chemical Modification of Asp at Position 2 Abolishes Its Lol Avoidance Function—To examine the importance of the Asp negative charge for the interaction with Lol protein, synthetic lipoproteins, SCLP(SS) and SCLP(DQ), were constructed (Fig. 3A). The former has no negative charge except for that of the C-terminal carboxylic acid, whereas the latter has Asp at position 2 followed by Gln, thereby having a strong Lol avoidance signal (5). Both lipoproteins have T7 and His6 tags at the C termini for their detection and purification. In vivo processing of the SCLP(SS) precursor to the mature form was inhibited by globomycin, an inhibitor of lipoprotein-specific signal peptidase (Fig. 3B). SCLP(SS) was localized in the outer membrane, whereas SCLP(DQ) was exclusively found in the inner membrane (Fig. 3C), indicating that membrane sorting of these synthetic lipoproteins takes place according to the “+2 rule” (4).

SCLP(SS) and SCLP(DQ) were purified and treated with carboxylate-specific cross-linker EDC in the presence of biotin-PEO-amine. After this treatment, both lipoproteins reacted with avidin-HRP, indicating that the C-terminal carboxylate was modified with biotin (data not shown). SCLP(SS) and SCLP(DQ) treated with or without EDC-biotin were reconstituted into proteoliposomes and then subjected to the release reaction (Fig. 4). Whether treated or not treated with EDC-biotin, SCLP(SS) was released from proteoliposomes on the addition of LolA, indicating that the C-terminal modification
Modification was terminated by the addition of 150 mM with the solubilized samples. with avidin-HRP or anti-Pal antibodies. The amounts of Pal molecules analyzed by SDS-PAGE were 5-fold for the reconstituted samples compared with the solubilized samples.

with biotin does not inhibit the release reaction. LolA did not induce the release of SCLP(DQ) that had not been treated with EDC-biotin. On the other hand, upon treatment with EDC-biotin, a significant portion of SCLP(DQ) was released on the addition of LolA, indicating that the carboxylic acid of Asp at position 2 was cross-linked with biotin, and therefore the negative charge required for the Lol avoidance function was lost.

Cys at Position 2 of Lipoproteins Anchored to Membranes Is Inaccessible from the Solvent—The accessibility of Cys at position 2, 3, or 4 of lipoproteins was examined in proteoliposomes. Six Pal derivatives possessing Cys (Table I) were modified with maleimide biotin in the presence of a detergent, sucrose monolaurate, and therefore detected with avidin-HRP (Fig. 5). When modification with this membrane-impermeable maleimide biotin was examined after reconstitution into proteoliposomes, Pal derivatives having Cys at position 2 were not modified, whereas those having Cys at position 3 or 4 were modified. An Asp residue introduced to position 2 or 3 or both did not affect the accessibility of Cys. These results suggest that when lipoproteins are anchored to membranes, Cys at position 2 is not exposed to the solvent.

Phosphatidylethanolamine Is Required for the Lol Avoidance Function of Asp at Position 2—The results presented above suggested that the residue at position 2 of lipoproteins was present in a lipid environment. Considering the importance of the negative charge of Asp, it seemed to be possible that amine-specific modification of PE affects the Lol avoidance function of Asp at position 2. To address this issue, E. coli phospholipids containing PE, phosphatidylglycerol, and CL as the major phospholipids were treated with amine-specific reagent SNA. Thin layer chromatography revealed that PE was specifically modified, i.e. the amount of PE decreased with the concomitant appearance of a new spot (data not shown). Proteoliposomes were reconstituted with SNA-treated or -untreated E. coli phospholipids. The LolA- and LolCDE-dependent release of Pal(wt) was not affected by the SNA treatment (Fig. 6A). Pal(2D) was not released from proteoliposomes reconstituted from SNA-untreated phospholipids as observed before. On the other hand, Pal(2D) was released from proteoliposomes in LolA- and LolCDE-dependent manners when SNA-treated phospholipids were used.

To further examine the requirement of PE for the Lol avoidance mechanism, proteoliposomes were reconstituted with CL alone (Fig. 6B). Pal(2D) was released from CL-proteoliposomes as efficiently as Pal(wt) was. This release was completely inhibited by orthovanadate, an inhibitor of LolCDE (7). On the other hand, the Lol avoidance signal functioned in proteoliposomes reconstituted with PC alone. Taken together, these results indicate that the positive charge of phospholipids is important for the Lol avoidance function of Asp at position 2.

**DISCUSSION**

The N-terminal second residue of lipoproteins has been thought to play a crucial role in the determination of membrane specificity (4, 5, 26). However, SH-specific introduction of nonprotein molecules to the second residue did not inhibit the release of outer membrane-specific lipoproteins. These results strongly indicate that LolCDE releases outer membrane-specific lipoproteins without recognizing the second residue. It was recently found that an apolipoprotein lacking the N-terminal acyl chain was not recognized by LolCDE (15). Therefore, LolCDE seems to only recognize the N-terminal Cys possessing three acyl chains, the sole common structure of lipoproteins.
What happens if lipoproteins have Asp at position 2? It seems plausible that Asp at position 2 in combination with certain residues at position 3 makes the N-terminal structure of lipoproteins distinctive, thereby preventing the recognition of the lipid-modified Cys by LolCDE.

Neither iodoacetate-modified Cys nor Glu (5) at position 2 functioned as the Lol avoidance signal, whereas oxidation of Cys at position 2 to cysteic acid resulted in generation of the Lol avoidance signal. Conversely, modification of the carboxylic acid of Asp at position 2 abolished its Lol avoidance function. These results, taken together, indicate that the Lol avoidance signal should have a negative charge that is within a certain distance from Cα of the second residue (Fig. 7), which is not accessible from the solvent. Amine-specific modification of PE and substitution of PE with CL abolished the Lol avoidance function of Asp at position 2. In contrast, the Lol avoidance signal functioned in proteoliposomes reconstituted with PC. These results, taken together, strongly suggest that the electrostatic and steric complementarity between Asp at position 2 and phospholipids having a positive charge is responsible for the Lol avoidance mechanism. It seems less likely that a positive charge of phospholipids is required for the proper functioning of LolCDE, although this cannot be completely excluded at present.

When Glu, Asp, Gln, or Asn is at position 3, Asp at position 2 becomes a very strong Lol avoidance signal (5). In contrast, His, Lys, Val, Ile, Ala, Cys, or Thr at position 3 significantly decreases the Lol avoidance function of Asp at position 2. Other residues also affect the potency of the Lol avoidance signal. Residues that strengthen the Lol avoidance function of Asp are acidic ones or their amide forms that do not undergo ionic interaction with Asp at position 2. It therefore seems likely that the third residue should not disturb the steric and electrostatic complementarity between Asp at position 2 and PE for the Lol avoidance mechanism. Interaction between Asp at position 2 and PE is likely to be strengthened by hydrogen bonds formed...
between Cys at position 1 and the PE molecule interacting with Asp at position 2 (Fig. 8). On the other hand, when Glu possessing a longer side chain is at position 2, the PE molecules involved in the electrostatic interaction with Glu and the hydrogen bond formation with Cys at position 1 would be different; therefore the Glu-PE interaction is not strengthened. The third residues that strengthen the Lol avoidance signal are also expected to form a salt bridge or hydrogen bond with the PE molecule interacting with Asp at position 2. Based on these considerations, we speculate that the Lol avoidance signal causes the formation of a tight lipoprotein-PE complex (Fig. 8). This complex has five acyl chains and cannot be accommodated in LolCDE. The release incompetence of the Pal(2C) dimer and apolipoprotein seems to indicate that the number of acyl chains is critical for the recognition by LolCDE. The LolCIA40PIDE complex is likely to be able to accommodate such a lipoprotein-PE complex, thereby causing the release of lipoproteins having the Lol avoidance signal. Arg at position 3 strengthened the Lol avoidance function of Asp at position 2, whereas Lys at position 3 did not (5). Perturbation of the electrostatic Asp-PE interaction by the positive charge at position 3 seems to be dependent on the side chain structure of the third residue.

Seydel et al. (26) reported that not only Asp but also residues such as Phe, Pro, and Trp at position 2 followed by Asn at position 3 cause the inner membrane retention of lipoproteins, although E. coli native lipoproteins do not have Phe, Pro, and Trp at position 2. We have reported that when the residue at position 3 is Ser, these residues at position 2 do not cause the inner membrane retention of lipoproteins (5). It seems likely that the mechanism of inner membrane retention caused by these hydrophobic residues at position 2 is different from the mechanism by which native lipoproteins remain in the inner membrane.

It should also be noted that Asp at position 2 may not always be the Lol avoidance signal in other bacteria, although all Lol proteins are conserved in various Gram-negative bacteria (7). For example, MexA in Pseudomonas species is an inner membrane lipoprotein possessing Gly and Lys at positions 2 and 3, respectively. In this particular case, the ionic interaction between PE and the second residue is not applicable to the Lol avoidance mechanism. Because membrane localization and sorting signals have been biochemically determined for only a few lipoprotein in other bacteria, it remains to be determined how widely the Lol avoidance mechanism shown here is applicable to the inner membrane-specific lipoproteins in bacteria. However, the Lol avoidance mechanism is likely to be required for the localization of PulA of Klebsiella oxytoca on the outer surface of the outer membrane. Pugsley and collaborators (2) examined the membrane localization of PulA in E. coli with or without a subset of pul genes comprising the Type II secretion pathway. Wild-type PulA having Asp at position 2 was localized on the outer surface of the outer membrane when expressed with the Type II secretion pathway. In contrast, PulA was exclusively localized on the periplasmic surface of the inner membrane in the absence of the Type II pathway, indicating that Asp at position 2 functions as the Lol avoidance signal in the absence of the Type II pathway. Substitution of Asp with another residue resulted in the localization of PulA on both the periplasmic surface and the outer surface of the outer membrane when the Type II secretion pathway was present. However, this PulA derivative was exclusively localized on the periplasmic surface of the outer membrane in the absence of the Type II secretion pathway. These observations and our findings, taken together, indicate that PulA expressed in E. coli and, presumably, K. oxytoca should have a Lol avoidance signal to be efficiently translocated to the outer surface of the outer membrane through the Type II pathway, otherwise the Lol system causes the localization of PulA to the periplasmic surface of the outer membrane.

The structural characteristics critical for the Lol avoidance function were revealed in this study. Because the structure of LolCDE also affects the Lol avoidance function (16), it is of great interest to identify the localized structure of LolCDE that determines the Lol avoidance.

Acknowledgments—We thank Kazuki Takeda (Riken-Harima Institute) for valuable discussions, Masatoshi Inukai (Sankyo Co.) for the globomycin, and Rika Ishihara for technical support.

REFERENCES
1. Hayashi, S., and Wu, H. C. (1990) J. Bioenerg. Biomembr. 22, 451–471
2. Pugsley, A. P. (1993) Microbiol. Rev. 57, 50–108
3. Sankaran, K., and Wu, H. C. (1994) J. Biol. Chem. 269, 19701–19706
4. Yamaguchi, K., Fujio, Y., and Inouye, M. (1988) Cell 53, 423–432
5. Terada, M., Kuroda, T., Matsuyama, S., and Tokuda, H. (2001) J. Biol. Chem. 276, 47690–47694
6. Yakushi, T., Yokota, N., Matsuyama, S., and Tokuda, H. (1998) J. Biol. Chem. 273, 32576–32581
7. Yakushi, T., Masuda, K., Narita, S., Matsuyama, S., and Tokuda, H. (2000) Nat. Cell Biol. 2, 212–218
8. Narita, S., Tanaka, K., Matsuyama, S., and Tokuda, H. (2002) J. Bacteriol. 184, 1417–1422
9. Matsuyama, S., Tajima, T., and Tokuda, H. (1995) EMBO J. 14, 3365–3372
10. Tajima, T., Yokota, N., Matsuyama, S., and Tokuda, H. (1996) FEBS Lett. 439, 51–54
11. Matsuyama, S., Yokota, N., and Tokuda, H. (1997) EMBO J. 16, 6947–6955
12. Tanaka, K., Matsuyama, S., and Tokuda, H. (2001) J. Bacteriol. 183, 6538–6542
13. Takeda, K., Miyatake, H., Yokota, N., Matsuyama, S., Tokuda, H., and Miki, K. (2003) EMBO J. 22, 3199–3209
14. Masuda, K., Matsuyama, S., and Tokuda, H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7390–7395
15. Fukuda, A., Matsuyama, S., Haru, T., Nakayama, J., Nagaawa, H., and Tokuda, H. (2000) J. Biol. Chem. 275, 43512–43518
16. Narita, S., Kanamaru, K., Matsuyama, S., and Tokuda, H. (2003) Mol. Microbiol. 49, 167–177
17. Tokuda, H., Shiozuka, K., and Mizushima, S. (1990) Eur. J. Biochem. 192, 583–589
18. Yakushi, T., Tajima, T., Matsuyama, S., and Tokuda, H. (1997) J. Bacteriol. 179, 2587–2592
19. Tani, K., Tokuda, H., and Mizushima, S. (1990) J. Bacteriol. 165, 17341–17347
20. Nishiyama, K., Mizushima, S., and Tokuda, H. (1995) EMBO J. 12, 3409–3415
21. Hirs, C. H. W. (1967) Methods Enzymol. 11, 197–199
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. Hussain, M., Ichihara, S., and Mizushima, S. (1980) J. Biol. Chem. 255, 3707–3712
24. Yamada, H., Matsuyama, S., Tokuda, H., and Mizushima, S. (1989) J. Biol. Chem. 264, 18577–18581
25. Gennity, J. M., and Inouye, M. (1991) J. Biol. Chem. 266, 16458–16464
26. Seydel, A., Gounon, P., and Pugsley, A. P. (1999) Mol. Microbiol. 34, 810–821
27. Engh, R. A., and Huber, R. A. (1991) Acta Crystallogr. Sect. A 47, 392–400