Roles of the Hydrophobic Cavity and Lid of LolA in the Lipoprotein Transfer Reaction in Escherichia coli**§

Received for publication, August 31, 2005, and in revised form, November 21, 2005. Published, JBC Papers in Press, December 14, 2005, DOI 10.1074/jbc.M509596200

Shoji Watanabe†, Shin-ichi Matsuyamaǂ, and Hajime Tokuda††

From the †Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan and the ‡Department of Life Science, Rikkyo University, 3-34-1 Nishi-ikebukuro, Toshima-ku, Tokyo 171-8501, Japan

LolA, a periplasmic chaperone, binds to outer membrane-specific lipoproteins released from the inner membrane through the action of an ATP-binding cassette transporter, LolCDE and then transfers them to the outer membrane receptor LolB, thereby mediating the inner to outer membrane transport of lipoproteins. The crystal structure of free LolA revealed that it has an internal hydrophobic cavity, which is surrounded by hydrophobic residues and closed by a lid comprising α-helices. The hydrophobic cavity most likely represents the binding site for the lipid moiety of a lipoprotein. It is speculated that the lid undergoes opening and closing upon the binding and transfer of lipoproteins, respectively. To determine the functions of the hydrophobic cavity and lid in detail, 14 residues involved in the formation of these structures were subjected to random mutagenesis. Among the obtained 21 LolA derivatives that did not support growth, 14 were active as to the binding of lipoproteins but defective in the transfer of lipoproteins to LolB, causing the periplasmic accumulation of a lipoprotein as a complex with a LolA derivative. A LolA derivative, I93G, bound lipoproteins faster than wild-type LolA did, whereas it did not transfer associated lipoproteins to LolB. When I93G and wild type LolA co-existed, lipoproteins were bound only to I93G; which therefore exhibited a dominant negative property. Another derivative, L59R, was also defective in the transfer of lipoproteins to LolB but did not exhibit a dominant negative property. Taken together, these results indicate that both the hydrophobic cavity and the lid are critically important for not only the binding of lipoproteins but also their transfer.

Escherichia coli has at least 90 lipoproteins (1–3), which are anchored through N-terminal lipids to the periplasmic side of either the inner or outer membrane. Lipoproteins synthesized as precursors are translocated through N-terminal lipids to the periplasmic side of either the inner or outer membrane. Lipoproteins synthesized as precursors are translocated through N-terminal lipids to the periplasmic side of either the inner or outer membrane. Lipoproteins synthesized as precursors are translocated through N-terminal lipids to the periplasmic side of either the inner or outer membrane.

**This work was supported by Grants 14037212 and 15208009 from the Ministry of Education, Science, Sports and Culture of Japan (to H. T.). 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.

†The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

†1 To whom correspondence should be addressed. Tel.: 81-3-5841-7830; Fax: 81-3-5841-8464; E-mail: htokuda@iam.u-tokyo.ac.jp.

‡1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

‡ Materials—FLAG M2 affinity gel and FLAG peptides were purchased from Sigma. Antibodies against LolA (12), Pal (18), Lpp (19), and

Experimental Procedures

Materials—FLAG M2 affinity gel and FLAG peptides were purchased from Sigma. Antibodies against LolA (12), Pal (18), Lpp (19), and
Hydrophobic Cavity and Its Lid of LolA

a hexahistidine tag (20) were raised in rabbits as described. Antibodies against maltose-binding protein were purchased from New England Biolabs. Tran\textsuperscript{35}S-label (a mixture of 70\% [\textsuperscript{35}S]Met and 20\% [\textsuperscript{35}S]Cys; 1000 Ci/mmol) was obtained from ICN. IgG Sorb was from Enzyme Center Inc.

Bacterial Strains and Media—E. coli K-12 strains XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacY1 proAB lacZM15 : Tn10 (Tetr)) (Stratagene), DLP7-36 (HfrC pps man lpp) (10), JE6505 (F\textsuperscript{−}, lpp pps his proAArgE thi gal lac ytl mtlsx2) (21), and TT016 (JE6505 lpp\textsuperscript{+} lacY1 proA + lolA) (15) were used. The last strain carries the chromosomal lolA gene under the control of the lactose promoter-operator. Expression of the chromosomal lolA gene was induced with 0.1 mM IPTG\textsuperscript{2} unless otherwise noted. L-broth was used as the standard medium. Labeling experiments were carried out in M63 minimal medium (22) supplemented with 0.5% NaCl, 0.2% maltose, 20 \( \mu \)g/ml thiamine, 20 \( \mu \)g/ml thymine, and 40 \( \mu \)g/ml all amino acids except methionine and cysteine. When required, chloramphenicol was added at a concentration of 25 \( \mu \)g/ml.

Construction of LolA Mutants—As reported for the construction of LolA derivatives R43L (15) and F47E (17), random mutagenesis of target amino acid residues was performed by means of PCR with a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol, with pAM201 carrying lolA-His under the control of P\textsubscript{BAD} (15) as a template and a pair of oligonucleotides (supplementary Table S1) as primers. The plasmid DNAs were amplified in the XL1-Blue strain lacking functional endonuclease R and then transformed into TT016. Chloramphenicol-resistant transformants were selected on L-broth plates supplemented with 0.1 mM IPTG, and then their growth in the absence of IPTG was examined on plates containing chloramphenicol. Some LolA derivatives containing FLAG-tagged LolA were adsorbed to an anti-FLAG M2 affinity column and eluted with 20 mM Tris-HCl (pH 8.0) containing 100 \( \mu \)g/ml FLAG peptide. Purified His-tagged or FLAG-tagged LolA proteins were dialyzed against 20 mM Tris-HCl (pH 8.0) overnight at 4\( ^\circ \)C and then kept frozen at \(-80\)\( ^\circ \)C.

Release of L10P from Spheroplasts—The reported method (12) was slightly modified to examine the release reaction after the completion of L10P maturation. Briefly, E. coli DLP7-36 cells harboring pYL10P (\textit{P\textsubscript{BAD}-L10P}) (10) were grown on M63 (0.5% NaCl), 0.2% maltose minimal medium at 37\( ^\circ \)C. At \(A\textsubscript{660}\) of 1.0, the cells were induced with 0.2\% arabinose for 5 min and then converted to spheroplasts. The spheroplasts were stabilized by the addition of 20 mM MgCl\textsubscript{2}, collected by centrifugation at 16,000 \( \times \) g for 2 min, and then resuspended in M63 (0.5% NaCl), 0.2% maltose minimal medium supplemented with 250 mM sucrose and 10 mM MgCl\textsubscript{2}. The suspension (300 \( \mu \)l) containing 5 \( \times \) \( 10^8\) spheroplasts was mixed with 750 \( \mu \)l of M63 (0.5% NaCl), 0.2% maltose minimal medium supplemented with 250 mM sucrose, 10 mM MgCl\textsubscript{2}, and 10 \( \mu \)Ci of Tran\textsuperscript{35}S-label, followed by 1-min pulse-labeling and a 10-min chase with 12 mM nonradioactive Met and Cys. His-tagged LolA proteins (2 \( \mu \)g/ml) were then added to induce the release of L10P. To terminate the reaction, the mixture was chilled in ice water, followed by centrifugation at 16,000 \( \times \) g for 2 min. The spheroplasts and supernatant thus obtained were subjected to trichloroacetic acid precipitation and immunoprecipitation with anti-Lpp antibodies as reported (12). Tran\textsuperscript{35}S-Labeled L10P was analyzed by SDS-PAGE and fluorography.

Inhibition of LolA-dependent Release by Derivatives—Spheroplasts prepared as described above were pulse-labeled with 10 \( \mu \)Ci of Tran\textsuperscript{35}S-label for 1 min in the presence of FLAG-tagged wild-type LolA (2 \( \mu \)g/ml) and His-tagged wild-type LolA, I93G, L59R, or R43L (0–2 \( \mu \)g/ml) in a total volume of 1,050 \( \mu \)l, followed by a 5-min chase with nonradioactive Met and Cys. The release reaction was terminated, and a spheroplast supernatant was then obtained as described above. The supernatant was diluted 10-fold with fresh M63 minimal medium supplemented with 0.5% NaCl, 0.2% maltose, and 250 mM sucrose. An equal volume of the diluted supernatant was then applied to affinity columns for FLAG-tagged proteins and His-tagged proteins. Proteins adsorbed to the columns were eluted with FLAG peptide or imidazole, as described for the purification of tagged LolA proteins, and then subjected to trichloroacetic acid precipitation and immunoprecipitation with anti-Lpp antibodies, followed by SDS-PAGE and fluorography.

In Vitro Outer Membrane Incorporation of L10P—LolB-dependent incorporation of L10P into outer membranes was examined using Tran\textsuperscript{35}S-labeled L10P released from spheroplasts, as described (13). Briefly, a spheroplast supernatant (1 ml) containing the LolA-L10P complex was centrifuged at 100,000 \( \times \) g for 30 min to remove insoluble materials. The supernatant was then incubated at 30\( ^\circ \)C for the indicated times in the presence of 0.2 mg/ml outer membranes. The outer membrane incorporation of L10P was terminated by chilling of the reaction mixture (200 \( \mu \)l) in ice water and analyzed after fractionation into a supernatant and pellet by centrifugation at 100,000 \( \times \) g for 30 min. The supernatant and pellet fractions thus obtained were analyzed by SDS-PAGE and fluorography after trichloroacetic acid precipitation.

Other Methods—Outer membranes were prepared from E. coli JE6505 cells as reported (13). Immunoprecipitation was performed as described (24). SDS-PAGE was carried out as described by Laemmli (25) and Hussain \textit{et al.} (19). Densitometric quantification was performed with an ATTO Densitograph.
RESULTS

Hydrophobic Cavity and Lid of LolA—Residues forming the hydrophobic cavity (Fig. 1A) are conserved among LolA homologs (italicized boldface letters in Fig. 1C). Arg43 forms hydrogen bonds with the main chain of Leu10 in the α1-helix, Val13 in the loop between the α1-helix and the β1-strand, and Ile93 and Ala94 in the α2-helix, thereby causing the tight fixation of the α1-helices to the β1-strands like a lid (Fig. 1B). The residues forming hydrogen bonds with Arg43 are aliphatic ones and are conserved among LolA homologues (boldface letters in Fig. 1C). The lid of LolA, comprising three α1-helices, is expected to undergo opening and closing upon the binding and transfer of lipoproteins, respectively (14).

To understand how the hydrophobic cavity and lid status affects the LolA function, 14 residues involved in the formation of the hydrophobic cavity and closing of the lid were subjected to random mutagenesis. LolA mutants that could not support the growth of E. coli were then screened for. Among 21 mutants thus isolated, five exhibited a dominant negative phenotype (i.e. expression of the mutant inhibited growth even in the presence of wild-type LolA) (Table 1). No defective mutant was obtained for Val13 and Tyr152 despite extensive attempts, suggesting that any residue can replace the original ones. Val13 forms a hydrogen bond with Arg43 in one crystal form (Protein Data Bank code 1UA8) of LolA, whereas it does not in another form (Protein Data Bank code 1UA8).

TABLE 1
Defective LolA mutants isolated in this study

| Function and residue | Mutant | Wild type | Mutant | Dominant negative^a | Periplasmic lipoprotein^b |
|----------------------|--------|-----------|--------|---------------------|--------------------------|
| Lid closing          | Leu10  | L10K CTG  | AAG    |                     |                          |
| Val13                | None   | GTC       |        |                     |                          |
| Ile93                | I93E ATT | GAG   |        |                     |                          |
| I93G                 | GGA    |          | +      |                     |                          |
| I93N                 | AAT    |          | +      |                     |                          |
| Ala94                | A94I GCC | ATT   |        |                     |                          |
| Ala94                | A94L TTA |       | +      |                     |                          |
| Cavity               | Phe20  | F20R TTC  | AGG    |                     |                          |
| Trp49                | W/49P TGG | CCG   |        |                     |                          |
| Leu59                | L59R CTG | CCA   |        |                     |                          |
| L59V                 | AGG    |          | +      |                     |                          |
| Phe90                | F90S TTG | TCT   |        |                     |                          |
| Met91                | M91A ATG | GCT   |        |                     |                          |
| Trp101               | W/101I TGG | ATT   |        |                     |                          |
| Ile106               | I106D ATC | GAT   |        |                     |                          |
| I106Q                | CAG    |          | +      |                     |                          |
| I106R                | CCA    |          | +      |                     |                          |
| Phe127               | F127P TTC  | CCG   |        |                     |                          |
| Tyr132               | None   |          | +      |                     |                          |
| ^a Expression of LolA derivatives with 0.2% arabinose inhibited the growth of T016 (lacPO-lolA) cells in the presence of 0.1 mM IPTG. ^b Expression of LolA derivatives caused the periplasmic accumulation of Pal and Lpp.

The structure of E. coli LolA is shown as gray globules, which are surrounded by 10 residues represented by gray sticks. Residues involved in the lid closing are depicted as a black ball-and-stick model and are indicated with boldface letters. The results of random mutagenesis and screening of LolA mutants are shown in Table 1. The structure around Arg at position 43 was visualized with PyMOL version 0.98 (available on the World Wide Web at pymol.sourceforge.net/index.php). Residues forming hydrogen bonds with Arg43 are shown as a stick model. Phe at position 140 interacts with Ile at position 93 and is also indicated in the stick model. The structural information on LolA (Protein Data Bank code 1UA8) was obtained from the RCSB Protein Data Bank (available on the World Wide Web at www.protein.osaka-u.ac.jp/pdb/). The sequences of LolA homologues were obtained from GenomeNet (available on the World Wide Web at www.genome.ad.jp) and aligned using ClustalW (available on the World Wide Web at clustalw.genome.jp). Arg at position 43 is highlighted in black. Boldface letters indicate residues forming hydrogen bonds with Arg43. The residues forming the hydrophobic cavity are indicated in italicized boldface letters, and α1-helices and β1-strands are indicated above the E. coli sequence. Asterisks indicate highly conserved residues. Eco, E. coli; Sty, Salmonella typhimurium; Ype, Yersinia pestis; Hin, Hemophilus influenzae; Vch, Vibrio cholerae.
Hydrophobic Cavity and Its Lid of LolA

1IWL) (14), suggesting that the hydrogen bonding between these two residues contributes little to the stabilization of the closed form of LolA. Among 10 residues surrounding the hydrophobic space, Tyr152 is located most distantly from the hydrophobic cavity (Fig. 1A). This may be the reason why no defective mutant was obtained for Tyr152.

Periplasmic Accumulation of Lipoproteins—Since the outer membrane localization of lipoproteins is very rapid, they are not detected in the periplasm under normal conditions (15). On the other hand, many residues introduced in place of Arg at position 43 were recently found to cause the periplasmic accumulation of lipoproteins as complexes with LolA derivatives (16). These mutations were found to increase the strength of the hydrophobic interaction between lipoproteins and LolA derivatives, thereby reducing the efficiency of lipoprotein transfer to LolB (16). Since we introduced mutations at residues involved in formation of the hydrophobic cavity or stabilization of the LolA lid, periplasmic accumulation of outer membrane-specific lipoproteins, Lpp and Pal, was examined in TTO16 cells expressing the respective LolA derivatives in the presence of wild-type LolA (Fig. 2).

When wild-type LolA was expressed from a plasmid, neither Lpp nor Pal was detected in the periplasm (Fig. 2, left lane). Maltose-binding protein (26) was examined as a control to confirm that similar amounts of periplasmic proteins were examined for the respective cultures. Among the defective LolA derivatives examined, 14 were found to cause the periplasmic accumulation of lipoproteins to various extents (Fig. 2 and Table 1), suggesting that these derivatives are active as to lipoprotein binding but defective in lipoprotein transfer. The properties of the I93G derivative were the most interesting among the defective derivatives, because it caused the most significant periplasmic accumulation of lipoproteins and exhibited a dominant negative phenotype, whereas two other defective derivatives, I93E and I93N, had neither property. Ile93 forms a hydrogen bond with Arg48 and undergoes a hydrophobic interaction with Phe140 (Fig. 1B), which is located near the hydrophobic cavity (Fig. 1A). Ile93 is therefore involved in closing of the LolA lid and, in addition, may affect the status of the hydrophobic cavity. Disruption of the hydrophobic interaction between Ile93 and Phe140 is most likely the reason why I93E and I93N are defective. Indeed, when the same mutations were introduced at Phe140, the resultant derivatives, F140E and F140N, were also defective. We then asked why I93G exhibits a dominant negative phenotype.

TTO16 cells harboring a plasmid carrying a gene for the wild type LolA or an Ile93 derivative were grown on L-broth containing 25 μg/ml chloramphenicol, 25 μg/ml kanamycin, and 0.1 mM IPTG. When the culture A660 reached 0.6, the cells were induced with 0.2% arabinose for 1.5 h and then converted into spheroplasts to prepare the periplasmic fraction corresponding to 10⁸ cells. The periplasmic fractions were analyzed by SDS-PAGE and Western blotting with antibodies against the specified proteins. MBP, maltose-binding protein.

The levels of Pal and Lpp accumulated in the periplasm increased with an increase in the amount of I93G (Fig. 3B). In contrast, two other Ile93 derivatives did not cause the periplasmic accumulation of lipoproteins even when they were expressed at maximum levels.

I93G Efficiently Binds Lipoproteins but Cannot Transfer Them to LolB—L10P is a derivative of the major outer membrane lipoprotein Lpp and is suitable for kinetic analysis of the release reaction, since its release from spheroplasts absolutely depends on LolA and takes place even after mature L10P has been kept anchored to the inner membrane for a long time (27). L10P was labeled with [35S]S and chased with nonradioactive amino acids in spheroplasts in the absence of LolA, and then its release was induced by the addition of LolA, I93G, or another Ile93 derivative (Fig. 4). [35S]L10P remained in the spheroplasts in the absence of LolA, whereas the addition of LolA caused the nearly complete release of L10P in 2 min (Fig. 4A). I93G also induced the L10P release into the supernatant, and little [35S]L10P remained in the spheroplasts at 1 min after the addition of I93G. On the other hand, neither I93E nor I93N induced the release of L10P. Densitometric quantification of [35S]L10P release revealed that I93G is considerably more active than the wild-type LolA as to the release reaction (Fig. 4). To determine whether or not this efficient release of lipoproteins from spheroplasts is characteristic of I93G, the release activity was examined with another derivative, in which a residue forming the hydrophobic cavity had been mutated. L59R possessed Arg in place of Leu at position 59, which is involved in the formation of the hydrophobic cavity (Fig. 1A). This derivative caused the periplasmic accumulation of lipoproteins but did not exhibit a dominant negative phenotype (Table 1). The rate of L10P release by L59R (Fig. 4C) was essentially the same as that by wild-type LolA (Fig. 4).

To examine the transfer of lipoproteins to the LolB-containing outer membrane, L10P was released with LolA, I93G, or L59R from spheroplasts as in Fig. 4, and the spheroplast supernatant containing the LolA-L10P, I93G-L10P, or L59R-L10P complex was incubated with the outer membrane. L10P released as a complex with LolA was nearly completely transferred to the outer membrane in about 30 min (Fig. 5A and C). In contrast, most L10P molecules released as a complex with I93G (Fig. 5A) or L59R (Fig. 5C) remained in the supernatant even after a 60-min incubation with the LolB-containing outer membrane. Densitometric quantification revealed that the transfer activities of I93G and L59R were about 10 and 3% of that of LolA at 20 min after the start of the reaction, respectively (Fig. 5B and D). Taken together, these results indicate that both I93G and L59R are defective in the transfer of associated lipoproteins to LolB. Moreover, it is suggested that the dominant negative phenotype of I93G is related to its very high activity to bind lipoproteins.

To determine whether or not LolA derivatives inhibits the binding of lipoproteins to wild-type LolA, we constructed and purified LolA pos-

2 S. Watanabe, S. Matsuyma, and H. Tokuda, unpublished observation.

FIGURE 2. Accumulation of lipoproteins in the periplasm. TTO16 (lacPO-lolA) cells harboring pAM201 (P_{lacPO}-lolA-his) or a mutant of it encoding the specified LolA derivative were grown on L-broth containing 25 μg/ml chloramphenicol, 25 μg/ml kanamycin, and 0.1 mM IPTG. When the culture A660 reached 0.6, the cells were induced with 0.2% arabinose for 1.5 h and then converted into spheroplasts to prepare the periplasmic fraction analyzed by SDS-PAGE and Western blotting with antibodies against the specified proteins. Lpp, lipoprotein; Pal, penta-lipoic acid; MBP, maltose-binding protein.
sessing a FLAG tag at its C terminus. Expression of this LolA-FLAG alone supported the growth of TT016 in the absence of IPTG (data not shown). Moreover, LolA-FLAG released L10P from spheroplasts (Fig. 6A) and then transferred L10P to LolB (data not shown). These results indicate that LolA-FLAG is functional both in vivo and in vitro. The release of [35S]L10P from spheroplasts was examined in the presence of a fixed amount (2 g) of LolA-FLAG and various amounts of His-tagged LolA, I93G, L59R, or R43L. R43L was used as a control, because this derivative causes the periplasmic accumulation of lipoproteins but does not exhibit the dominant negative property (15). L10P released as complexes with LolA proteins into the spheroplast supernatant was adsorbed to His tag and FLAG tag affinity columns and then eluted with imidazole and FLAG peptide, respectively. [35S]L10P co-eluted with the respective LolA proteins was analyzed by fluorography (Fig. 6A). LolA-FLAG and LolA-His were found to be equivalent as to the release activity, and almost the same amounts of L10P were co-eluted with LolA-FLAG and LolA-His when the same amounts of the two LolA protein species were added. The activities of R43L and L59R were also nearly the same as that of the wild-type LolA. On the other hand, when I93G-His was added, the amount of L10P co-eluted with LolA-FLAG was significantly decreased. The results were plotted as a function of the amount of His-tagged LolA protein (Fig. 6, B and C). The amounts of L10P bound to 2 µg of LolA-FLAG decreased to 50% upon the addition of 2 µg of LolA-His, I93G-His, or L59R-His. On the other hand, 1 µg of I93G-His completely inhibited L10P binding to 2 µg of LolA-FLAG (Fig. 6B). The amounts of L10P bound to the His tag affinity column (Fig. 6C)
FIGURE 4. Release of lipoproteins by I93G is faster than that by LolA. [%sup-35S]L10P was released from spheroplasts with 5 μg of wild-type LolA, the indicated I93 derivative (A and B), or L59R (C and D) at 30 °C. A and C, at the indicated times, the reaction mixture was fractionated into spheroplasts (P) and a supernatant (S), followed by SDS-PAGE and fluorography after immunoprecipitation with anti-Lpp antibodies. B and D, the fluorographs shown in A and C were quantified with an ATTO Densitograph, and the percentages of L10P released into the supernatant were plotted as a function of time.

FIGURE 5. I93G is defective in the transfer of L10P to LolB in the outer membrane. [%sup-35S]L10P was released from spheroplasts by the addition of wild-type LolA, I93G (A and B), or L59R (C and D) as in Fig. 4. Spheroplast supernatants containing the LolA-L10P, I93G-L10P, or L59R-L10P complex were incubated for the indicated times with 0.2 mg/ml outer membrane containing LolB as described under “Experimental Procedures.” A and B, the reaction mixtures were fractionated into pellets (P) and supernatants (S) by centrifugation. L10P in each fraction was detected by SDS-PAGE and fluorography. B and D, the fluorograph shown in A and B was quantified as in Fig. 4, and the percentages of L10P incorporated into the outer membrane were plotted as a function of time.
I93G inhibits formation of the LolA-L10P complex. Release of [35S]L10P from spheroplasts was carried out at 30 °C for 5 min in the presence of 2 µg of LolA-FLAG and various amounts of either LolA-His, R43L-His, I93G-His, or L59R-His. The reaction mixture was fractionated into a supernatant and pellet by centrifugation. An equal volume of the supernatant was adsorbed to affinity columns for FLAG-tagged and His-tagged proteins and then eluted from the columns as described under “Experimental Procedures.” A, [35S]L10P was released with 2 µg of LolA-FLAG (F) and the indicated amounts of His-tagged (H) LolA, R43L, I93G, or L59R. L10P co-eluted with LolA proteins adsorbed to a FLAG tag affinity column (F), and a His tag affinity column (H) was immunoprecipitated with anti-Lpp antibodies and then detected by SDS-PAGE and fluorography. B and C, the amount of L10P released as a complex with LolA-FLAG (B) or the indicated His-tagged LolA protein (C) was determined and expressed as a percentage, taking the amount in the absence of His-tagged proteins as 100. Open circles, LolA-His; closed diamonds, R43L-His; closed circles, I93G-His; closed triangles, L59R.

I93G Does Not Take Up L10P That Is Already in a Complex with LolA—The results presented above indicate that I93G has considerably higher affinity for lipoproteins than does the wild-type LolA. To determine whether or not I93G can take up L10P from the LolA-L10P complex, [35S]L10P was released from spheroplasts with the wild-type LolA possessing no tag. The spheroplast supernatant containing the LolA-L10P complex was incubated with various amounts of I93G-His at 30 °C for 30 min. Each reaction mixture was then applied to a His tag affinity column. The column was eluted with 20 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. Pass-through fractions (T) and eluate fractions (E) were respectively collected and precipitated with trichloroacetic acid, followed by immunoprecipitation with anti-Lpp antibodies. Equivalent amounts of the respective fractions were analyzed by SDS-PAGE and fluorography or immunoblotting with anti-LolA or -hexahistidine antibodies.

StAR-related lipid transfer (START) domains (28), which are lipid-binding domains involved in intracellular lipid transport, lipid metabolism, and cell signaling and found in an extensive protein family in eukaryotic cells (29). The prototype is StAR (steroidogenic acute regulatory protein), which transfers cholesterol to the inner membrane of mitochondria in steroid hormone-producing cells. Human phosphatidylcholine transfer protein is a START domain family protein. The crystal structure of phosphatidylcholine transfer protein containing lipid inside the hydrophobic cavity has been reported (30). The hydrophobic cavity of LolA most likely represents a binding site for the lipid moiety of lipoproteins, whereas no amino acid sequence similarity exists between START domains and LolA. The structure of the LolA-lipoprotein complex is not yet known. The details of the function of the hydrophobic cavity therefore remain to be determined. In contrast, recent biochemical analyses suggested that the strength of the hydrophobic interaction between LolA and lipoprotein is critical for efficient lipoprotein transfer to LolB and is affected by residues introduced at position 43 (16). To understand the functional importance of the hydrophobic cavity and closing of the LolA lid, amino acid residues expected to be involved in these processes were mutagenized.

Of 21 defective LolA mutants, 14 caused periplasmic accumulation of lipoproteins to various extents (Fig. 2), indicating that these 14 mutations did not abolish the lipoprotein-binding activity but impaired the lipoprotein transfer activity. Three mutations were of residues involved in closing of the lid, and the other 11 were of residues forming the hydrophobic cavity (Table 1). It should be noted that the residues involved in the lid closing also form the hydrophobic cavity (Fig. 6B). If the hydrophobic interaction between LolA and a lipoprotein is too strong, lipoprotein transfer from LolA to LolB is inhibited (16). Therefore, the 14 mutations might increase the strength of the hydrophobic interaction. Alternatively, the mutations impaired the interaction with LolB. Among these mutations, five exhibited a dominant negative property. These mutants are especially interesting because they are expected to inhibit the function of wild-type LolA. Moreover, dominant negative mutants exhibiting lipoprotein-releasing activity have not been isolated before. On the other hand, seven mutants neither exhibited a dominant negative property nor caused periplasmic accumulation of lipoproteins. It may be noteworthy that five of them were isolated as sole mutants as to the respective residues despite our exhaustive mutant isolation. It seems likely that these five residues are less important for the LolA
function, and only mutations causing significant structure alteration, such as the mutation to Pro, were isolated. Therefore, most mutants seemed to have completely lost the LolA function.

Mutations of the residues involved in the lid closing are likely to affect the hydrophobic interaction between LolA and lipoproteins, since these residues form the hydrophobic cavity (Fig. 1A). Indeed, three mutants, I93G, A94I, and A94L, exhibited a dominant negative property and caused periplasmic lipoprotein accumulation, suggesting that these mutants have higher affinity for lipoproteins and inhibit the wild-type LolA. The increase in the hydropathy scale (31) upon mutation from Ala (+1.8) at position 94 to Ile (+4.5) or Leu (+3.8) most likely caused the increase in the affinity for lipoproteins. On the other hand, another dominant negative mutation caused a hydropathy scale change from Ile (+4.5) to Gly (−0.4) at position 93. Therefore, an increase in the hydrophobic interaction between I93G and lipoproteins is difficult to assume. As observed with F20R, L59R, M91S, I106D, I106Q, and I106R, mutations impair the interaction with LolB, thereby preventing the transfer of lipoproteins to LolB. In any event, these mutants exhibited an unexpected phenotype and are worthy of further analyses.

Properties of Leu and Arg differ significantly. It is not immediately clear why R43L and L59R exhibited the same phenotype (i.e. active in the lipoprotein binding but defective in the lipoprotein transfer) although directions of the amino acid substitutions were opposite between the two derivatives. L59R seems to be a useful derivative for further examination of the function of the hydrophobic cavity.

Acknowledgments—We thank Kazuki Takeda for valuable discussion and Rika Ishihara for technical support.

REFERENCES

1. Juncker, A. S., Willenbrock, H., von Heijne, G., Brunak, S., Nielsen, H., and Krogh, A. (2003) Protein Sci. 12, 1652–1662
2. Brox, S. J., Ellison, M., Locke, T., Bortorff, D., Frost, L., and Weiner, J. H. (2004) J. Bacteriol. 186, 3254–3258
3. Miyadai, H., Tanaka-Masuda, K., Matsuyama, S., and Tokuda, H. (2004) J. Biol. Chem. 279, 39807–39813
4. Hayashi, S., and Wu, H. C. (1990) J. Bioenerg. Biomembr. 22, 451–471
5. Pugsley, A. P. (1993) Microbiol. Rev. 47, 50–108
6. Sankaran, K., and Wu, H. C. (1994) J. Biol. Chem. 269, 19701–19706
7. Yamaguchi, K., Yu, F., and Inouye, M. (1988) Cell 53, 423–432
8. Hara, T., Matsuyama, S., and Tokuda, H. (2003) J. Biol. Chem. 278, 40408–40414
9. Okuda, A., Gounon, P., and Pugsley, A. P. (1999) Mol. Microbiol. 34, 810–821
10. Terada, M., Kuroda, T., Matsuyama, S., and Tokuda, H. (2001) J. Biol. Chem. 276, 47680–47694
11. Yakushi, T., Masuda, K., Narita, S., Matsuyama, S., and Tokuda, H. (2000) Nat. Cell Biol. 2, 212–218
12. Matsuyama, S., Tajima, T., and Tokuda, H. (1995) EMBO J. 14, 3365–3372
13. Matsuyama, S., Yokota, N., and Tokuda, H. (1997) EMBO J. 16, 6947–6955
14. Takeda, K., Miyatake, H., Yokota, N., Matsuyama, S., Tokuda, H., and Miki, K. (2003) EMBO J. 22, 3199–3209
15. Miyamoto, A., Mastuyama, S., and Tokuda, H. (2001) Biochem. Biophys. Res. Commun. 287, 1125–1128
16. Taniguchi, N., Matsuyama, S., and Tokuda, H. (2005) J. Biol. Chem. 280, 34481–34488
17. Miyamoto, A., Matsuyama, S., and Tokuda, H. (2002) FEBS Lett. 528, 193–196
18. Tajima, T., Yokota, N., Matsuyama, S., and Tokuda, H. (1998) FEBS Lett. 439, 51–54
19. Hussain, M., Ichihara, S., and Mizushima, S. (1980) J. Biol. Chem. 255, 3707–3712
20. Ikeyari, K., Nishihara, A., Matsuyama, S., and Tokuda, H. (2004) Bacterial. Biotechnol. Biochem. 69, 1595–1602
21. Hirota, Y., Suzuki, H., Nishimura, Y., and Yasuda, S. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1417–1420
22. Miller, J. H. (1972) Experiments in Molecular Genetics, p. 431, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972
24. Tsukiyama, S., Fujita, Y., and Mizushima, S. (1993) EMBO J. 12, 265–270
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Hazelmayer, G. L. (1975) J. Bacteriol. 122, 206–214
27. Yakushi, T., Yokota, N., Matsuyama, S., and Tokuda, H. (2001) J. Biol. Chem. 276, 32576–32581
28. Ponting, C. P., and Aravind, L. (1999) Trends Biochem. Sci. 24, 130–132
29. Socci, N. R., and Breslow, J. L. (2003) J. Biol. Chem. 278, 22183–22186
30. Roderick, S. L., Chan, W. W., Agate, D. S., Olsen, L. R., Vetting, M. W., Rajashankar, K. R., and Cohen, D. E. (2002) Nat. Struct. Biol. 9, 507–511
31. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
32. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. 47, 45–148