Opening and Closing of the Hydrophobic Cavity of LolA Coupled to Lipoprotein Binding and Release*

Received for publication, June 20, 2008. Published, JBC Papers in Press, July 9, 2008, DOI 10.1074/jbc.M804736200

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Outer membrane-specific lipoproteins of Escherichia coli are released from the inner membrane through the action of LolCDE, which leads to the formation of a complex between the lipoprotein and LolA, a periplasmic chaperone. LolA then transfers lipoproteins to LolB, a receptor in the outer membrane. The structures of LolA and LolB are very similar, having an incomplete β-barrel covered with an α-helical lid forming a hydrophobic cavity inside. The cavity of LolA, but not that of LolB, is closed and thus inaccessible to the bulk solvent. Previous studies suggested that Arg at position 43 of LolA is critical for maintaining this closed structure. We show here, through a crystallographic study, that the cavity of the LolA(R43L) mutant, in which Leu replaces Arg-43, is indeed open to the external milieu. We then found that the binding of a fluorescence probe distinguishes the open/close state of the cavity. Furthermore, it was revealed that the hydrophobic cavity of LolA opens upon the binding of lipoproteins. Such a liganded LolA was found to be inactive in the release of lipoproteins from the inner membrane. On the other hand, the liganded LolA became fully functional when lipoproteins were removed from LolA by detergent treatment or transferred to LolB. Free LolA thus formed was inacces- sible to a fluorescence probe. These results, taken together, reveal the LolA cycle, in which the hydrophobic cavity under- goes opening and closing upon the binding and release of lipoproteins, respectively.

Periplasmic molecular chaperone LolA (1) binds to bacterial lipoproteins released from the inner membrane through the action of an ATP-binding cassette transporter LolCDE complex (2), and then transfers lipoproteins to receptor LolB in the outer membrane (3). LolA thus transports hydrophobic acylated proteins from the inner to the outer membrane through the hydrophilic periplasm. About 80 species of Escherichia coli lipoproteins are sorted to the outer membrane by the Lol sys- tem composed of LolCDE, LolA, and LolB (4, 5). Only the step catalyzed by the LolCDE complex requires ATP, the transfer of lipoproteins from LolA to LolB and LolB to the outer membrane efficiently occurring without energy input, and an intermediate LolA-lipoprotein complex is not detected in the periplasm under normal conditions. In contrast, the LolA(R43L) mutant causes periplasmic accumulation of lipoproteins, because it can bind to lipoproteins but cannot transfer them to LolB (6).

The crystal structures of LolA and LolB are very similar to each other despite their dissimilar sequences (7). Both proteins comprise an incomplete β-barrel with an α-helical lid, which together form a hydrophobic cavity. The cavity is most likely the binding site for the acyl chains of lipoproteins. The crystal structure of LolA revealed an interesting function of the Arg at position 43, which was mutated to Leu in the LolA(R43L) mutant. Arg-43 is located in the loop connecting the β2 and β3 strands, and forms hydrogen bonds with some residues in the α-helical lid. Therefore, Arg-43 is speculated to play a critical role in the closing of the hydrophobic cavity of LolA. On the other hand, such a residue is not present in LolB, the hydrophobic cavity of which is open to the external milieu. From these observations, it seemed likely that the opening and closing of the LolA lid plays a critical role in the efficient energy-independent lipoprotein transfer to LolB (7). To address this issue, we mutated Arg-43 to other residues and examined the activities of the resultant derivatives. The results were unexpected; Arg-43 was found to be dispensable for the release of lipoproteins from the inner membrane but important for the transfer of lipoproteins to LolB. Arg-43 weakens the hydrophobic interaction between LolA and lipoproteins. In contrast, Leu in place of Arg-43 causes a significant increase in the strength of the hydrophobic interaction with lipoproteins, thereby inhibiting the lipoprotein transfer to LolB (8). Because Val or Ile could replace Arg-43 with only a marginal effect, the importance of hydrogen bonding between Arg-43 and lid residues was not clarified. Moreover, it has not yet been demonstrated that the LolA lid undergoes opening and closing upon the binding and release of lipoproteins, respectively.

* This work was supported by grants 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 atomic coordinates and structure factors (codes 2ZPC and 2ZPD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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or when LolA or LolA(R43L) was denatured and re-natured in the presence of lipoproteins.4 These observations suggest that the hydrophobic cavity is closed even in the LolA(R43L) mutant and that its opening is essential for lipoprotein binding. To elucidate the dynamic properties of the cavity lid, we solved the crystal structure of LolA(R43L) and then established an assay system that can distinguish the open/close state of the LolA hydrophobic cavity.

EXPERIMENTAL PROCEDURES

Materials—Talon Co2+ affinity resin (Clontech) was used to purify hexahistidine-tagged proteins. Antibodies against LolA (1) and Lpp (10) were raised in rabbits as described. Tran35S-label (a mixture of 70% [35S]Met and 20% [35S]Cys, 1000 Ci/mol) was obtained from MP Biomedicals. IgGsorb was purchased from Enzyme Center Inc.

Bacterial Strains and Media—E. coli K-12 strains, XL1-Blue (Stratagene), MC4100 (11), and TT016 (6), were used. The last strain carried the chromosomal lolA gene under the control of the lactose promoter-operator. E. coli B strain BL21(DE3) (12) was used for high level expression of His-tagged LolA proteins. Cells were grown on LB broth (Difco) or 2YT broth (12). When required, 25 μg/ml chloramphenicol, 50 μg/ml spectinomycin, or 50 μg/ml ampicillin was added.

Construction of Plasmids—For fluorescent bis-ANS5 binding assays, plasmids allowing higher level expression of His-tagged LolA(R43L) were constructed. For this, the R43L mutation was introduced into LolA-His by means of PCR with pSS2 (P17lac-lola-His) (13) as a template and a pair of primers, R43L-F (5′-cagggctgtcggggaaggacctcaattcggtccat-3′) and R43L-R (5′-atgcttcgttagaattttgcaAATTccacccagatcctgccg-3′), in which the uppercase letters denote Leu. The mutant plasmid, pYOR43L (P17lac-lola(R43L)-His), thus constructed was amplified in E. coli XL1-Blue, and then the mutation was confirmed by sequencing of both strands.

Purification of LolA Proteins—For crystallographic studies, His-tagged LolA(R43L) was overexpressed in TT016 cells harboring pAMR43L, which carried the gene for LolA(R43L)-His under the control of the arabinose promoter, and then purified as described previously (6). The cells were grown on LB-broth supplemented with 1 mM IPTG and 25 μM IPTG. Purification of His-tagged LolA proteins was examined by SDS-PAGE and Coomassie Brilliant Blue staining, and then dialedyzed against 20 mM sodium phosphate (pH 7.2) overnight at 4 °C.

Purification of mLolB—A soluble derivative of LolB, mLolB (3), was purified from the periplasm of E. coli MC4100 cells harboring pYKT102 (P17lac-mloloB). The cells were grown on 2

4 N. Yokota and H. Tokuda, unpublished observation.
5 The abbreviations used are: bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; sucrose monocaprate, β-o-fructopyranosyl-α-c-glucopyranosidemonodecanoate; IPTG, isopropyl-β-D-thiogalactopyranoside.
YT broth containing 50 μg/ml ampicillin at 37 °C. When the culture $A_{660}$ reached 0.8, the cells were induced with 1 mM IPTG for 1.5 h. Purification of mLolB was carried out on a cation exchange Mono S column, which had been equilibrated with 25 mM sodium acetate (pH 5.0). The column was developed with a linear gradient of NaCl. Fractions containing mLolB were then applied on an anion exchange Mono Q column equilibrated with 25 mM Tris-HCl (pH 8.0). Purified mLolB was obtained in the pass-through fraction and then dialyzed against 20 mM sodium phosphate (pH 7.2).

Preparation of Free LolA Proteins from Liganded LolA Proteins—Free LolA proteins were generated from FLAG-tagged LolA proteins complexed with His-tagged Pal by a detergent wash or incubation with mLolB. To remove Pal by washing with a detergent, the liganded LolA was incubated with 2% sucrose monocaprate at 30 °C for 1 h. To transfer Pal to mLolB, the LolA-Pal complex was incubated with a double amount of mLolB at 30 °C for 1 h. The reaction mixtures were then passed through a Talon column. Free LolA was purified from the Talon column-unbound fractions on a Mono Q column as described for the purification of His-tagged LolA proteins.

Release of Lpp from Spheroplasts—The release of the major outer membrane lipoprotein, Lpp, from spheroplasts was examined as reported (1). Briefly, E. coli MC4100 cells were converted into spheroplasts in the presence of 1 mM EDTA and 33 μg/ml lysozyme. The spheroplast suspension (300 μl) was kept on ice for 3 min with the specified LolA proteins. M63 minimal medium (750 μl) comprising 0.25 M sucrose, 20 μg/ml thiamine, 20 μg/ml thymine, 40 μg/ml each of all amino acids except Met and Cys, and 10 μCi of Tran35S-label was then added for 2-min labeling at 30 °C. The labeling was chased for 2 min by the addition of non-radioactive Met and Cys (each at 12 mM). The release reaction was terminated by chilling the reaction mixture in ice water, followed by fractionation into spheroplasts and medium by centrifugation at 16,000 × g for 2 min, and then analysis by SDS-PAGE and fluorography as reported (1).

Binding of bis-ANS—LolA proteins at the specified concentrations were incubated in 20 mM sodium phosphate (pH 7.2) in the presence of 5 μM bis-ANS for 10 min at room temperature. Fluorescence was then measured with a Shimadzu spectrofluorometer RF-5000. The dye was excited at the wavelength of 390 nm. Emission spectra were collected in the range of 420–620 nm.

Other Methods—SDS-PAGE was carried out according to Laemmli (15) or, in the case of Lpp, Hussain et al. (10). Densitometric quantification was performed with an ATTO Densitograph.

RESULTS

Structure of the LolA(R43L) Derivative—Two forms of LolA(R43L) crystals, trigonal and monoclinic ones (Fig. 1, A and B, respectively), were obtained under the same conditions,
and the structures were refined to resolutions of 2.35 and 1.85 Å (Table 1), respectively. The structure of the trigonal crystal of R43L was almost identical to that of LolA. The value of the root mean square deviation against the trigonal crystal of LolA (PDB ID: 1UA8) was 0.52 Å. Therefore, the trigonal crystal of LolA(R43L) was a closed form.

The strands forming the molecular framework of the monoclinic crystal of LolA(R43L) could be well superimposed on those of the trigonal form of LolA(R43L), whereas the positions of the α2 and α3 helices of the two crystals were significantly different (Fig. 1, C and D). In particular, the α2 helix plugging the cavity had moved outward by ~6 Å in the monoclinic form (Fig. 1E), indicating that this crystal is an open form. The loop connecting the β8 and β9 strands (loop 8–9) also showed a large deviation of ~5 Å.

Aromatic residues (Phe-20, Phe-47, Trp-49, Phe-90, Trp-101, Tyr-104, Phe-127, Phe-140, and Tyr-152) cluster around the entrance to the cavity of LolA (Fig. 2, A and B). These residues are significantly conserved among LolA homologues (7). In the closed form, Phe-90 located in the α2 helix interacts with other aromatic residues in the β-sheet region such as Phe-47 and Trp-49 (Fig. 2A). Consequently, the entrance is shielded from the bulk solvent by these residues. On the other hand, these interactions are abolished in the open state (Fig. 2B), rendering the cavity open to the external milieu.

Irrespective of whether LolA was the closed or open form, the peptide bond between Leu–43 and Pro–44 remained in the cis-type conformation (Fig. 2, C and D), which places the side chain of Arg–43 inside of the wild-type LolA molecule (7). However, because the side chain of Leu–43 with no polar atoms cannot fix the helices through hydrogen bonding, the temperature factors for the main-chain atoms exhibited significant deviations (data not shown). Thus, the crystal structure of LolA(R43L) revealed that Arg–43 indeed plays an important role in the closing of the hydrophobic cavity of LolA, as previously speculated. However, because R43L also existed in the closed form, residues other than Arg–43 also contribute to the formation of a closed form.

**Binding of bis-ANS in the Hydrophobic Cavity of LolA**—The crystal structure of LolA(R43L) revealed that the derivative exists in not only a closed conformation but also an open conformation (Figs. 1 and 2). On the other hand, the formation of the LolA(R43L)-lipoprotein complex required the lipoprotein release reaction involving LoCDE as the formation of the LolA-lipoprotein complex did (8). Therefore, the physiological significance of the open form of LolA(R43L) remained unclear. To establish an assay system that can easily distinguish the open/closed structures of LolA and LolA(R43L), we examined the binding of some fluorescent probes and found that the binding of bis-ANS was significantly different between LolA and LolA(R43L). The fluorescence of bis-ANS has been reported to significantly increase when it binds to a hydrophobic environment (16). The fluorescence intensity of bis-ANS was very low in the absence of LolA proteins (Fig. 3A, inset). Moreover, the emission maximum was observed at different wavelengths in the presence and absence of LolA proteins. The increase in the bis-ANS fluorescence was only marginal with wild-type LolA, whereas it was remarkable with the LolA(R43L) derivative (Fig. 3A), indicating that bis-ANS can gain access to the hydrophobic cavity of LolA(R43L) but not that of LolA.

We previously reported large scale preparation of the LolA-lipoprotein complex (13). To examine whether or not the fluorescence intensity of bis-ANS is affected by lipoprotein binding to LolA proteins, homogenous LolA-Pal and LolA(R43L)-Pal complexes were purified (Fig. 3B). The fluorescence intensity was then examined in the presence of these complexes (Fig. 3A). Both complexes significantly increased the bis-ANS fluorescence to essentially the same level. Moreover, the fluorescence intensities with these complexes were almost the same as that with free LolA(R43L) (Fig. 3A). These results indicate that the major fraction of LolA(R43L) exists in the open conformation. Moreover, because the fluorescence intensities induced by LolA(R43L) were essentially the same whether Pal was bound or not, it is clear that bis-ANS binds to the same site of LolA(R43L) irrespective of the presence or absence of Pal. It should be worthy of note that the fluorescence intensity in the presence of the LolA-Pal complex was unchanged even after 1-h incubation, indicating that bis-ANS binds to the hydrophobic cavity but does not compete with lipoproteins for the binding site.

**Regeneration of the Closed Conformation of LolA Proteins**—Because the homogenous LolA-lipoprotein complex was prepared only recently, it was not known whether or not a liganded LolA is active in the lipoprotein release reaction. Furthermore, it was also unknown whether or not free LolA generated from the liganded LolA is active in the release reaction. To address these issues, the release of Lpp from spheroplasts was examined in the presence of various LolA proteins (Fig. 4A). The release of Lpp was absolutely dependent on LolA, as previously reported (1). The liganded LolA or LolA(R43L) was completely inactive in the release reaction. Lipoproteins bound to LolA can be removed by washing with a detergent, sucrose monolipate (13), or by lipoprotein transfer reaction to mLoIB, a soluble version of LoIB (3). The LolA-Pal complex was subjected to
these treatments, and then free LolA regenerated was used for the lipoprotein release assays (Fig. 4A). Regenerated free LolA was found to be completely active as to the lipoprotein release reaction.

The bis-ANS fluorescence intensities were examined in the presence of the regenerated free LolA used in Fig. 4A. The high fluorescence intensities observed with the LolA-Pal complex were near completely abolished with free LolA regenerated from the complex on treatment with sucrose monocaprate or mLolB (Fig. 4B). All these results, taken together, indicate that the hydrophobic cavity of LolA opens upon lipoprotein binding and then closes upon lipoprotein release and that only free LolA is active as to the lipoprotein release reaction.

**DISCUSSION**

The structures of LolA and LolB are characteristic comprising an incomplete β-barrel covered by an α-helical lid, which together form a hydrophobic cavity (7). Crystallographic analysis revealed that LolA(R43L) exists in both open and closed forms (Fig. 1), the latter of which is essentially identical to that of wild-type LolA. These results indicate that Arg at position 43 is indeed important for stabilization of the closed conformation of LolA, as previously speculated (7, 8). The open/close state of the hydrophobic cavity was found to be possible to monitor by means of fluorescent bis-ANS binding (Fig. 3). The fluorescence increase induced by LolA(R43L) was almost instantaneous, whereas the fluorescence of bis-ANS did not increase even when it was incubated with wild-type LolA for a long period (data not shown). Moreover, the fluorescence intensity scarcely increased even when LolA(R43L) bound to Pal (Fig. 3), indicating that the open conformation comprises the major fraction of LolA(R43L). However, the formation of the LolA(R43L)-lipoprotein complex still required ATP hydrolysis by LolCDE. It seems therefore likely that the LolCDE-dependent ATP hydrolysis is required for the correct transfer of lipoproteins from the inner membrane to LolA and LolA(R43L). It was suggested recently that the energy liberated on ATP hydrolysis is transmitted to membrane subunits LolC and/or LolE and then utilized for the lipoprotein transfer to LolA (17).

Taking advantage of the convenient bis-ANS binding assay, the hydrophobic cavity of wild-type LolA was shown to undergo opening and closing upon the binding and release of lipoproteins, respectively (Fig. 4B). Furthermore, liganded forms of LolA and
LolA(R43L) were found to be inactive as to the lipoprotein release reaction, whereas regeneration of free LolA caused recovery of the activity (Fig. 4A). We recently found that free LolA does not inhibit the transfer of lipoproteins from the liganded LolA to LolB (13). Taken together, these results indicate that LolA undergoes a conformational change upon the binding of lipoproteins. This conformational change is critical for specific interaction between liganded LolCDE and free LolA and between liganded LolA and LolB.

We recently established an experimental system by which a large amount of the LolA-lipoprotein complex can be purified (13). The crystal structure of the complex is of great importance for understanding the mechanisms underlying the transport and sensing of the complex lipid materials in the bacterial periplasm.

Three other proteins were recently found to have very similar structures to those of LolA and LolB. LppX (PDB ID: 2BYO) is a lipoprotein of Mycobacterium tuberculosis and has been speculated to accommodate complex lipids in its hydrophobic cavity for their transport to the outer layers of the cell (18). The second one is the N-terminal domain of E. coli RseB (PDB ID: 2V42, 2V43, and 2P4B), which constitutes a signal transduction pathway leading to the induction of $\sigma^B$ expression. It is speculated that mislocalized lipoproteins bind to the hydrophobic cavity of the N-terminal domain of RseB, which is critical for activation of the periplasmic quality control mechanism (19, 20). The third one is VioE (PDB ID: 3BMZ, 2ZF3, and 2ZF4) of Chromobacterium violaceum, which is speculated to play a key role in the biosynthesis of violacein (21, 22), a purple pigment with antibacterial and cytotoxic properties. The functions of the hydrophobic cavities of these proteins remain only speculation. We revealed here that the hydrophobic cavity of LolA is the binding site for lipoproteins and its opening is essential for the LolA function. We are currently examining bis-ANS binding to the hydrophobic cavity of LolB with or without lipoproteins.

Acknowledgment—We thank Rika Ishihara for technical support.

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