# Dominant negative mutant of a lipoprotein-specific molecular chaperone, LolA, tightly associates with LolCDE

Atsuki Miyamoto, Shin-ichi Matsuyama, Hajime Tokuda*

*Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*

Received 4 July 2002; revised 19 August 2002; accepted 21 August 2002

First published online 30 August 2002

Edited by Giudo Tettamanti

**Abstract Periplasmic molecular chaperone LolA and the inner membrane ATP binding cassette transporter LolCDE are essential for ATP-dependent release of outer membrane-directed lipoproteins from the inner membrane of *Escherichia coli*. A LolA(F47E) mutant carrying a Phe to Glu mutation at position 47 was defective in the release of lipoproteins from spheroplasts and proteoliposomes reconstituted with LolCDE. When incubated with proteoliposomes containing LolCDE, LolA remained in the supernatant whereas LolA(F47E) bound to proteoliposomes. This tight association of LolA(F47E) with LolCDE caused a dominant negative phenotype in vivo, suggesting that the LolA–LolCDE interaction is critical for lipoprotein release.** 

*Key words:* Lipoprotein; Molecular chaperone; Membrane anchoring; Proteoliposome; ATP binding cassette transporter; Periplasm

## 1. Introduction

*Escherichia coli* possesses more than 90 lipoproteins, most of which are anchored to the outer membrane through N-terminal lipids [1]. Lipid modification and processing to mature lipoproteins occur on the periplasmic surface of the inner (cytoplasmic) membrane [2]. An ATP binding cassette (ABC) transporter, LolCDE, in the inner membrane releases outer membrane-specific lipoproteins that possess amino acid residues other than Asp at position 2, leading to the formation of a LolA–lipoprotein complex [3,4]. When this complex interacts with outer membrane receptor LolB, the associated lipoprotein is transferred from LolA to LolB, followed by anchoring to the outer membrane [5,6]. Asp at position 2 of lipoproteins functions as a LolCDE avoidance signal, thereby causing the retention of lipoproteins in the inner membrane [1].

Five Lol proteins are essential for *E. coli* growth [7–9] and widely conserved in Gram-negative bacteria [3,10]. In addition to lipoprotein binding and interaction with LolB, LolA is expected to interact with LolCDE at the step of lipoprotein release. However, this has not been demonstrated. In contrast, $MalFGK_2$, an ABC transporter mediating maltose uptake, was recently shown to bind a periplasmic maltose binding protein, MalE, at the transition step of the uptake reaction [11].

We recently constructed LolA derivatives carrying single mutations at conserved residues. LolA(R43L) was found to be defective in the transfer of associated lipoproteins to LolB, causing the accumulation of the LolA–lipoprotein complexes in the periplasm [10]. Among the isolated LolA mutants only LolA(F47E) exhibited a dominant negative phenotype, i.e. the expression of LolA(F47E) inhibited growth even in the presence of wild-type LolA. We report here that LolA(F47E) is inactive in the release reaction and tightly associates with LolCDE.

## 2. Materials and methods

### 2.1. Materials

LolA and its F47E derivative having a His-tag at its C-terminus were purified to homogeneity with Talon metal affinity resin (Clontech). LolCDE was purified as reported [1]. Restriction enzymes were purchased from Takara Shuzo Co. Isopropyl β-D-thiogalactopyranoside (IPTG) was from Sigma. Antibodies against LolA [4], Pal [7], Lpp [12], and OmpA [13] were prepared as described. Tran$^{35}$S label was obtained from ICN.

### 2.2. Bacteria and media

*E. coli* TT016 carried the chromosomal *lolA* gene under the control of the lactose promoter–operator [10]. Expression of the chromosomal *lolA* gene was induced with 1 mM IPTG. MC4100 [14] was used to prepare spheroplasts as described [4]. *E. coli* strains were grown at 37°C on L-broth or M63 minimal medium supplemented with 0.2% maltose.

### 2.3. Mutagenesis of Phe at position 47 of LolA

As reported for the construction of LolA(R43L) [10], Phe at position 47 of LolA was mutagenized by means of polymerase chain reaction using a Quick change site-directed mutagenesis kit (Stratagene) with pAM201 encoding LolA-His [10] as a template and a pair of oligonucleotides, 5′-GGGTGAAACGTCCAAACTTANNNAACTGGCATATGACACAACC-3′ and 5′-GGTTGTGTCATATGCCAGTTNNNTAAGTTTGGACGTTTCACCC-3′, as primers, in which each N represents the mixture of four nucleotides introduced for mutagenesis. The plasmid DNAs were amplified in the XL1-Blue strain lacking functional endonuclease R, and then transformed into TT016 (*lacPO-lolA*). Chloramphenicol-resistant transformants were selected on L-broth plates supplemented with 1 mM IPTG, and then their growth in the absence of IPTG was examined on plates containing arabinose. The plasmids harbored by transformants that did not grow on these plates were expected to carry non-functional *lolA* and therefore were sequenced to determine the mutations. To

*Corresponding author. Fax: (81)-3-5841 8464.
*E-mail address:* htokuda@imcbns.iam.u-tokyo.ac.jp (H. Tokuda).

*Abbreviations:* ABC, ATP binding cassette; LolCDE, a complex containing one molecule each of LolC and LolE, and two molecules of LolD; LolA, a periplasmic molecular chaperone for lipoproteins; LolB, lipoprotein receptor in the outer membrane; $MalFGK_2$, a complex containing one molecule each of MalF and MalG, and two molecules of MalK

0014-5793/02/$22.00 
PII: S0014-5793(02)03305-7

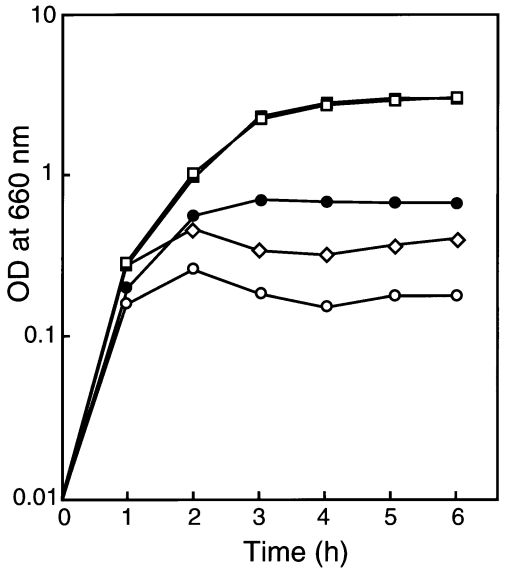


Fig. 1. LolA(F47E) is a dominant negative mutant. TT016 (P*lac-lolA-his*) cells harboring pAM201 ($P_{BAD}$-*lolA-his*, diamonds and squares) or pAF47E ($P_{BAD}$-*lolA(F47E)-his*, circles) were grown at 37°C in the presence and absence of IPTG and/or arabinose, as specified. The growth of cells was monitored by following the turbidity of cultures at 660 nm. The data obtained from a single experiment are shown. Open diamonds, −arabinose/−IPTG; open squares, +arabinose/−IPTG; closed squares, +arabinose/+IPTG; open circles, +arabinose/−IPTG; closed circles, +arabinose/+IPTG.

assure the mutations, DNA sequences were determined on both strands. Where specified, growth of TT016 cells harboring the specified plasmids was examined in the presence of both 30 μM IPTG and 0.2% arabinose.

### 2.4. Release of Lpp from spheroplasts

The LolA-dependent release of lipoproteins from spheroplasts was examined as described [4]. A suspension (300 μl) containing $5\times10^8$ spheroplasts was kept on ice for 3 min in the presence and absence of LolA. M63 medium (750 μl) containing 0.25 M sucrose and 10 μCi of Tran$^{35}$S label (1000 Ci/mmol as [$^{35}$S]Met) 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 of lipoproteins was terminated by chilling of the reaction mixture in ice water, and analyzed after fractionation into spheroplasts and medium by centrifugation at 16 000×*g* for 2 min.

### 2.5. In vitro membrane incorporation of Pal

LolB-dependent incorporation of Pal into outer membranes was examined using $^{35}$S-labeled Pal released from spheroplasts as described [5,6]. The membrane incorporation of Pal was terminated by chilling of the reaction mixture in ice water, and analyzed after fractionation into a supernatant and pellet by centrifugation at 100 000×*g* for 30 min.

### 2.6. Immunoprecipitation, SDS–polyacrylamide gel electrophoresis (PAGE), and Western blot analyses

$^{35}$S-labeled lipoproteins were immunoprecipitated with antibodies against the respective lipoproteins and then analyzed by SDS–PAGE, followed by fluorography with Enlightning (NEN Life Science Products) as described [4]. The amounts of lipoproteins were determined with an ATTO Densitograph. Western blot analyses were performed as described [6]. The blots were densitometrically quantitated at least twice within 5% deviation.

### 2.7. Reconstitution of proteoliposomes

Proteoliposomes were reconstituted with LolCDE (4 μg), L10P (1 μg), and 0.8 mg *E. coli* phospholipids in the presence of ATP as reported [1,3]. The release of L10P from the proteoliposomes was examined in the presence of 2 mM ATP and 10 μg/ml LolA or LolA(F47E) at 30°C for 20 min. The reaction mixture was fractionated into proteoliposomes and a supernatant by centrifugation at 100 000×*g* for 30 min, followed by SDS–PAGE and immunoblotting.

## 3. Results

### 3.1. LolA(F47E) inhibits the growth of E. coli

TT016 (*lacPO-lolA*) cells required the induction of LolA with IPTG for growth (Fig. 1). Expression of LolA from both chromosome and plasmid by the addition of IPTG and arabinose had no effect on growth (compare the open and closed squares). As reported previously for LolA(R43L) [10], mutagenized *lolA* was expressed from a plasmid by the addition of arabinose, and defective LolA mutants that did not support the growth of TT016 in the absence of IPTG were isolated. Among these mutants, one carried on pAMF47E was found to inhibit the growth of TT016 not only in the absence (open circles) but also in the presence (closed circles) of IPTG,

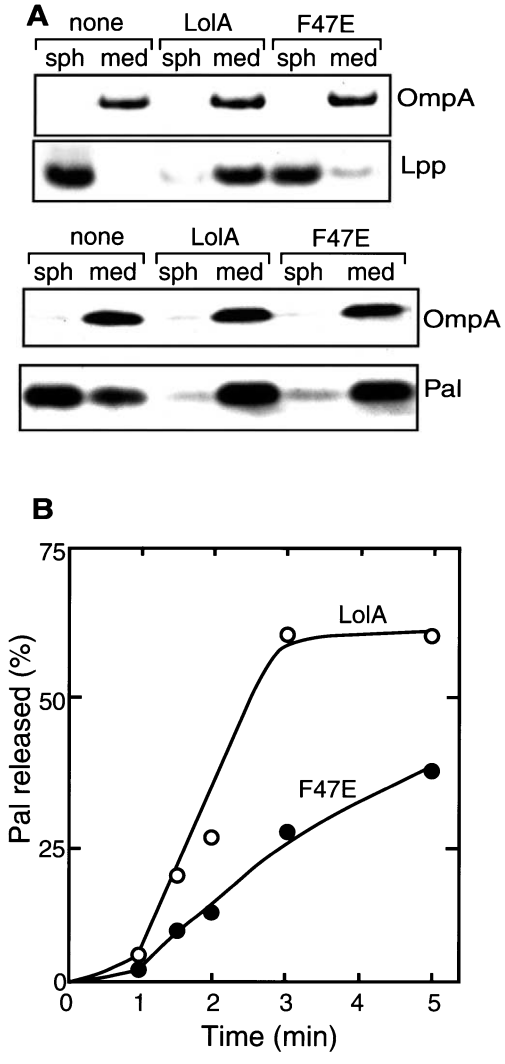


Fig. 2. LolA(F47E) is defective in lipoprotein-releasing activity. Spheroplasts were prepared from MC4100 cells harboring pTAN21 [6], which carried P*tac-pal*, induced for 5 min with 1 mM IPTG. A: Pulse-labeling and the chase were performed as described under Section 2 in the presence of 10 μg/ml LolA or LolA(F47E). B: Spheroplasts were pulse-labeled for 1 min in the presence of 3 μg/ml LolA or LolA(F47E), and then chased for the indicated times. The data obtained from a single experiment are shown.

indicating a dominant negative phenotype. This mutant (F47E) was found to carry a Phe (TTC) to Glu (GAA) mutation at position 47. Other mutants, which have Lys (AAG), Asp (GAT) or Pro (CCA) in place of Phe at position 47, were also isolated as defective mutants but did not exhibit a dominant negative phenotype.

### 3.2. LolA(F47E) is defective in the release reaction

The release of outer membrane lipoproteins Lpp and Pal from spheroplasts was examined in the presence of 10 μg/ml LolA or LolA(F47E) (Fig. 2A). Outer membrane protein OmpA, which is not modified by lipids, was secreted into the medium irrespective of the presence or absence of LolA, whereas that of Lpp absolutely required LolA as reported [4,6]. When LolA(F47E) was used in place of LolA, only a marginal amount of Lpp was released. In contrast to Lpp, a small amount of Pal was released in a LolA-independent manner and the addition of LolA caused the complete release of Pal as reported [6]. LolA(F47E) was found to be as active as wild-type LolA as to the release of Pal. It was shown previously that the release of Pal was more efficient than that of Lpp [6]. Since these results suggested that LolA(F47E) was a leaky mutant, the release of Pal was examined in the presence of a subsaturating amount (3 μg/ml) of LolA or LolA(F47E) (Fig. 2B). Under these conditions, the release of Pal induced by LolA(F47E) was less efficient than that by LolA, indicating that LolA(F47E) is generally defective in the lipoprotein releasing activity.

### 3.3. LolA(F47E) can transfer associated lipoproteins to LolB

Pal was released from spheroplasts on the addition of 10 μg/ml LolA or LolA(F47E). As a control, wild-type LolA [4] having no His-tag was also used for the release assay. The spheroplast supernatant containing released Pal was then applied on a metal-chelating column (Fig. 3). Neither LolA nor Pal bound to the resin when the release reaction had been performed with LolA having no His-tag (lane 3). In contrast, when the release reaction had been carried out with LolA-His, not only LolA but also Pal bound to the resin (lane 6), indicating the formation of a LolA–Pal complex. Pal released in the presence of LolA(F47E) also bound to the resin, indicating that this mutant is able to form a complex with Pal (lane 9). However, the amounts of Pal released into the medium (compare lanes 4 and 7) and bound to the resin (lanes 6 and 9) were smaller with LolA(F47E) than with wild-type

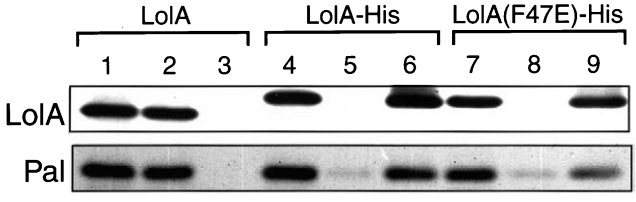


Fig. 3. LolA(F47E) forms a complex with released Pal. The release of $^{35}$S-labeled Pal from spheroplasts was performed for 5 min in the presence of 10 μg/ml LolA, LolA-His or LolA(F47E)-His as in Fig. 2. The spheroplast supernatants (lanes 1, 4 and 7) were applied on a metal-chelating column. Unbound fractions (lanes 2, 5 and 8) were obtained by washing the column with 10 mM Tris–HCl (pH 7.5). Bound fractions (lanes 3, 6 and 9) were eluted with 50 mM Tris–HCl (pH 7.5) containing 250 mM imidazole. Equivalent amounts of the fractions were analyzed by SDS–PAGE and immunoblotting for LolA or fluorography for Pal.

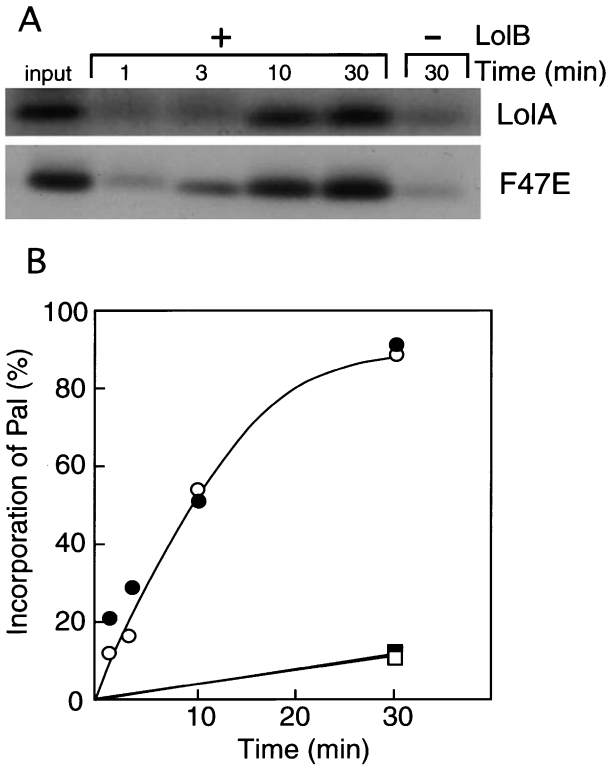


Fig. 4. LolA(F47E) is active as to the transfer of associated Pal to LolB. Spheroplast supernatants containing $^{35}$S-labeled Pal as a complex with LolA-His (open circles) or LolA(F47E)-His (closed circles) were prepared as in Fig. 3 and then incubated with 0.2 mg/ml outer membrane at 30°C for the specified times. After the reaction, Pal recovered in the outer membrane fraction was detected by fluorography (A) and quantitated (B). Where specified, LolB-depleted outer membranes prepared as reported [6] were used as a control (squares).

LolA. Moreover, a small amount of Pal did not bind to the resin when the release reaction had been performed with LolA(F47E). Taken together, these results indicate that LolA(F47E) is a leaky mutant defective in the release of lipoproteins. Since the release of Lpp more strongly depends on the LolA function than that of Pal [6], the defect of this mutant seemed to be more clearly observed in the release of Lpp.

It was previously reported that LolA(R43L) was defective in the transfer of associated lipoproteins to LolB [10]. To

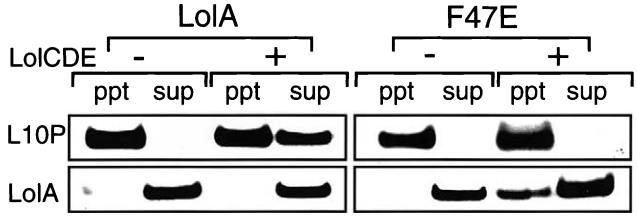


Fig. 5. LolA(F47E) tightly associates with LolCDE. Proteoliposomes were reconstituted with LolCDE, L10P, and *E. coli* phospholipids as described under Section 2. The release of L10P was induced by the addition of LolA or LolA(F47E). After fractionation into proteoliposomes and a supernatant, L10P and LolA or LolA(F47E) were analyzed by SDS–PAGE and immunoblotting with anti-Lpp or anti-LolA antibodies. One-fifth of the pellet material and one-fifth of the supernatant were applied to the gel for the analysis of L10P and LolA, respectively. Proteoliposomes reconstituted without LolCDE were used as a control.

examine the lipoprotein transfer activity of the LolA(F47E) mutant, a spheroplast supernatant containing the Pal–LolA or Pal–LolA(F47E) complex was incubated with outer membranes (Fig. 4). The ability to incorporate Pal into LolB-containing outer membranes was essentially the same for LolA and LolA(F47E). When LolB-depleted outer membranes were used, the incorporation of Pal was negligible.

### 3.4. Tight association of LolA(F47E) with LolCDE

To clarify the properties of LolA(F47E) in more detail, proteoliposomes were reconstituted with LolCDE, L10P, and *E. coli* phospholipids. L10P, a derivative of Lpp, is suitable for in vitro analysis of the release reaction [15]. L10P release was then examined in the presence of LolA or LolA(F47E) (Fig. 5). LolA(F47E) did not induce the release of L10P from proteoliposomes, whereas LolA induced the release of L10P in a LolCDE-dependent manner. Immunoblotting with anti-LolA antibodies revealed that LolA(F47E) was recovered in the proteoliposome fraction after the release reaction. The recovery of LolA(F47E) in this fraction was dependent on LolCDE. In contrast, LolA was always recovered in the supernatant. These results, taken together, suggest that LolA(F47E) tightly associates with LolCDE, thereby inhibiting the LolA-dependent release of lipoprotein in vivo (Fig. 1).

## 4. Discussion

When maltose uptake is inhibited by vanadate, MalE tightly binds to $MalFGK_2$, representing the transition state of the maltose transporter [16]. In contrast, inhibition of lipoprotein release by vanadate did not cause the tight association of LolA and LolCDE (unpublished observation). The ATPase activity of LolCDE was stimulated by lipoproteins but not affected by LolA [1]. Moreover, the LolCDE-dependent release of Pal occurred in the absence of LolA, albeit at a reduced rate [6]. Taken together, these observations appeared to suggest that the release of lipoproteins by LolCDE, and the formation of a complex between the released lipoprotein and LolA were separate events. However, the dominant negative phenotype of LolA(F47E) strongly suggests that LolA and LolCDE interact with each other when lipoproteins are released as a complex with LolA. The tight association between LolA(F47E) and LolCDE most likely inhibits this interaction, thereby causing growth inhibition even in the presence of wild-type LolA. However, it is not completely clear at present why LolA(F47E) was partially active as to the release of Pal, since our preliminary results suggest that this tight association is independent of lipoproteins.

The details of the molecular events occurring at the lipoprotein release step are largely unknown. It would be of great interest to determine how ATP energy is utilized to release lipoproteins anchored to the inner membrane and then transferred to form the LolA–lipoprotein complex. LolA(F47E) is expected to be very useful for determining the details of the lipoprotein release reaction.

*Acknowledgements:* We thank Rika Ishihara for the technical support. This work was supported by grants to H.T. from CREST of the Japan Science and Technology Corporation, and the Ministry of Education, Science, Sports and Culture of Japan.

## References

[1] Masuda, K., Matsuyama, S. and Tokuda, H. (2002) Proc. Natl. Acad. Sci. USA 99, 7390–7395.
[2] Puglsey, A.P. (1993) Microbiol. Rev. 57, 50–108.
[3] Yakushi, T., Masuda, K., Narita, S., Matsuyama, S. and Tokuda, H. (2000) Nat. Cell Biol. 2, 212–218.
[4] Matsuyama, S., Tajima, T. and Tokuda, H. (1995) EMBO J. 14, 3365–3372.
[5] Matsuyama, S., Yokota, N. and Tokuda, H. (1997) EMBO J. 16, 6947–6955.
[6] Yokota, N., Kuroda, T., Matsuyama, S. and Tokuda, H. (1999) J. Biol. Chem. 274, 30995–30999.
[7] Tajima, T., Yokota, N., Matsuyama, S. and Tokuda, H. (1998) FEBS Lett. 439, 51–54.
[8] Tanaka, K., Matsuyama, S. and Tokuda, H. (2001) J. Bacteriol. 183, 6538–6542.
[9] Narita, S., Tanaka, K., Matsuyama, S. and Tokuda, H. (2002) J. Bacteriol. 184, 1417–1422.
[10] Miyamoto, A., Maytsuyama, S. and Tokuda, H. (2001) Biochem. Biophys. Res. Commun. 287, 1125–1128.
[11] Davidson, A.L. (2002) J. Bacteriol. 184, 1225–1233.
[12] Hussain, M., Ichihara, S. and Mizushima, S. (1980) J. Biol. Chem. 255, 3707–3712.
[13] Tani, K., Tokuda, H. and Mizushima, S. (1990) J. Biol. Chem. 265, 17341–17347.
[14] Casadaban, M.J. (1976) J. Mol. Biol. 104, 541–555.
[15] Yakushi, T., Yokota, N., Matsuyama, S. and Tokuda, H. (1998) J. Biol. Chem. 273, 32576–32581.
[16] Chen, J., Sharma, S., Quiocho, F.A. and Davidson, A.L. (2001) Proc. Natl. Acad. Sci. USA 98, 1525–1530.