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Review

Sorting of lipoproteins to the outer membrane in *E. coli*

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Abstract

Escherichia coli lipoproteins are anchored to the periplasmic surface of the inner or outer membrane depending on the sorting signal. An ATP-binding cassette (ABC) transporter, LolCDE, releases outer membrane-specific lipoproteins from the inner membrane, causing the formation of a complex between the released lipoproteins and the periplasmic molecular chaperone LolA. When this complex interacts with outer membrane receptor LolB, the lipoproteins are transferred from LolA to LolB and then localized to the outer membrane. The structures of LolA and LolB are remarkably similar to each other. Both have a hydrophobic cavity consisting of an unclosed β-barrel and an α-helical lid. Structural differences between the two proteins reveal the molecular mechanisms underlying the energy-independent transfer of lipoproteins from LolA to LolB. Strong inner membrane retention of lipoproteins occurs with Asp at position 2 and a few limited residues at position 3. The inner membrane retention signal functions as a Lol avoidance signal and inhibits the recognition of lipoproteins by LolCDE, thereby causing their retention in the inner membrane. The positive charge of phosphatidylethanolamine and the negative charge of Asp at position 2 are essential for Lol avoidance. The Lol avoidance signal is speculated to cause the formation of a tight lipoprotein–phosphatidylethanolamine complex that has five acyl chains and therefore cannot be recognized by LolCDE.

Keywords: Lipoprotein; Lol protein; Outer membrane; Sorting signal; ABC transporter

1. Introduction

Escherichia coli, a Gram-negative bacterium, consists of four compartments; the cytoplasm, the inner (cytoplasmic) membrane, the periplasm and the outer membrane. Periplasmic and outer membrane proteins are synthesized as precursors with a signal peptide at their N-termini in the cytoplasm, and are then translocated across the inner membrane by Sec translocase [1–5]. The signal peptide is essential for translocation, but does not determine the final destinations of translocated proteins. Distinct mechanisms catalyze the sorting and transport of translocated proteins to the final destinations, i.e. the periplasm or outer membrane.

The outer membrane is composed of proteins, phospholipids and lipopolysaccharide (LPS) (Fig. 1). LPS is present exclusively in the outer leaflet of this membrane [6], where it serves as a permeability barrier to hydrophobic substances [7]. In contrast, phospholipids are mostly localized in the inner leaflet of the outer membrane. This asymmetrical bilayer contains a few species of abundant proteins such as OmpA, OmpC, OmpF, LamB and PhoE, and a number of lipid-modified proteins, so-called lipoproteins. These Omps span the outer membrane, whereas lipoproteins are anchored to the outer membrane through their attached lipids. *E. coli* possesses at least 90 lipoprotein species, most of which seem to be localized in the outer membrane (Matsuyama, S., Tanaka, K., and Tokuda, H., unpublished observation). No outer membrane-specific lipoproteins have hydrophobic stretches that function as stop transfer or signal anchor sequences [8]. It is also known that outer membrane proteins have no hydrophobic stretches but span the membrane with amphipathic β-strands possessing alternating hydrophobic residues, which does not cause the retention of proteins in the inner membrane. Lipid modification of lipoproteins occurs on the outer surface of the inner membrane [9] and therefore does not inhibit the translocation [10]. Thus, both the β-structure and lipid modification are characteristic of outer membrane-associated proteins.

The complete genome sequence revealed many putative lipoproteins in various bacteria, for example, 114 are pre-

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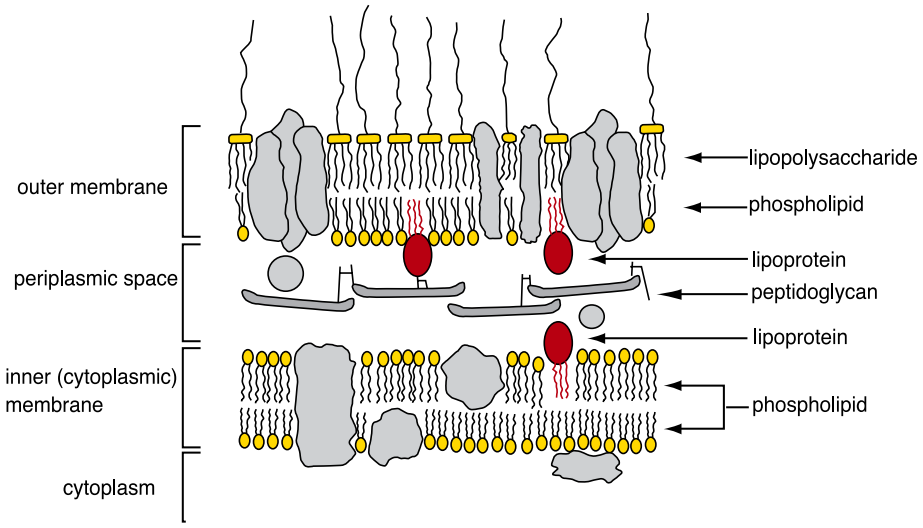

Fig. 1. Schematic representation of the *E. coli* envelope structure. Note that the outer membrane is an asymmetrical bilayer containing LPS in the outer leaflet and phospholipids in the inner leaflet. At least 90 lipoproteins (indicated in red) are present in *E. coli*, most of which are specific to the outer membrane. Lipoproteins are anchored to the periplasmic surface of either the inner or outer membrane in *E. coli*.

dicted for Gram-positive *Bacillus subtilis* [11], and 105 for Lyme disease spirochete *Borrelia burgdorferi* [12]. The presence of many lipoproteins in bacteria suggests that various membrane-associated activities are dependent on lipoproteins. However, the majority of *E. coli* lipoproteins have no known function.

2. Biogenesis of lipoproteins

Processing of a lipoprotein precursor, which has a consensus sequence called a lipobox or lipoprotein box around the signal peptide cleavage site [13], to its mature form sequentially takes place on the periplasmic side of the inner membrane (Fig. 2); formation of a thioether linkage between the N-terminal Cys residue of the mature region and diacylglycerol by phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt), cleavage of the signal peptide by prolipoprotein signal peptidase (LspA or signal peptidase II), and aminoacylation of the N-terminal Cys residue by phospholipid/apolipoprotein transacylase (Lnt) [14]. The mature lipoproteins thus formed have a lipid-modified Cys at the N-terminus. Globomycin [15] inhibits LspA and causes the accumulation of diacylglyceryl prolipoproteins in the inner membrane [16]. The three lipoprotein-processing enzymes Lgt, LspA and Lnt are widely conserved in Gram-negative bacteria, whereas Lnt homologs have not been found in Gram-positive bacteria, although a result suggesting the aminoacylation of *Staphylococcus aureus* lipoproteins has been reported [17].

In *E. coli*, lipoproteins are anchored to the periplasmic side of either the inner or outer membrane through N-terminal lipids depending on the lipoprotein-sorting signal [9]. Some Gram-negative bacteria are known to possess lipoproteins on the outer surface of the outer membranes [18]. In Gram-positive bacteria, lipoproteins are anchored to the outer leaflet of the cytoplasmic membrane.

3. Lipoprotein sorting signals

Yamaguchi et al. [19] first revealed the importance of the residue at position 2 for the sorting of *E. coli* lipoproteins.

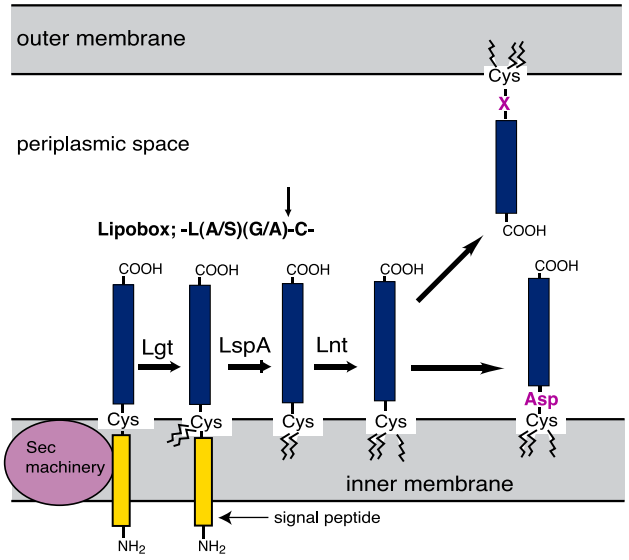

Fig. 2. Biogenesis of lipoproteins. Consensus lipobox sequences are indicated with a signal cleavage site indicated by an arrow. X represents a residue other than Asp. Lgt, phosphatidylglycerol/prolipoprotein diacylglyceryl transferase; LspA, prolipoprotein signal peptidase (also called Spase II); Lnt, phospholipid/apolipoprotein transacylase.

They showed that replacement of Ser at position 2 of an outer membrane-specific lipoprotein by Asp caused the protein to remain in the inner membrane. Furthermore, replacement of Asp at position 2 of an inner membrane-specific lipoprotein by another residue caused outer membrane localization of the protein. These results indicated that Asp at position 2 functions as an inner membrane retention signal for lipoproteins, whereas other residues cause outer membrane localization. However, they later found that when His or Lys was present at position 3, some lipoproteins were localized in the outer membrane even though Asp was present at position 2 [20].

Seydel et al. [21] reported a sensitive method for examining the inner membrane localization of lipoprotein derivatives. They constructed a maltose binding protein (MalE) derivative having a lipid-modified Cys at the N-terminus (lipoMalE) and then expressed it in a chromosomal *malE* deletion mutant. When Asp was at the N-terminal second position, lipoMalE was localized in the inner membrane, where it functioned as a maltose binding protein, thereby enabling the mutant to grow in the presence of maltose. In contrast, lipoMalE localized in the outer membrane was not functional and did not support *malE* mutant growth. Systematic substitution of the residue at position 2 of lipoMalE revealed that in addition to Asp, five other residues (Phe, Trp, Tyr, Gly and Pro) supported mutant growth, indicating that Asp at position 2 is not the sole inner membrane retention signal. However, *E. coli* native lipoproteins do not have Phe, Trp, Tyr, Gly and Pro at position 2. Since limited degradation of lipoMalE releases a small amount of a periplasmic form that can function in maltose transport, the results of this assay are not always conclusive.

4. Lol proteins

4.1. LolA and LolB

E. coli spheroplasts, in which the outer membrane is disrupted by EDTA and lysozyme, secrete various protein species that are destined for the periplasm or outer membrane. In contrast, the major outer membrane lipoprotein Lpp was not secreted but remained in the inner membrane of spheroplasts as a mature form. When periplasmic materials were added externally, Lpp was secreted from spheroplasts, indicating that a periplasmic factor is required for the release of Lpp from the inner membrane. The Lpp-releasing factor (20 kDa) was purified from the periplasm and named LolA [22]. LolA was then shown to release other outer membrane lipoproteins such as Pal, NlpB, Slp and RlpA, whereas inner membrane lipoproteins AcrA and NlpA were not released even in the presence of LolA [23], indicating that LolA plays a critical role in the sorting of lipoproteins. Lipoproteins released from spheroplasts in the presence of LolA were found to exist as a water-soluble complex with LolA. When this complex was incubated with the outer membrane, lipoproteins were incorporated into the outer membrane. An outer membrane protein required for this lipoprotein incorporation was identified by using proteoliposomes reconstituted from solubilized outer membrane proteins and *E. coli* phospholipids. The protein (23 kDa), named LolB, was identified as a novel outer membrane lipoprotein possessing receptor activity for lipoproteins [24]. A lipoprotein–LolB complex was formed upon incubation of the lipoprotein–LolA complex and LolB, indicating the transfer of lipoprotein from LolA to LolB. The release and outer membrane localization of LolB per se are also dependent on LolA and LolB, respectively [23]. Lipoproteins are highly hydrophobic owing to their N-terminal lipids, whereas both the lipoprotein–LolA and –LolB complexes are water-soluble, suggesting that the N-terminal lipid-modified region of lipoproteins is shielded from the aqueous environment by LolA or LolB.

LolA [22] and LolB [24] each function in a monomeric form. The LolA–lipoprotein complex is stable both in vivo [25] and in vitro [24] only in the absence of the LolB function, suggesting that the affinity for lipoproteins is lower for LolA than for LolB. Indeed, LolA easily transfers associated lipoproteins to LolB and returns to the free form [24]. These results indicate that the lipoprotein transfer occurs due to the affinity difference for the lipoproteins between the two proteins.

4.2. LolCDE

The LolA-dependent release of lipoproteins from right-side-out membrane vesicles was found to require ATP as well as an outer membrane sorting signal [26]. The inner membrane proteins conferring the release activity were purified by monitoring the activity reconstituted into proteoliposomes. The LolCDE complex thus identified has a subunit stoichiometry of $C_1D_2E_1$ with an expected molecular weight of 139,483 [10]. The genes encoding the three proteins form an operon. LolD possesses Walker A and B motifs with the consensus sequence of the ATP-binding cassette (ABC) transporter protein called the ABC signature motif [27]. Both LolC and LolE span the membrane four times and have a large domain exposed to the periplasm. The LolCDE complex is therefore an ABC transporter but mechanistically differs from all other ABC transporters in that it is not involved in the transmembrane transport of substrates. ABC transporters have at least 10 transmembrane stretches [28], while only 8 are predicted for the LolCDE complex. This may be related to the unique function of the LolCDE complex.

The amino acid sequences of membrane subunits LolC and LolE are similar to each other, the identity being 26%. Moreover, the N-terminal 60 residues of the two proteins exhibit 55% identity. Despite this sequence similarity, LolC and LolE are both essential for the release of lipoproteins [29]. Inhibition of the LolCDE function does not affect the translocation of a lipoprotein precursor across the inner

membrane and subsequent processing to the mature lipoprotein [10,29], indicating that the Sec-dependent translocation and modification of lipoprotein precursors are completely independent of and not affected by Lol-mediated reactions.

Five Lol proteins, A to E, are highly conserved in various Gram-negative bacteria. The sorting and localization of lipoproteins in many, if not all, Gram-negative bacteria therefore seem to be mediated by a Lol system comprising the LolCDE complex in the inner membrane, LolA in the periplasm and LolB in the outer membrane (Fig. 3). So far as we examined, LolB seems to be absent from bacteria such as *Helicobacter pylori*, *Campylobacter jejuni*, *Rickettsia prowazekii*, *Treponema pallidum* and *B. burgdorferi*, the last of which has been reported to possess more than 100 lipoproteins [12]. All Lol proteins are essential for *E. coli* growth [25,29,30].

E. coli is predicted to possess many ABC transporters [31,32]. As far as it is known, LolCDE [29] and MsbA [33] are the only essential ABC transporters of *E. coli*. MsbA is proposed to be involved in the transport of LPS and lipids from the inner to the outer membrane [34]. Therefore, these two essential ABC transporters appear to be involved in the biogenesis of the *E. coli* envelope.

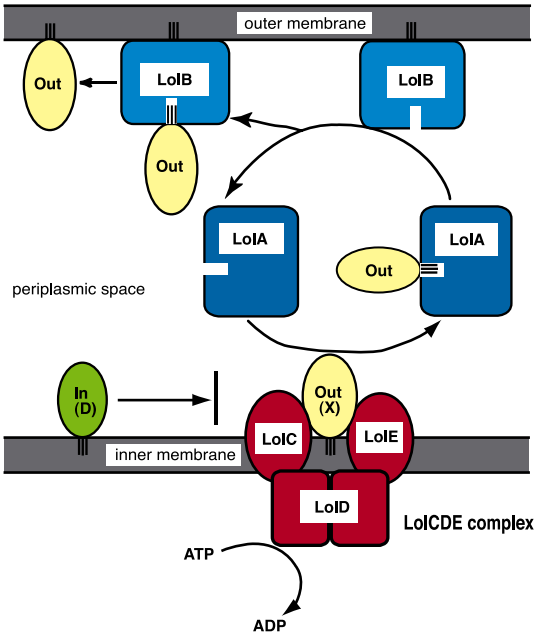

Fig. 3. Sorting and outer membrane localization of lipoproteins by the Lol system. "In" and "Out" represent inner membrane-specific and outer membrane-specific lipoproteins, respectively. An ABC transporter, LolCDE, releases outer membrane-specific lipoproteins from the inner membrane, causing the formation of a complex between the released lipoproteins and the periplasmic molecular chaperone LolA. When this complex interacts with outer membrane receptor LolB, the lipoproteins are transferred from LolA to LolB and then localized to the outer membrane. The inner membrane retention signal Asp at position 2 inhibits the recognition of lipoproteins by LolCDE, thereby causing their retention in the inner membrane. For more details, see the text.

4.3. Crystal structures of LolA and LolB

Although there is no apparent homology between the amino acid sequences of LolA and LolB, the structures of LolA and LolB solved at 1.65 and 1.9 Å resolution, respectively, are strikingly similar to each other [35]. Both have a hydrophobic cavity consisting of an unclosed β-barrel and an α-helical lid (Fig. 4). The inner surface of the β-sheet and three α-helices consists of hydrophobic residues. The hydrophobic cavity represents a possible binding site for the lipid moieties of lipoproteins. Structural differences between the two proteins provide significant insights into the molecular mechanisms underlying the energy-independent transfer of lipoproteins from LolA to LolB.

Though X-Pro *cis* peptide bonds are found in about 5% of all proteins [36,37], interestingly, while two of the seven X-Pro bonds in LolA, Arg43–Pro44 and Gln53–Pro54, have the *cis* conformation. The side chain of Arg43 is oriented toward the interior of the molecule due to this *cis* peptide bond (Fig. 4). The side chain N atoms of Arg43 are hydrogen bonded to the main chain carbonyls of residues in the α1- and α2-helices, thereby fixing the helices to the β2-strand as a lid. Based on the crystallographic data, Arg43 is the only residue to function as a lock disconnecting the hydrophobic cavity of LolA from the solvent region. In contrast, the hydrophobic cavity of LolB is not disconnected from the solvent by its loops. The "lid" of LolA is expected to undergo opening and closing upon the accommodation and release of lipoproteins, respectively. The LolA structure explains the phenotypes of LolA mutants, R43L [38] and F47E [39]. The LolA(R43L) mutant possesses Leu in place of Arg at position 43, a critical residue for lid closing, and causes the accumulation of the LolA(R43L)–lipoprotein complex in the periplasm. This mutant is as active as wild-type LolA as to the release of lipoproteins from spheroplasts. In marked contrast, the transfer of lipoproteins from LolA(R43L) to LolB is completely inhibited. Hydrogen bonds between the β-sheet and lid helices cannot be formed in R43L, thereby decreasing the stability of the free form relative to that of the bound form. The other mutant, LolA(F47E), carrying a Phe to Glu mutation at position 47 is defective in the release of lipoproteins from spheroplasts. Additional hydrogen bonds may be formed between the β-sheet and the lid helices in the F47E mutant, making opening of the lid more difficult. When LolA is incubated with proteoliposomes containing LolCDE, wild-type LolA remains in the supernatant, whereas LolA(F47E) binds to the proteoliposomes [39]. This tight association of LolA(F47E) with LolCDE is presumably responsible for its dominant negative phenotype, suggesting that the LolA–LolCDE interaction is critical for lipoprotein release.

The structures of LolA and LolB further extend the understanding of the mechanism underlying Lol system-dependent lipoprotein transfer; when the LolCDE complex interacts with outer membrane-specific lipoproteins, LolD

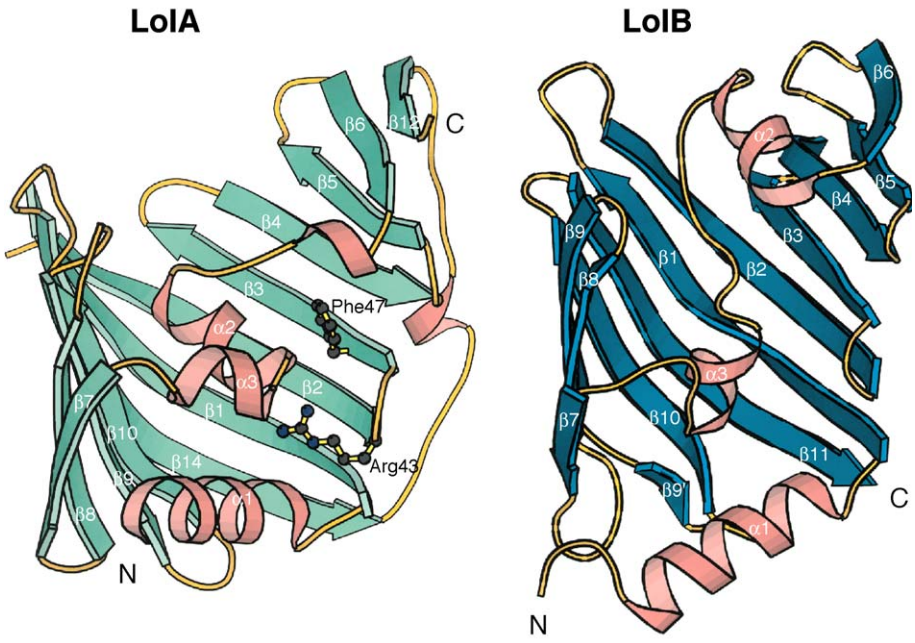

Fig. 4. Crystal structures of LolA and LolB. The LolA and LolB molecules are each shown as a ribbon model, in which α-helices (α1–α3) and β-strands are shown in red and green (for LolA) or blue (for LolB), respectively. Loops and short 310 helices are shown in yellow [35]. The residues of LolA which had been mutagenized [38,39] are shown as a ball-and-stick model.

releases the ATP energy on the cytoplasmic side of the membrane. This energy is transferred from LolD to LolC/LolE, and utilized to release lipoproteins from the outer leaflet of the membrane, leading to the formation of a LolA–lipoprotein complex in the periplasm. This requires the opening of the LolA lid through disruption of the hydrogen bonds between Arg43 and the lid-helices of LolA. The ATP energy released on the cytoplasmic side of membranes is thus utilized on the periplasmic side of the membrane to form a LolA–lipoprotein complex. The free form of LolA is expected to be more stable than the lipoprotein-bound form. Lipoproteins are transferred from LolA to LolB due to the affinity difference between their hydrophobic cavities for lipoproteins. LolB anchored to the outer membrane then transfers the associated lipoproteins to the inner leaflet of the outer membrane to which lipoproteins are stably anchored through three acyl chains.

5. Function of lipoprotein sorting signals

5.1. Inner membrane retention signals

Since lipoproteins released from spheroplasts on the addition of LolA are efficiently incorporated into the outer membrane in a LolB-dependent manner [23], the sorting signals for lipoproteins can be evaluated by examining the LolA-dependent release reaction. When the residue at position 3 is Ser, only Asp at position 2 causes the retention of lipoproteins in spheroplasts [40], confirming the importance of Asp at position 2 for the inner membrane retention of lipoproteins. However, the release of lipoproteins having Asp at position 2 is differentially affected by residues at position 3. Strong inner membrane retention occurs with a small number of different residues at position 3 (Fig. 5), indicating that Asp at position 2 alone is not sufficient for a strong inner membrane retention signal. The strongest inner membrane retention signals are Asp–Asp, Asp–Glu and Asp–Gln. These signals have been found for native lipoproteins having Asp at position 2, whereas ambiguous sorting signals causing less efficient retention or release, and hence localization in both membranes [20,21] have not been found for native lipoproteins. Since Asp–Asn is also a potent inner membrane retention signal, although this signal is not found in native lipoproteins, an acidic residue or its amide form at position 3 seems to make Asp at position 2 the strongest inner membrane retention signal. It remains to be clarified why Asp–Arg is a strong inner membrane signal but Asp–Lys rather functions as an outer membrane signal.

5.2. Lipid modification is not affected by sorting signals

Lnt catalysis of the last step of lipoprotein maturation (Fig. 2) is not found in Gram-positive bacteria despite the high conservation of the other two lipoprotein-processing enzymes, Lgt and LspA. This raised the possibility that the last step of lipoprotein modification, conversion of an apolipoprotein into a mature lipoprotein, is required only for lipoproteins destined for the outer membrane [21]. Asp at position 2 therefore might inhibit aminoacylation of apolipoproteins, causing the generation of different species

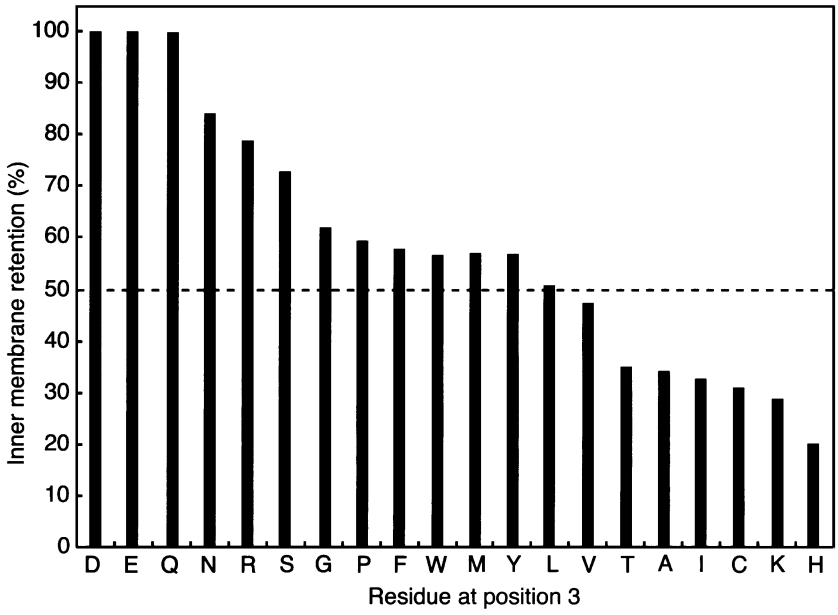

Fig. 5. Effect of the residue at position 3 on the inner membrane retention potency of Asp at position 2. The LolA-dependent release of lipoproteins possessing Asp at position 2 and the indicated residues at position 3 was determined in spheroplasts [40]. The amounts of lipoproteins retained in the inner membrane are shown as percentages of the total amounts. The amino acid residues at position 3 are expressed as a one-letter code.

of lipoproteins that are specific to the inner membrane. However, in vitro aminoacylation of apolipoproteins with purified Lnt and mass spectrometrical examination of lipoproteins purified from the inner membrane revealed that Asp at position 2 does not inhibit aminoacylation [41]. It was also found that the aminoacylation of lipoproteins is essential for LolCDE-dependent release.

The in vitro aminoacylation with purified Lnt revealed that this enzyme exhibits broad specificity to acyl chain donors as to phospholipid and fatty acid species [41]. Lnt can transfer the acyl chain of phosphatidylcholine, which is not present in *E. coli*. Moreover, Lnt transfers acyl chains of various lengths and degrees of saturation. Considering the broad specificity of Lnt to acyl donors, any fatty acid present in *E. coli* phospholipids is expected to be transferred to lipoproteins. Therefore, LolCDE should not exhibit strict specificity to aminoacyl chains in order to efficiently release lipoproteins from the inner membrane. On the other hand, the number of acyl chains attached to Cys at position 1 seems to be important for the recognition by LolCDE because apolipoproteins are not substrates for LolCDE.

5.3. Lol avoidance

The LolA-dependent release of outer membrane-specific lipoproteins from proteoliposomes reconstituted with LolCDE was inhibited on reconstitution with other outer membrane-specific, but not inner membrane-specific, lipoproteins. Moreover, outer membrane-specific lipoproteins stimulated ATP hydrolysis by LolCDE, whereas inner membrane-specific ones did not. These results revealed a novel function of Asp at position 2, i.e. lipoproteins having this signal avoid being recognized by LolCDE, thereby remaining in the inner membrane [42]. Thus, the mechanism underlying the Asp-dependent retention of lipoproteins in the inner membrane was found to be simple. It seems likely that inner membrane-specific lipoproteins selected the Lol avoidance signal after the Lol system had been developed as a mechanism to localize lipoproteins in the outer membrane.

For further understanding of the Lol avoidance mechanism with Asp at position 2, a mutant that can release lipoproteins having Asp and Gln at positions 2 and 3, respectively, was isolated [43]. The mutant carried an Ala to Pro mutation at position 40 of LolC. A significant portion of an inner membrane lipoprotein was localized to the outer membrane when the LolC mutant was expressed. LolA formed a complex with the released lipoprotein, which was subsequently incorporated into the outer membrane in a LolB-dependent manner, indicating that the inner membrane retention signal only functions with LolCDE.

5.4. Mechanism underlying Lol avoidance

Since the proposal by Yamaguchi et al. [19], it has been believed that the residue at position 2 functions as a lipoprotein-sorting signal. On the other hand, it was revealed that any residue other than Asp at position 2 allowed the recognition of lipoproteins by LolCDE, suggesting that the second residue is not the active lipoprotein-sorting signal. 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 [44]. SH-specific introduction of non-protein

molecules to Cys did not inhibit the LolCDE-dependent release. These results indicate that LolCDE releases outer membrane-specific lipoproteins without recognizing the second residue. Therefore, LolCDE only recognizes an N-terminal Cys possessing three acyl chains, the sole common structure of lipoproteins. SH-specific introduction of a negative charge to Cys did not cause the retention of lipoproteins, whereas oxidation of Cys to cysteic acid resulted in generation of the Lol avoidance signal. Conversely, modification of carboxylic acid of Asp at position 2 abolished its Lol avoidance function. These results indicate that the Lol avoidance signal should have a negative charge that is within a certain distance from Cα of the second residue. Furthermore, amine-specific modification of phosphatidylethanolamine (PE) and substitution of PE with cardiolipin abolished the Lol avoidance function of Asp at position 2, whereas the Lol avoidance signal functioned in proteoliposomes reconstituted with phosphatidylcholine. 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 (Fig. 6). When Asp, Glu, Gln or Asn is at position 3, Asp at position 2 becomes a very strong Lol avoidance signal (Fig. 5). In contrast, His, Lys, Cys, Ile, Ala 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. 6). When the side chain of Glu at position 2 is longer, 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. Residues at position 3 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 that has five acyl chains and cannot be accommodated in LolCDE. The release incompetence of apolipoproteins also seems to indicate that the number of acyl chains is critical for the recognition by LolCDE. The LolC(A40P)DE complex can presumably accommodate such a lipoprotein–PE complex, thereby causing the release of lipoproteins having the Lol avoidance signal [43]. Arg at position 3 strengthens the Lol avoidance function of Asp at position 2, whereas Lys at position 3 does not (Fig. 5). Perturbation of the electrostatic Asp–PE interaction by a positive charge at position 3 seems to be dependent on the side chain structure of the third residue.

It has been 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 [21], although *E. coli* native lipoproteins do not have these

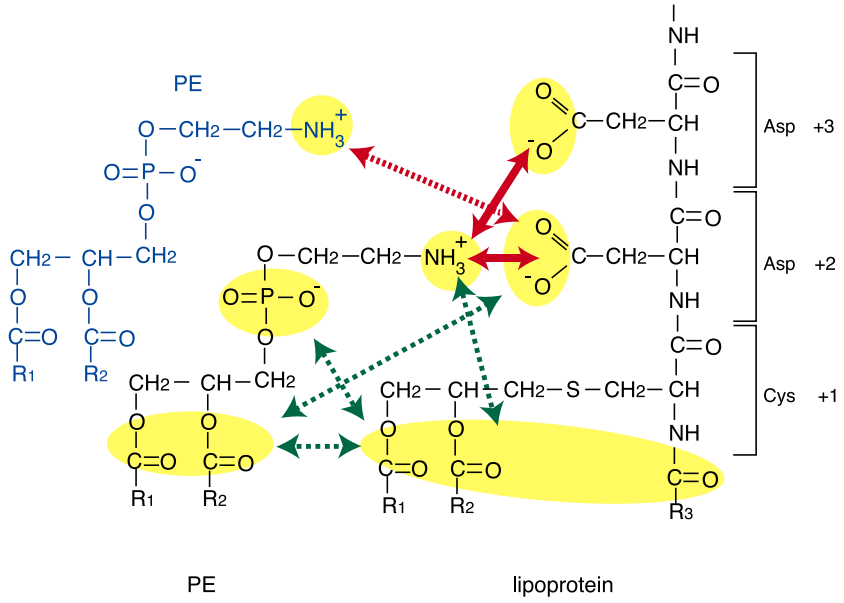

Fig. 6. A possible Asp–PE complex that causes Lol avoidance. The electrostatic and steric complementarity between Asp at position 2 and PE (solid red lines) is strengthened by hydrogen bonds (dotted green lines), causing the formation of a tight lipoprotein–PE complex possessing five acyl chains [44]. When Asp is also present at position 3, an electrostatic interaction is formed with PE, as indicated. When the side chain of Glu at position 2 is longer, the PE molecule (blue) involved in the electrostatic interaction (dotted red line) with Glu is different from the PE molecule forming the hydrogen bond with Cys at position 1. For more details, see the text.

residues at position 2. 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.

6. Lipoprotein sorting in other Gram-negative bacteria

Asp at position 2 may not always be the Lol avoidance signal in other bacteria, although Lol proteins are conserved in various Gram-negative bacteria. MexA in *Pseudomonas* species is an inner membrane lipoprotein possessing Gly and Lys at positions 2 and 3, respectively. The ionic interaction between PE and the second residue is not applicable to the Lol avoidance mechanism in this particular case. Since membrane localization and sorting signals have been biochemically determined for only a few lipoproteins in other bacteria, it remains to be determined how widely the Lol avoidance mechanism mentioned above 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 oxitoca* on the outer surface of the outer membrane. Pugsley [9] 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 is localized on the outer surface of the outer membrane when expressed with the Type II secretion pathway. In contrast, PulA is 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 a Lol avoidance signal in the absence of the Type II pathway. Substitution of Asp with another residue results in the localization of PulA on both the periplasmic surface and the outer surface of the outer membrane when the Type II secretion pathway is present. However, this PulA derivative is exclusively localized on the periplasmic surface of the outer membrane in the absence of the Type II secretion pathway. Therefore, PulA expressed in *E. coli* and, presumably, *K. oxitoca* should have a Lol avoidance signal in order 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.

A similar mechanism may function in other bacteria having lipoproteins on the outer surface of the outer membrane. *B. burgdorferi*, the Lyme disease spirochete, has been reported to possess more than 100 lipoproteins, some of which are on the outer surface of the outer membrane [18]. Since this bacterium also possesses Lol homologs, lipoproteins on the outer surface of the outer membrane might have a Lol avoidance signal, which may not be Asp in this bacterium. Alternatively, the Lol system may not be complete since a LolB homolog has not been found in this bacterium. In any event, since spirochetal lipoproteins have been reported to cause an immunoresponse of host cells [45], it is important to clarify the mechanism underlying the sorting of more than 100 lipoproteins in *B. burgdorferi*.

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