BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2010,** *49,* 4565–4567 4565
DOI: 10.1021/bi100493e

Proteins Required for Lipopolysaccharide Assembly in *Escherichia coli* Form a Transenvelope Complex†

Shu-Sin Chng,‡ Luisa S. Gronenberg,‡ and Daniel Kahne*

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
‡*These authors contributed equally to this work.*

Received April 2, 2010; Revised Manuscript Received May 4, 2010

ABSTRACT: The viability of Gram-negative organisms is dependent on the proper placement of lipopolysaccharide (LPS) in the outer leaflet of its outer membrane. LPS is synthesized inside the cell and transported to the surface by seven essential lipopolysaccharide transport (Lpt) proteins. How these proteins cooperate to transport LPS is unknown. We show that these Lpt proteins can be found in a membrane fraction that contains inner and outer membranes and that they copurify. This constitutes the first evidence that the Lpt proteins form a transenvelope complex. We suggest that this protein bridge provides a route for LPS transport across the cell envelope.

Lipopolysaccharide (LPS)[1] is a glycolipid found in the outer leaflet of the outer membrane (OM) of Gram-negative bacteria (Figure 1) (*1*, *2*). The presence of LPS on the cell surface improves the barrier function of the OM, making many antibiotics used to treat Gram-positive infections ineffective against Gram-negative pathogens (*3*). The pathways by which LPS is synthesized at the cytoplasmic leaflet of the inner membrane (IM) and translocated to the periplasmic leaflet have been well characterized (*4*). The mechanism by which LPS is transported across the cell envelope to the surface is much less well understood (*5*). LPS must be released from the IM, transported across the aqueous periplasm, and assembled into the outer leaflet of the OM. Seven essential Lpt (lipopolysaccharide transport) proteins mediate these final steps in LPS assembly (Figure 1) (*6*, *7*).

Extracting LPS from the phospholipid (PL) bilayer of the IM requires energy. Four Lpt proteins, LptB, LptC, LptF, and LptG (formerly YhbG, YrbK, YjgP, and YjgQ, respectively), have been proposed to form a complex that couples ATP hydrolysis to the release of LPS from the IM (*8*−*12*). This complex has been purified and shown to exhibit ATP hydrolytic activity (*11*). Two additional proteins, LptD and LptE (formerly Imp and RlpB, respectively), form a complex at the OM that is responsible for the correct insertion of LPS into the outer leaflet of the OM (*13*−*16*). LptE has been shown to bind LPS in a specific manner and may receive LPS from the periplasm (*16*). The periplasmic protein

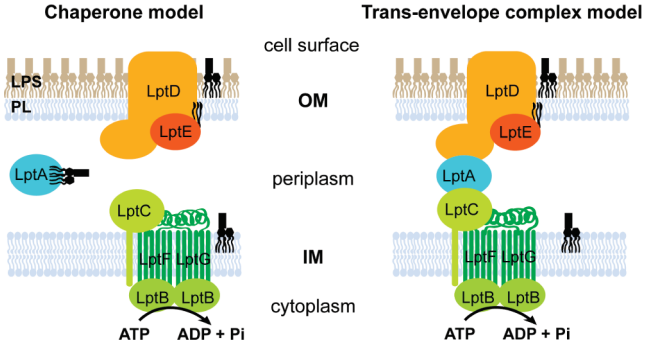

FIGURE 1: Two models for the transport of LPS across the periplasmic compartment.

LptA (formerly YhbN) is believed to mediate LPS transport across the aqueous compartment, somehow coordinating the functions of the IM and OM Lpt complexes (*8*, *9*, *17*−*19*). Consistent with this role, LptA has been reported to interact with LPS (*19*).

Two models for how the Lpt proteins facilitate LPS transport across the aqueous periplasm have been considered (Figure 1) (*5*−*7*). In one model, LptA forms a soluble complex with LPS to shuttle it from the IM to the OM (Figure 1, left). As such, LptA would function in a fashion analogous to that of LolA, the chaperone involved in OM lipoprotein trafficking (*20*). In another model, LPS transport occurs through protein (Figure 1B, right) or membrane bridges found in zones of adhesion between the IM and the OM (*5*). These adhesion zones were first observed in electron micrographs (EM) by Bayer more than 40 years ago (*21*, *22*). Newly synthesized LPS has been observed to appear in patches in the OM close to these "Bayer junctions" (*23*). Furthermore, LPS appears to transiently accumulate in a novel OM_L fraction [a less dense, "light", OM (vide infra)] that can be isolated during membrane fractionation (*24*). This OM_L fraction contains the IM and the OM, reminiscent of Bayer junctions. The most elegant experiment that supports the "bridge" model demonstrated that LPS transport to the OM continues in spheroplasts, despite the fact that most periplasmic contents were lost (*25*).

Here we provide evidence of direct physical interaction among the seven Lpt proteins. We show that all Lpt proteins cofractionate with the OM_L fraction and that they can be copurified together, suggesting these proteins can form a continuous connection between the IM and the OM (Figure 1, right).

Whole cell lysates obtained from wild-type *Escherichia coli* cells were fractionated by sedimentation on sucrose density gradients, and the localization of Lpt proteins was examined via

†This work was supported by National Institutes of Health Grant AI081059 (to D.K.) and a National Science Foundation fellowship (to L.S.G.).
*To whom correspondence should be addressed. Phone: (617) 496-0208. Fax: (617) 496-0215. E-mail: kahne@chemistry.harvard.edu.
[1]Abbreviations: OM, outer membrane; IM, inner membrane; LPS, lipopolysaccharide; PL, phospholipid; OM_L, light OM; OM_H, heavy OM; wt, wild-type; P_i, phosphate; NADH, reduced β-nicotinamide adenine dinucleotide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy; MS, mass spectrometry.

Published on Web 05/06/2010

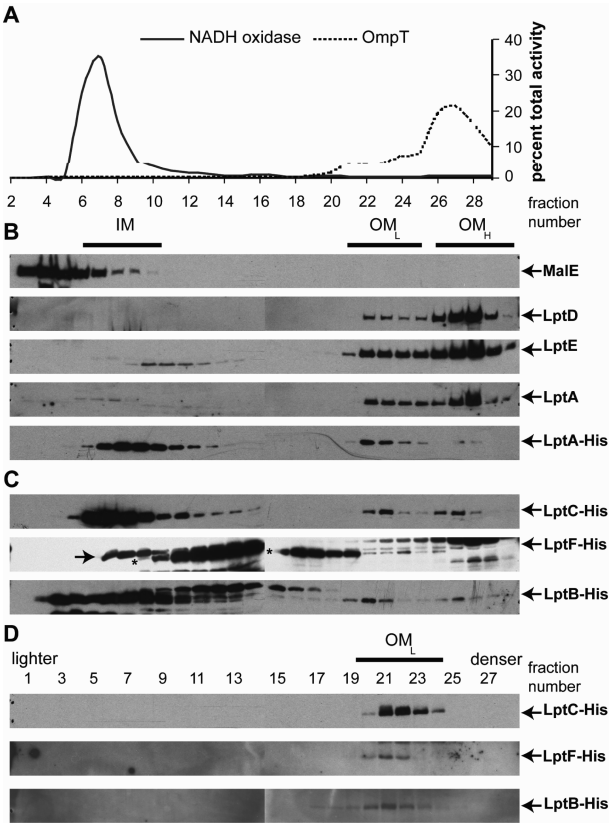

FIGURE 2: Lpt proteins are found in the OM_L, a fraction containing both IM and OM. (A and B) Sucrose gradient fractionation using wild-type cells or cells expressing His-tagged LptA. (A) Enzymatic activity of IM and OM markers detected in each fraction expressed as a percentage of total activity detected across all fractions. (B) Immunoblot analysis of fractions for Lpt proteins, as indicated. (C) Sucrose gradient fractionation using cells expressing His-tagged Lpt protein. Immunoblot analysis of fractions for His-tagged IM Lpt proteins, as indicated. The arrow on the left denotes LptF-His in the IM fractions. Asterisks denote a major band that cross-reacts with the α-His antibody. (D) Immunoblot analysis of floatation gradient fractions of pooled OM_L-containing fractions (fractions 21−23) from panel C. The position of OM_L (indicated) corresponds to fractions containing major OM proteins as judged by Coomassie blue staining (not shown).

immunoblotting (Figure 2). Under the conditions used, the IM and OM fractionated at buoyant densities of 1.11−1.16 g/mL (fractions 6−9) and 1.22−1.27 g/mL (fractions 21−29), respectively, as judged by activities of the IM enzyme, NADH oxidase, and the OM protease, OmpT (Figure 2A). Separation of the IM from the OM was achieved with no detectable cross contamination. As indicated by the distribution of OmpT activity, the OM further separated into OM_L (fractions 21−23) and OM_H (bulk, "heavy", OM, fractions 25−29) fractions (*24*). Antibodies directed against LptD and LptE revealed that these proteins are both associated with the OM_L and OM_H fractions (Figure 2B). Remarkably, LptA was also found to cofractionate almost exclusively with these OM fractions (Figure 2B). LptA has been reported to be found in the soluble periplasmic fraction when overexpressed as a C-terminally His-tagged protein (LptA-His) (*7*, *17*, *18*). In our fractionation, where untagged LptA is expressed at native levels, it is apparently not a freely soluble protein (compare to the distribution of the soluble periplasmic maltose-binding protein MalE).[2]

[2]The LptA ortholog in *Neisseria meningitidis* has been suggested to associate with membranes (unpublished observation in ref *5*).

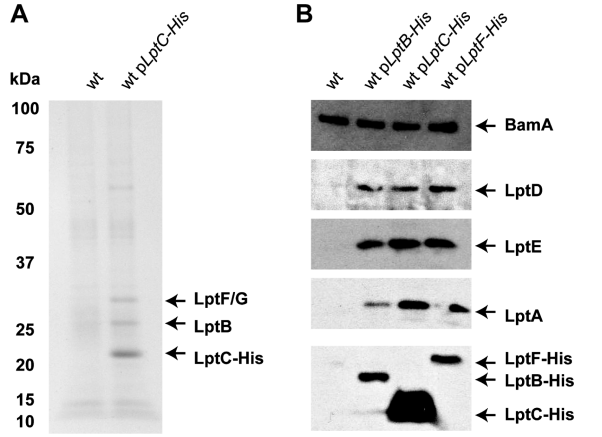

FIGURE 3: All seven Lpt proteins physically interact. (A) SDS−PAGE analysis of samples from TALON affinity purification and immunoprecipitation of DDM-solubilized total membranes containing LptC-His. Copurified proteins LptB, LptF, and LptG were identified by tandem MS of the major bands; levels of amino acid coverage for the three proteins were 83, 49, and 29%, respectively. (B) Immunoblot analysis of samples from affinity purification experiments using a His-tagged component of the IM Lpt complex. OM protein BamA was used as a loading control.

We also found that even LptA-His expressed at low levels associates with membranes. However, LptA-His localizes almost exclusively to the IM (Figure 2B), suggesting that LptA can also interact with the IM. It appears that the His tag interferes with its interaction with the OM, and when such interaction is inhibited, LptA still preferentially fractionates with membranes, rather than with soluble proteins.

We also examined the localization of the IM Lpt proteins. To detect LptB, LptC, and LptF, we engineered viable *E. coli* strains containing a chromosomal knockout while exogenously expressing functional His-tagged protein from a plasmid (we could not obtain a functional LptG-His construct). The distribution profiles of these proteins are shown in Figure 2C. As expected, all of them fractionated with the IM fractions. Interestingly, however, small amounts of each of these proteins cofractionated with the OM fractions as well (Figure 2C, fractions 21−29). We do not know if there is enrichment of IM Lpt proteins in the OM_L over the IM as compared to NADH oxidase. However, LptB, LptC, and LptF were enriched in the OM_L over the OM_H as compared to the OmpT activity in these fractions. This result is consistent with the report that the OM_L predominantly contains double-membrane fragments consisting of the IM and the OM, while the OM_H predominantly contains single-membrane OM fragments (*24*). We pooled the OM_L fractions containing the His-tagged proteins and performed a second floatation sucrose gradient (Figure 2D). Each of them still associated with the OM_L fractions, suggesting that their presence in the OM_L was not a result of contamination from the IM. These results show that the OM_L fraction contains both the IM and OM Lpt proteins required for LPS transport. This is consistent with the idea that the OM_L may provide a route for LPS transport to its final destination (*24*).

To determine if this route involves a protein bridge that connects the inner and outer membrane machinery of the Lpt proteins, we examined if the Lpt proteins physically interact. We performed affinity purification experiments (Figure 3). His-tagged LptC, expressed at low levels, copurifies with three endogenous proteins, as judged by additional bands on SDS−PAGE (Figure 3A). MS sequencing revealed that these bands contain

LptB, LptF, and LptG. This result was expected on the basis of a recent report showing that these four proteins form a stable complex when overexpressed (*11*). Perhaps, what was unexpected is that we are also able to pull down the three OM-associated Lpt proteins using any His-tagged component in the IM Lpt complex (Figure 3B). Cell lysates containing His-tagged LptB, LptC, or LptF were subjected to affinity chromatography. Based on immunoblot analysis, all three OM-associated proteins, LptD, LptE, and LptA, copurified with these IM proteins. In contrast, the OM protein BamA (formerly YaeT) was not selectively enriched relative to the control sample that did not contain any His-tagged protein (*26*). These results show that the IM and OM components of the LPS transport machinery physically interact.

That all Lpt proteins can be found in the double-membrane OM_L fraction and that they directly interact constitute the first evidence of a physical transenvelope complex of Lpt proteins that could provide the bridge for LPS transport. We do not know if these bridges form only transiently or are always present in the cell. Nevertheless, genetic evidence suggests that the transenvelope complex is relevant in vivo. Depletion of any member of the Lpt complex causes LPS to accumulate in the periplasmic leaflet of the IM (*9*, *10*, *17*); removing any of the individual components breaks the machine and disrupts the entire transport process. Whether this transenvelope machine maintains the proposed membrane bridge in a Bayer junction or actually itself mediates LPS transport is not clear (*5*, *6*). In support of the second interpretation, it has recently been shown that LptA can interact with LPS (*19*).

How the IM LptBCFG complex is physically connected to LptA and the OM LptDE complex may provide us with insights into the mechanism of LPS transport. The crystal structure of LptA reveals that multiple LptA molecules can stack in a head-to-tail fashion to form a fibril containing a hydrophobic groove running through its entire length (*18*). It has been pointed out that LptC and the N-terminal periplasmic domain of LptD both belong to the same OstA superfamily as LptA (*5*, *18*, *27*, *28*). As previously suggested, LptC may connect with LptD through one or more copies of LptA (Figure 1, right) (*18*). Our finding that LptA can interact with both the IM and the OM is consistent with this model. The continuous hydrophobic groove created by the OstA-like domains may shield the lipid A portion of LPS molecules from the aqueous environment as they traverse the periplasm.

Maintenance of the permeability barrier of the OM is essential for the viability of Gram-negative organisms. Because this barrier function depends on the proper assembly of LPS, the Lpt proteins represent a new opportunity for antibiotic development (*12*, *29*). A better mechanistic understanding of how the Lpt proteins facilitate LPS assembly would help us learn how to interfere with the pathway. In this paper, we provide biochemical evidence of the existence of a transenvelope Lpt protein complex that is involved in the transport of LPS across the periplasm. That the Lpt complex spans two separate membranes poses a major challenge for studying the mechanism of LPS transport. The development of new tools may be needed to determine the detailed molecular mechanism, and what individual roles each of these seven essential Lpt proteins plays in the process.

ACKNOWLEDGMENT

We thank the staff of the Taplin MS Facility at Harvard Medical School for help with protein identification.

SUPPORTING INFORMATION AVAILABLE

Detailed molecular cloning and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Mühlradt, P. F., and Golecki, J. R. (1975) *Eur. J. Biochem. 51*, 343–352.
2. Kamio, Y., and Nikaido, H. (1976) *Biochemistry 15*, 2561–2570.
3. Nikaido, H. (2003) *Microbiol. Mol. Biol. Rev. 67*, 593–656.
4. Raetz, C. R. H., and Whitfield, C. (2002) *Annu. Rev. Biochem. 71*, 635–700.
5. Bos, M. P., Robert, V., and Tommassen, J. (2007) *Annu. Rev. Microbiol. 61*, 191–214.
6. Ruiz, N., Kahne, D., and Silhavy, T. J. (2009) *Nat. Rev. Microbiol. 7*, 677–683.
7. Sperandeo, P., Dehò, G., and Polissi, A. (2009) *Biochim. Biophys. Acta 1791*, 594–602.
8. Sperandeo, P.; et al. (2007) *J. Bacteriol. 189*, 244–253.
9. Sperandeo, P.; et al. (2008) *J. Bacteriol. 190*, 4460–4469.
10. Ruiz, N., Gronenberg, L., Kahne, D., and Silhavy, T. J. (2008) *Proc. Natl. Acad. Sci. U.S.A. 105*, 5537–5542.
11. Narita, S., and Tokuda, H. (2009) *FEBS Lett. 583*, 2160–2164.
12. Gronenberg, L. S., and Kahne, D. (2010) *J. Am. Chem. Soc. 132*, 2518–2519.
13. Braun, M., and Silhavy, T. J. (2002) *Mol. Microbiol. 45*, 1289–1302.
14. Bos, M. P., Tefsen, B., Geurtsen, J., and Tommassen, J. (2004) *Proc. Natl. Acad. Sci. U.S.A. 101*, 9417–9422.
15. Wu, T., McCandlish, A. C., Gronenberg, L. S., Chng, S. S., Silhavy, T. J., and Kahne, D. (2006) *Proc. Natl. Acad. Sci. U.S.A. 103*, 11754–11759.
16. Chng, S. S., Ruiz, N., Chimalakonda, G., Silhavy, T. J., and Kahne, D. (2010) *Proc. Natl. Acad. Sci. U.S.A. 107*, 5363–5368.
17. Ma, B., Reynolds, C. M., and Raetz, C. R. H. (2008) *Proc. Natl. Acad. Sci. U.S.A. 105*, 13823–13828.
18. Suits, M. D., Sperandeo, P., Dehò, G., Polissi, A., and Jia, Z. (2008) *J. Mol. Biol. 380*, 476–488.
19. Tran, A. X., Trent, M. S., and Whitfield, C. (2008) *J. Biol. Chem. 283*, 20342–20349.
20. Tokuda, H. (2009) *Biosci., Biotechnol., Biochem. 73*, 80778-1-9.
21. Bayer, M. E. (1968) *J. Gen. Microbiol. 53*, 395–404.
22. Bayer, M. E. (1991) *J. Struct. Biol. 107*, 268–280.
23. Mühlradt, P. F., Menzel, J., Golecki, J. R., and Speth, V. (1973) *Eur. J. Biochem. 35*, 471–481.
24. Ishidate, K.; et al. (1986) *J. Biol. Chem. 261*, 428–443.
25. Tefsen, B., Geurtsen, J., Beckers, F., Tommassen, J., and de Cock, H. (2005) *J. Biol. Chem. 280*, 4504–4509.
26. Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J., and Kahne, D. (2005) *Cell 121*, 235–245.
27. Finn, R. D.; et al. (2008) *Nucleic Acids Res. 36*, D281–D288.
28. Hu, K.-Y., and Saier, M. H., Jr. (2006) *Curr. Genomics 7*, 447–461.
29. Srinivas, N.; et al. (2010) *Science 327*, 1010–1013.