Concentration-dependent oligomerization and oligomeric arrangement of LptA

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Abstract: Gram-negative bacteria such as *Escherichia coli* have an inner membrane and an asymmetric outer membrane (OM) that together protect the cytoplasm and act as a highly selective permeability barrier. Lipopolysaccharide (LPS) is the major component of the outer leaflet of the OM and is essential for the survival of nearly all Gram-negative bacteria. Recent advances in understanding the proteins involved in the transport of LPS across the periplasm and into the outer leaflet of the OM include the identification of seven proteins suggested to comprise the LPS transport (Lpt) system. Crystal structures of the periplasmic Lpt protein LptA have recently been reported and show that LptA forms oligomers in either an end-to-end arrangement or a side-by-side dimer. It is not known if LptA oligomers bridge the periplasm to form a large, connected protein complex or if monomeric LptA acts as a periplasmic shuttle to transport LPS across the periplasm. Therefore, the studies presented here focus specifically on the LptA protein and its oligomeric arrangement and concentration dependence in solution using experimental data from several biophysical approaches, including laser light scattering, crosslinking, and double electron electron resonance spectroscopy. The results of these complementary techniques clearly show that LptA readily associates into stable, end-to-end, rod-shaped oligomers even at relatively low local protein concentrations and that LptA forms a continuous array of higher order oligomeric end-to-end structures as a function of increasing protein concentration.

Keywords: LptA; LPS binding protein; protein oligomerization; EPR spectroscopy; light scattering; periplasmic β -sheet protein; endotoxin

Introduction

Gram-negative bacteria such as *Escherichia coli* have an inner membrane (IM) and an asymmetric outer membrane (OM) that together protect the cytoplasm and act as a highly selective permeability barrier. The outer leaflet of the OM is comprised mainly of LPS,¹ also referred to as endotoxin, which is essential for the survival of nearly all Gram-negative bacteria and is a major factor in the protection of bacteria from adverse environmental stresses encountered during infection or host colonization.^{2,3}

Lipid A, the lipid base for LPS, is synthesized in the cytoplasm and inserted into the inner leaflet of the IM, where the core sugars are added to form R_a LPS. The ABC transporter MsbA then flips R_a LPS to the outer leaflet (Fig. 1)^{4–6} where the WaaL ligase then attaches the O-antigen to create LPS.⁴ Although transport of LPS across the periplasm to the outer leaflet of the OM is less well understood, recent advances include the identification of the putative IM protein complex LptFGB₂, which has been classified as an ABC transporter of unknown specific function, the IM-associated protein LptC, the periplasmic protein LptA, and two OM proteins known to form an LptDE complex (Fig. 1).^{2,7–15} These seven Lpt proteins are thought to comprise the LPS transport system,^{11,16} however it is still unclear how they interact to accomplish LPS transport across the periplasm and the OM. It has been shown that LptA and LptB are essential to bacterial survival,^{17,18} and

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Figure 1. Cartoon showing the shuttle (left) and bridge (right) LPS transport theories. Following synthesis in the cytoplasm and insertion into the inner membrane (IM), lipid A (gray) is transported across the IM by the MsbA homodimer. The O-antigen is added by WaaL (not shown) to create LPS and the Lpt system transports it across the periplasm (~ 100–300 Å wide) and to the outer leaflet of the outer membrane (OM).

both LptA and the soluble portion of LptC have been crystallized.^{19,20} It has also been shown that the LptFGB₂ and LptC proteins are associated with the IM⁷ and the LptD and LptE proteins are associated with the OM,¹³ however it is not known if LptA oligomers bridge the periplasm to form a large, connected protein complex (“bridge” hypothesis), or if monomeric LptA acts as a periplasmic shuttle (“shuttle” hypothesis; Fig. 1).

The studies presented here focus specifically on the LptA protein and its oligomeric structure in solution. Mature LptA is comprised of amino acids 28–185, formed by the cleavage of its periplasmic signal

sequence, and has a molecular weight (MW) of 18.1 kDa.²¹ The crystal structures of the reported LptA dimers are shown in Figure 2 (2R19). The LptA monomer represents a novel fold, largely comprised of a β -sheet folded in half and twisted 90° from end-to-end.¹⁹ Electron density was not observed for the C-terminal residues 165–185 of one monomer within the crystal dimer or 172–185 of the second LptA monomer. On the basis of crystallographic symmetry, either an end-to-end dimer (where the resolved C-terminal β -strand of one monomer interacts with the N-terminal β -strand of the second monomer; Fig. 2A) or a side-by-side dimer (where the folded

Figure 2. Crystal structures of LptA (2R19).¹⁹ (A) End-to-end and (B) side-by-side dimer orientations are shown with the N-(V28) and C-termini (L164 or D171) labeled. The locations of the double cysteine pairs used to analyze these two arrangements by crosslinking are indicated by CPK representations in (A) I36C–L164C and (B) T64C–M98C.

Figure 3. Summary of the LS results for WT LptA. The concentration of LptA in each SEC-LS elution peak is plotted against the average molecular weight of the protein complex. Error bars indicate the $\pm 5\%$ accuracy of the SEC-LS technique.²⁸ The minor tick marks represent multiples of 18.1 kDa, the molecular weight of monomeric WT LptA. The dotted line represents the best fit to the data to estimate the average molecular weight of the oligomers in the 20–60 μM range using the equation $y = ax^b$, where y is the average molecular weight, $a = 40 \pm 1.5$, x is the protein concentration, and $b = 0.53 \pm 0.01$.

N-terminal helix and β -turn loops interact with each other; Fig. 2B) was formed. The oligomeric arrangements of LptA reported in these crystal structures have not been studied in solution. Here, experimental data from several biophysical approaches are presented to address the questions regarding the oligomeric number and arrangement of LptA in solution.

Results

Oligomerization of LptA is concentration dependent

Size-exclusion chromatography laser light scattering (SEC-LS) was carried out for the first time on LptA to determine the weight-average MW of LptA as a function of concentration (Fig. 3). The hydrodynamic size (Stock's radius, R_h), the root mean square radius (radius of gyration, R_g ; Table 1), and the calculated MWs (Fig. 3) all varied considerably with protein concentration, clearly indicating that the protein oligomers are in rapid equilibrium and vary in size with concentration. Furthermore, the UV traces (absorption at 280 nm vs. elution time) did not show individual peaks identifying distinct oligomer populations (data not shown). The data also suggest that at least 20-mers are formed at concentrations of 66 μM and above, and that the average MW of a 1 μM solution of LptA is at least a dimer. These data clearly indicate that at concentrations greater than approximately 1 μM , LptA forms oligomers that increase in size as the protein concentration increases.

The ratio of $R_g:R_h$, which is known as the ρ factor²² and reports on the shape of the oligomers in

solution, was calculated to be 2.0 for LptA at the highest concentrations analyzed (Table I). This distinctly indicates a rod-like shape for the oligomer and suggests a length:diameter ratio of at least 30:1.²² Given that LptA is 40 Å in length and nearly 30 Å wide, a 30:1 ratio suggests 22-mers, aligning well with the average oligomer size of approximately 20-mers calculated above from the average MWs of the highest concentration peak analyzed. For the lowest concentration of LptA that ρ was able to be reliably determined, 11.8 μM , the length:diameter ratio is approximately 11:1 based on its calculated ρ value of 1.4. This ρ value corresponds to octamers of LptA stacked end-to-end, and the average MW of the 11.8 μM elution peak also corresponds to octamers, again supporting the concentration dependence and the end-to-end arrangement of LptA oligomerization.

In contrast, the side-by-side arrangement of LptA would be expected to exhibit a ρ factor corresponding to a sphere-like shape ($\rho = 0.774$) due to its dimeric arrangement with an approximate length of 40 Å and width of 60 Å. Similarly, the average MW for the side-by-side arrangement in solution would have had a maximum value of 36.2 kDa, corresponding to terminal dimer structures unable to form higher order oligomers. Neither of these results is observed in the experimental data, ruling out the possibility that the side-by-side dimer of LptA observed in one of the crystallographic models (Fig. 2B) represents the oligomeric structure of LptA in solution.

These data demonstrate that LptA oligomerization is concentration dependent, with dimers forming at concentrations less than 1 μM and at least 20-mers forming by concentrations of approximately 66 μM . In addition, the rod-like shape of the oligomers as well as the increasing average MW as a function of protein concentration support the end-to-end stacking of the monomers of LptA as reported in one of the published crystallographic models (Fig. 2A).

Distance measurements and crosslinking patterns between monomers indicate an end-to-end oligomeric arrangement of LptA

To further investigate the oligomeric arrangement of LptA in solution, single and double cysteine mutants

Table I. Comparison of LptA Values of ρ to Values for Spherical- ($\rho = 0.78$), Coil- ($\rho = 0.82$) and Rod- ($\rho = 1.7$) Shaped Oligomers²² Clearly Indicates that LptA Forms Concentration-Dependent Rod-Shaped Oligomers

[LptA] (μM)	R_g (Å)	R_h (Å)	ρ
71	305	140	2.2
66	280	140	2.0
17	140	90	1.6
12	110	78	1.4

R_h refers to the hydrodynamic size or Stock's radius. Values for the radius of gyration, R_g , were not reliably determined for concentrations below 12 μM (octamers).

Figure 4. LptA monomer with the locations of the spin labeled single cysteine sites identified. Distance distributions with insets showing the background-corrected DEER spectroscopy data (gray lines) overlaid with fits (black lines) for each spin labeled LptA cysteine analyzed. The center of the distance distribution and the half-width at half-height value for each dataset is listed in Table 2.

of LptA were created and analyzed by double electron resonance (DEER) spectroscopy and cross-linking studies. Using a short-range crosslinker with an 8 Å spacer [i.e., bis-maleimidoethane (BMOE)] allows the identification of sites within LptA that are within this range by SDS-PAGE and Western blot analysis, whereas DEER spectroscopy directly reports on long-range distances between spin labeled cysteine sites separated by at least 20 Å.

On the basis of the crystal structure of LptA, five single cysteine sites were selected at various positions on LptA (I36C, M98C, S110C, L144C, and L164C) to report on its oligomeric conformation (Fig. 4). Each single cysteine mutant was purified, spin labeled, and analyzed by DEER spectroscopy. This pulsed EPR technique directly detects the distance-dependent dipolar interaction between introduced spin labels in the range of approximately 20–55 Å. Given that the predicted distances between the selected sites in the two models are 31–53 Å apart, this is an ideal method for analyzing the oligomeric arrangement of LptA in solution and is complementary to the short-range interactions detectable by crosslinking.

In the case of the end-to-end oligomeric arrangement observed in the reported crystal structures,¹⁹

each of the five introduced spin labels is expected to be approximately 40 Å from the same spin labeled site in an adjacent monomer (Table II). In the case of the side-by-side oligomeric arrangement of LptA observed in the crystal lattice, the distances between the selected cysteine sites vary from 5 to 53 Å (Table II). As shown in Figure 4 and summarized in Table II, each of the spin labeled mutants analyzed reported distances very similar to the expected distances for the end-to-end arrangement. In contrast, the distances expected for the side-by-side arrangement were not observed.

To further investigate the end-to-end oligomeric arrangement, the double cysteine mutant I36C–L164C, where the cysteines are expected to be 5 Å from each other (see Fig. 2A), was created. This cysteine pair shows a ladder of oligomeric species up to at least octamers when allowed to react with the crosslinker BMOE and analyzed by SDS-PAGE and Western blot (Fig. 5A). Even in the absence of crosslinker, stable oligomeric species are observed by SDS-PAGE and Western blot indicating that oligomers are spontaneously formed during purification. The disappearance of the oligomer bands on addition of β-mercaptoethanol (β-ME) confirms that the oligomers formed during purification are due to covalent disulfide bonds between monomers. The double cysteine pair, T64C–M98C (see Fig. 2B), where the cysteines are expected to be within 5 Å of each other in the side-by-side arrangement, was also created and similarly analyzed. In contrast to the end-to-end pair, this cysteine pair did not form significant amounts of disulfide bonded dimers under any of the tested conditions as would be expected if the side-by-side dimer arrangement was a stable form of LptA in solution (Fig. 5B).

Discussion

Although great progress has been made on the genetic level to identify the key players in LPS transport, and structurally in the determination of

Table II. The Expected Distances Between the C_{β} Atoms of the Indicated Sites on LptA in the End-to-End and Side-by-Side Crystallographic Oligomer Models¹⁹ Are Shown for Comparison to the Experimental Distance Data Collected Using Cysteine-Attached Spin Labels and DEER Spectroscopy (Fig. 4)

	Crystal end-to-end	Experimental DEER data	Crystal side-by-side	Location
I36	37 Å	39 ± 2.5 Å	31 Å	N-term. β-strand
M98	42 Å	44 ± 7 Å	5 Å	Central loop
S110	40 Å	43 ± 8.5 Å	50 Å	Central fold
L144	40 Å	45 ± 9 Å	35 Å	Central loop
L164	38 Å	40 ± 3.5 Å	53 Å	C-term. β-strand

The center and the half-width at half-height values of the experimental distance distributions are listed.

Figure 5. Crosslinking experiments in the presence and absence of BMOE as visualized by SDS-PAGE and Western blot against the 6xHis tag on LptA. Double cysteine mutants (A) I36C–L164C and (B) T64C–M98C are expected to be within 5 Å of each other in the end-to-end and side-by-side LptA crystallographic models, respectively. Lane 1: as purified; Lane 2: in the presence of excess β-ME; Lane 3: after 2 h in the presence of BMOE; Lane 4: after 4 h in the presence of BMOE. LptA monomers (M) and dimers (D) are labeled and each higher molecular weight band represents increasingly higher order oligomeric species.

the crystal structures for LptA, very little is known about LptA in solution beyond two binding studies showing that LptA and LptC interact *in vitro*²³ and that LptA and LptE do not interact.²⁴ The studies presented here on the structural arrangement of LptA in solution are the critical next steps in understanding its role in LPS transport.

The LS data, which show a concentration dependent increase in the average MW of LptA, indicate that the LptA monomers likely associate with each other one at a time, forming chains that include all possible oligomeric sizes rather than distinct sets of oligomers such as dimers or tetramers. Assuming that each monomer:monomer interface is identical, as suggested by at least one of the crystallographic models, it is logical that there is an array of oligomers present in solution as shown in the UV traces of the LS experiment. Due to the array of oligomeric species up to at least 20-mers observed by LS, the data plotted in Figure 3 were not fit to a distribution of distinct oligomeric species, but rather to an equation simply relating the average MW as a function of protein concentration for the range analyzed. LptA oligomer formation is likely noncooperative, based on the nonsigmoidal shape of the data shown in Figure 3.

In the presence of LPS, the LptA crystals formed an eight-molecule structure comprised of two end-to-end tetramers contacting each other once (2R1A; not shown) but did not include electron density for LPS.¹⁹ This dimer of tetramers is not likely to occur in solution, based on the ρ values

determined from the LS data indicating very long and narrow rod-shaped oligomers. In addition, the expected distances between spin labels corresponding to this interaction model were not observed in the DEER experiments. In contrast, the distances observed by DEER spectroscopy show a remarkable correlation with the distances expected only in the end-to-end oligomer arrangement. A possible exception is that the distance distribution observed for spin labels at S110C, although centered at 43 Å, is wide enough to include the 50 Å distance expected for the side-by-side model. However, the side-by-side model is not supported by the other four data points in Figure 4 nor by the complementary LS and crosslinking data presented and is thus not considered to be present in solution. Instead, the combined data presented here clearly indicate that the end-to-end oligomeric LptA structure is the one formed in solution.

The distance distributions differ in width between the five positions studied in LptA, with several showing wide ranges of distances between spin labels and others showing a very narrow range of distances between the labeled sites. This is likely due to the fact that the introduced spin label is expected to be relatively restricted in motion when involved in an oligomeric interaction interface and relatively flexible when not in contact with another LptA monomer. Thus, the fact that the two spin labeled sites located on the end-to-end oligomeric interfaces (I36 and L164) show narrow distance distributions, that is, the spin label is locked into a relatively specific position due to tertiary contacts at the monomer–monomer interface, further supports the end-to-end oligomer model. Similarly, the spin labels at the three positions shown not to be located on end-to-end oligomeric interfaces (M98, S110, and L144) are not restricted by protein–protein contacts and are thus able to sample a wider range of positions, resulting in a broad distribution of observed distances between these sites in the LptA oligomer.

Although the average MW of LptA suggests oligomer sizes of greater than dimers as the average species observed even at the lowest protein concentration (0.9 μM) analyzed by LS, extrapolation of the data based on the equation used to fit the data points suggests that monomers would be the predominant species at a concentration value of approximately 0.2 μM in solution. If the concentration of LptA in the periplasm is <<1 μM, then the monomeric shuttle theory of LPS transport across the periplasm could be correct, though monomers were not directly observed in our data. In contrast, the periplasm is known to be very viscous and packed with high concentrations of protein, thus diffusion of monomeric shuttle proteins is expected to be slow. Though it is difficult to estimate the local concentration of LptA in the periplasmic space, it is likely

that LptA is present at concentrations sufficient to form oligomers large enough to readily bridge the periplasm, which is estimated to be at least 100 Å and up to >300 Å.^{25–27} LptA end-to-end oligomers from dimers up to octamers would fit within these parameters to continuously and directly span the estimated space between the membranes, and would be accommodated by periplasmic LptA concentrations of <1–12 μM based on the LS results. Thus, our data showing that LptA readily associates into stable, end-to-end, rod-shaped oligomers even at relatively low local protein concentrations, together with the clear end-to-end distances reported by the DEER spectroscopy data, suggest that the bridge model is the more likely mechanism for LptA's role within the periplasm.

Materials and Methods

Protein purification and mutagenesis

The DNA encoding the LptA protein was PCR amplified from the chromosome of *E. coli* BL21(DE3) cells using flanking primers that inserted NdeI and XhoI restriction sites at the beginning and end of the gene, respectively. The *lptA* gene was ligated into pET21b (Novagen, Merck Chemicals, Germany), which encodes for a C-terminal 6xHis tag, and the proper insertion and sequence verified by DNA sequencing (Retrogen, San Diego, CA). Single and double cysteine mutants of LptA, which contains no native cysteines, were introduced using the Stratagene Quikchange Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and their DNA sequences verified by sequencing. All plasmids encoding recombinant LptA were transformed into BL21(DE3) cells for purification.

BL21(DE3) cells containing plasmid-encoded WT or mutant LptA were grown overnight in 10 mL of Luria Broth (LB) and ampicillin (100 μg/mL) at 37°C and 5 mL subcultured into 2 L of fresh LB/ampicillin. After overnight growth at 37°C, cells were induced for 3 h with 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside), pelleted by centrifugation and washed with 50 mM sodium phosphate, pH 7.0, and 300 mM NaCl buffer (buffer A). The washed cells were broken by passage through a Thermo-Spectronic French Pressure cell, unbroken cells were pelleted by low speed centrifugation (10 min at 1157*g* in an Eppendorf 5810R), and the membrane fraction was pelleted by centrifugation at 40,905*g* for 1 h in a Sorvall Evolution RC. LptA was purified from the soluble fraction by cobalt affinity chromatography using 0.5 mL Talon resin (Clontech, Mountain View, CA). The protein-bound resin was washed and eluted in buffer A using the imidazole concentrations as previously described.²¹ Each of the ten 1 mL elution fractions were monitored by 15% SDS-PAGE and either used immediately or stored at

–20°C. Proteins were concentrated using Amicon Ultra 10 K concentrators and the protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) with lysozyme (Sigma–Aldrich, St. Louis, MO) as a protein standard or by reading the absorbance at 280 nm (WT LptA $\epsilon_{\text{molar}} = 7450 \text{ M}^{-1} \text{ cm}^{-1}$).

Laser light scattering

Purified WT LptA was analyzed by size exclusion chromatography multiangle laser light scattering at the Keck Biotechnology Resource Laboratory at Yale University (<http://medicine.yale.edu/keck/biophysics/index.aspx>).^{28,29} In brief, 0.22 μm-filtered LptA samples (500 μL) in the range of 0.01–10 mg/mL in buffer A were passed over a Pharmacia Superdex 200 HR10/30 (GE Healthcare Bio-Sciences, Piscataway, NJ) size exclusion column equilibrated with 20 mM HEPES, pH 8, 150 mM NaCl, and 1 mM EDTA immediately before detection by a DAWN-EOS light detector (Wyatt Technologies, Santa Barbara, CA). The concentrations of LptA in the peak slices analyzed for the ASTRA calculations are indicated in the *x*-axis of Figure 3.

Crosslinking and Western blot analysis

Ten micrograms of purified LptA I36C–L164C and T64C–M98C proteins were incubated with a 40-fold molar excess of BMOE (8 Å spacer; Pierce Thermo Scientific, Rockford, IL) at room temperature for 2 and 4 h. Protein samples containing crosslinker were separated by 10% SDS-PAGE along with controls in the absence of crosslinker and in the presence of 10 mM β-ME. The separated proteins were transferred to 0.45 μm nitrocellulose paper (Biorad, Hercules, CA) using a Biorad Mini Trans-Blot Cell at 100 V for 1.5 h. The C-terminally 6xHis-tagged LptA protein was detected by Western blot using a 1:1000 dilution of the Penta-His HRP conjugate (Qiagen, Valencia, CA) and visualized using TMB Stabilized Substrate for HRP (Promega, Madison, WI).

Spin labeling and EPR spectroscopy

Fractions of purified LptA cysteine mutants were pooled, incubated with 4 M urea for 1 h, followed by 10× DTT for 15 min at room temperature, and spin labeled with a 50-fold excess of MAL-6 (4-maleimido TEMPO; Sigma–Aldrich, St. Louis, MO). The labeling reactions proceeded at 4°C for 5 h or overnight and excess label and denaturant were removed by extensive dialysis against buffer A. The maleimide-based MAL-6 spin label was used in place of the more commonly used methanethiosulfonate (MTS)-based spin label (MTSL) because we were focusing on frozen distance measurements rather than motional analysis of the spin labeled sites (MTSL is preferred for mobility studies because it produces

less motionally restricted spectra than sites labeled with MAL-6), it forms a nonreducible thio-ether bond with cysteine (rather than a reducible disulfide bond), which allows greater amounts of reducing agent to be present during the labeling reaction without reversing productive labeling of the protein, and it allows for addition of a greater excess of spin label without forming unproductive spin label dimers in aqueous solution, all of which combine to yield a higher overall labeling efficiency of the introduced cysteines.

DEER spectroscopy was carried out on a Bruker ELEXSYS E580 at X-band using a four-pulse sequence³⁰ at 80 K, as described previously.³¹ The data were analyzed using DeerAnalysis2011³² and Gaussian or Tikhonov regularization models depending on which gave the best fit to the background-corrected dipolar evolution data. Samples typically contained 300–400 μ M LptA and 20% deuterated glycerol as a cryoprotectant, were 25–30 μ L in volume and flash frozen in an acetone/dry ice slurry.

Conclusions

The LS, DEER, and crosslinking data presented above all clearly demonstrate for the first time that LptA forms end-to-end oligomers in solution. Our data are also consistent with one of the two proposed crystallographic models for LptA oligomerization, and show for the first time that LptA forms a continuous array of higher order oligomeric end-to-end structures as a function of increasing protein concentration.

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