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Dimerization of isolated *Pseudomonas aeruginosa* lipopolysaccharide transporter component LptA

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ABSTRACT

LptA is a soluble periplasmic component of the lipopolysaccharide (LPS) transport system of Gram-negative bacteria that transports newly synthesized LPS from the inner membrane to the outer leaflet of the outer membrane. LptA links the inner membrane components (LptBFGC) to the outer membrane components (LptDE), but it is uncertain whether LptA is a freely moving LPS shuttle or part of a stable trans-periplasm structure. *Escherichia coli* LptA forms highly polymerized head-to-tail oligomers in solution, but dimers in vivo. We studied the oligomerization of purified *Pseudomonas aeruginosa* LptA. Size-exclusion chromatography showed that *P. aeruginosa* LptA, unlike *E. coli* LptA, is a dimer over a wide range of concentrations. Chemical crosslinking with bis(sulfosuccinimidyl) suberate confirmed that dimers were the predominant species even at sub-micromolar LptA concentrations, which was unaffected by LPS binding. Mass spectrometry of crosslinked dimers showed that crosslinks occurred between the N-terminal α-amino group and either Lys-172 or Lys-173 near the C-terminus. These results support a hypothetical structure for the dimer of isolated *P. aeruginosa* LptA in which the N-terminus of one monomer is in close proximity to the C-terminus of the other, and the same surface of each monomer forms the interface between them, preventing further oligomerization.

1. Introduction

The lipid component of the outer leaflet of the outer membrane of Gram-negative bacteria consists primarily of lipopolysaccharide (LPS), also known as endotoxin, instead of phospholipids. LPS is composed of a glycolipid called lipid A attached to one of several core oligosaccharides, which is in turn attached to an O-antigen polysaccharide that varies between strains [1,2]. The physical properties of LPS contribute to the relative impermeability of Gram negative bacteria to the entry of drugs [3]. Endotoxin is responsible for a severe, dangerous immune response in human patients with Gram negative infections [4], caused mainly by lipid A. Genes required for lipid A and inner core oligosaccharide biosynthesis are essential for several pathogenic bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* [5]. Drugs that inhibit production of LPS or its transport to the surface of the bacteria would be valuable for antibacterial therapy, both to kill the bacteria and to reduce the amount of endotoxin released into the bloodstream of infected patients. As proof of the therapeutic potential of LPS biogenesis inhibition, compounds that inhibit LpxC, the enzyme that catalyzes the first committed step in lipid A biosynthesis, have Gram-negative antibacterial activity [6]. Additionally, peptidomimetic compounds with antibacterial activity against *P. aeruginosa* have been described that target LptD, an outer membrane component of the LPS transport system [7,8].

LPS is synthesized in the cytoplasm and on the periplasmic surface of the inner membrane [9]. Completed LPS is transported to the outer leaflet of the outer membrane by the 7-component ATP-dependent lipopolysaccharide transport (Lpt) system [10], which has been characterized in *E. coli*. LptBFG is an ATP Binding Cassette (ABC) transporter that transfers LPS from the outer leaflet of the inner membrane to LptC, an integral membrane protein of the inner membrane [11,12]. Inhibitors of the ATPase activity of purified *E. coli* LptB have been reported that inhibit growth of *E. coli* in culture [13]. LptC transfers LPS to the soluble periplasmic protein LptA [12,14,15]. LptA, which can form rod-like oligomers [16–18], either forms a bridge to the outer membrane or acts as a shuttle for LPS between the two membranes [19,20]. X-ray crystallography shows that LptA and LptC are structurally homologous, consisting largely of β-strands in a jellyroll-like fold [15,18]. LptDE is an outer membrane complex that guides LPS into place in the outer leaflet. LptD is a channel-like β-barrel protein [21]. LptE is

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MRF-VNTLPLIFGLTAA-LGSSM-ALALPSDREQPIRVQADSAELDDKQG-VAVYRGDVV
MKFKTNKLSLNLVLASSLLAASIPAFAVTGDTDQPIHIESDQQSLD-MQGNVVTFTGNVI
* *  * * *   *    *  *  * *   *  ***    *   **  ** *    * *
VTQGSTKLTGNTVTL-KQ-DKNGDIEVVTSVGKPA-YYEQKPAPDKDVTKAYGLTIQYFV
VTQGTIKINADKVVVTRPGGEQGK-EVIDGYGKPATFYQMQDN-GKPV-EGHASQMHYEL
****  *     *          *  **    ****  *       * *          *
TQNRVVLIDQAKVIQEGNTFEGEKIVYDTQRQIVNAGRATGSQVTSPRPRIDMVIQPK-K
AKDFVVLTGNAYLQQVDSNIKGDKITYLVKEQKMQAFSDKGKRVTTVL--VPSQLQDKNN
    ***   *   *      * ** *    *   *    *  **          * *
KAQ--------
KGQTPAQKKGN
* *
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Fig. 1. Alignment of *P. aeruginosa* (top) and *E. coli* (bottom) LptA amino acid sequences. Identical residues are indicated by asterisks.

required for assembly of LptD [22], occupies a space at least partially enclosed by LptD in a plug-and-barrel arrangement [23], and binds LPS [24]. Transport of LPS through this system is driven by cytoplasmic ATP hydrolysis.

The Lpt system has not been investigated in Gram negative pathogens other than *E. coli*.[1] Sequence homology between the *E. coli* and *P. aeruginosa* genes is fairly low, except for LptB, for which there is 66% sequence identity. There is 33% sequence identity between the *lptA* genes of the two species (57 out of 175 residues of *P. aeruginosa lptA*), with no run of more than 4 identical residues (Fig. 1). Here, we report the results of an investigation into the oligomerization of *P. aeruginosa* LptA. Unlike the highly oligomeric *E. coli* LptA, purified *P. aeruginosa* LptA is dimeric over a wide range of concentrations. Chemical crosslinking showed that the dimers adopt a configuration in which the N-terminus of one monomer is in close proximity to the C-terminus of the other.

2. Materials and methods

2.1. Cloning, expression and purification of P. aeruginosa LptA

The *lptA* gene from *P. aeruginosa* was codon-optimized for expression in *E. coli* and custom-synthesized with N-terminal His_6 purification tag, FLAG epitope tag, and TEV protease cleavage site (GenScript, Piscataway, NJ). The 24-amino acid N-terminal secretion signal was deleted. The optimized gene was cloned into pET-24a(+) (Novagen Biosciences, Madison, WI) using NdeI and XhoI restriction sites to create plasmid pNG056.

For protein expression, the plasmid was transformed into BL21-Gold(DE3) (Agilent Technologies, Santa Clara, CA) and plated on Luria–Bertani (LB) medium containing 25 μg/ml kanamycin at 37 °C overnight. A single colony of BL21-GOLD(DE3)/pNG056 was inoculated into a 100-ml culture of LB containing 25 μg/ml kanamycin and grown overnight at 37 °C. The overnight culture was diluted to OD_{600} = 0.1 in 4 × 1 L of LB containing 25 μg/ml kanamycin and grown at 37 °C with aeration to mid-logarithmic phase (OD_{600} = 0.6). The culture was transferred to 30 °C. IPTG was added to 0.5 mM. After a 3-h induction at 30 °C, the cells were harvested by centrifugation at 5000*g* for 15 min at 25 °C. Cell paste was stored at −20 °C.

Frozen cell paste from 4 L of cell culture was suspended in 50 ml of Buffer A consisting of 25 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 5% (v/v) glycerol, supplemented with 1 EDTA-free protease inhibitor cocktail tablet (Roche Molecular Biochemical, Indianapolis, IN). Cells were disrupted by French Press at 18,000 psi twice at 4 °C, and the crude extract was centrifuged at 150,000*g* for 30 min at 4 °C. The supernatant was applied at a flow rate of 2.0 ml/min onto a 5-ml HiTrap Ni^{2+}-chelating column (GE Healthcare Life Sciences, Piscataway, NJ) pre-equilibrated with Buffer A. The column was washed with Buffer A, and LptA was eluted by a linear gradient from 0 to 0.5 M imidazole in Buffer A. Fractions containing LptA were pooled, and incubated with Turbo TEV protease (Eton Bioscience Inc., San Diego, CA) at a ratio of 1:100 TEV/LptA (w/w) overnight at 4 °C while dialyzing against 2 L of Buffer B, consisting of 25 mM Tris–HCl (pH 8.0), 0.1 M NaCl, and 5% (v/v) glycerol. The dialyzed sample was applied at a flow rate of 2.0 ml/min onto a 5-ml HiTrap Ni^{2+}-chelating column pre-equilibrated with Buffer A. The flow-through fractions were pooled and dialyzed against 1 L of 25 mM HEPES-NaOH (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.15 M NaCl, and 5% (v/v) glycerol and concentrated by Amicon® Ultracel-10K centrifugal ultrafiltration device (Millipore, Billerica, MA). The protein concentration was determined by the Bradford method [25] and characterized for purity by SDS–PAGE and mass by LC–MS. The protein was stored at −80 °C. His_6-tagged LptA was prepared as above, omitting the TEV protease cleavage.

2.2. LPS binding

Samples (180 μl) of His_6- and untagged *P. aeruginosa* LptA at 25 μM were incubated with 2.5 mg/ml *P. aeruginosa* LPS (Sigma) in binding buffer consisting of 50 mM sodium phosphate (pH 8.0) and 50 mM NaCl at room temperature for 1 h. The samples were mixed with 85 μl settled volume of His-Select Ni^{2+} affinity resin (Sigma) in 160 μl of buffer, incubated for another hour at room temperature with constant mixing, and centrifuged at 1000*g* for 1 min. The supernatant was fraction 0. Buffer (200 μl) was added to the pellets, which were mixed for 1 min then centrifuged as above. The supernatants were fraction 1. This step was repeated 3 more times, yielding fractions 2–4. The pellets were mixed with 200 μl of 0.3 M imidazole in buffer, mixed for 5 min, and centrifuged. The supernatants were fraction 5. This step was repeated, yielding fraction 6. The pellets were mixed with 200 μl of 0.5 M imidazole in buffer, mixed for 5 min, and centrifuged. The supernatants were fraction 7. The samples were prepared for SDS–PAGE by mixing with 1/3 volume of 4X NuPAGE LDS sample buffer (Life Technologies/Novex) and heating for 10 min at 70 °C. A portion of each sample (10 μl) was separated on each of two 4–12% acrylamide Bis-Tris NuPAGE SDS–PAGE mini-gels at 200 V with MES running buffer. Protein molecular mass markers were SeeBlue® Plus 2 prestained standards (Life Technologies/Novex). Since LptA contains no cysteine residues, no reducing agent was used. One gel was stained for protein with InstantBlue colloidal Coomassie Blue (Expedeon, San Diego, CA). The other gel was stained for LPS with a Pro-Q Emerald 300 lipopolysaccharide gel stain kit (Life Technologies) according to the manufacturer's instructions. Fluorescent staining was imaged with an AlphaImager (ProteinSimple, Santa Clara, CA).

2.3. Chemical crosslinking

LptA was transferred into crosslinking buffer consisting of 50 mM sodium phosphate (pH 7.5) and 150 mM NaCl by passing 70 μl of 1.7 mg/ml LptA through 2 consecutive Micro Bio-Spin™ P-6 centrifugal gel filtration columns (Bio-Rad, Hercules, CA)

[1] The x-ray crystal structures of LptDE from *Shigella flexneri* and *Salmonella typhimurium* were recently published ([30] and [31], respectively).

equilibrated 8 times with the same buffer, according to the manufacturer's instructions. The concentration of the resulting protein sample was measured using the Bradford method [25] using bovine serum albumin as the standard. The crosslinker bis(sulfosuccinimidyl) suberate (BS^3) (Thermo Scientific/Pierce Biotechnology, Rockford, IL) was dissolved in water immediately before use. LptA and BS^3 were mixed together and reacted for 30 min at room temperature. The reactions were quenched with Tris–HCl (pH 8.0) at 0.1 M for 15 min at room temperature. LptA was precipitated by trichloroacetic acid (TCA) (Sigma, St. Louis, MO) and sodium deoxycholate (NaDOC) (EMD Millipore/Calbiochem, Billerica, MA) at 7.2% (w/v) and 0.015% (w/v), respectively. Samples were incubated on ice for 10 min, then centrifuged at 16,000*g* for 10 min at room temperature. The pellets were dissolved in 1X NuPAGE LDS sample buffer and heated for 10 min at 70 °C prior to electrophoresis as above. Gels were stained with colloidal Coomassie Blue.

2.4. Sample preparation for mass spectrometry

Coomassie-stained cross-linked dimer bands were excised and sliced into 1 mm^3 pieces. The gel pieces were washed with distilled H_2O, destained with methanol:50 mM NH_4HCO_3 (1:1 v/v) and dehydrated in acetonitrile:50 mM NH_4HCO_3 (1:1 v/v) followed by 100% acetonitrile. After air drying, the gel pieces were rehydrated in freshly prepared 25 mM dithiothreitol in 50 mM NH_4HCO_3 and incubated for 20 min at 56 °C. The supernatant was removed. Freshly prepared 55 mM iodoacetamide in 50 mM NH_4HCO_3 was added to the gel pieces and incubated in the dark for 20 min at room temperature. The gel pieces were washed with distilled H_2O, then dehydrated with acetonitrile:50 mM NH_4HCO_3 followed by 100% acetonitrile. Dried gel pieces were rehydrated and digested in 12 ng/μl Trypsin Gold (Promega, Madison, WI) in 0.01% ProteaseMAX surfactant (Promega):50 mM NH_4HCO_3 for 10 min. The same volume of 0.01% ProteaseMAX surfactant:50 mM NH_4HCO_3 was added and the gel pieces were incubated for 2 h at 37 °C with gentle mixing. The supernatant was collected. The gel pieces were washed with 100 μl of 1% formic acid with mixing for 10 min and the supernatant was combined with the first supernatant. The digest was dried in vacuum and stored at −20 °C until LC/MS analysis.

2.5. NanoLC-tandem mass spectrometry analysis

LC/MS analysis was performed on an LTQ OrbitrapVelos mass spectrometer (Thermo, Waltham, MA) equipped with a Thermo nanospray source and a Thermo easy-NanoLC system. Separation of the peptide mixtures was achieved on a 75 μm ID × 150 mm PicoFrit ProteoPep2 C18 column (New Objective, Woburn, MA) with a Thermo C18 Easy-column 100 μm ID × 20 mm pre-column and a gradient of acetonitrile (5–45%) in 0.1% formic acid for 80 min at a flow rate of 300 nl/min following 5 min at 5%.

LC–MS/MS data were acquired in the data-dependent mode. The full-scan spectra were collected in Fourier Transform (FT) positive mode for *m*/*z* from 400 to 1600 at a resolution of 60,000. MS/MS spectra were acquired by FTMS at a resolution of 7500. The top 10 most intense ions were selected for high-energy collisional dissociation (HCD) fragmentation at a normalized collision energy of 45%. Dynamic exclusion duration was 60 s. Singly charged, doubly charged and unassigned ions were excluded from MS/MS, and +3 charge state was selected as default for MS/MS.

2.6. LC/MS data analysis and crosslinked peptide identification

The LC/MS Xcalibar raw file was converted to a Mascot generic format (mgf) file with Proteome Discoverer 1.4 (Thermo), then processed with pLink software 1.15 [26]. Tolerances of the precursor and fragment mass were 10 and 20 ppm, respectively. BS^3 was selected as the crosslinker, with crosslink monoisotopic shift of 138.0680786 Da and monolink mass shift of 156.0786442 Da. The maximal number of missed cleavage sites was set at 2. Cysteine carbamidomethylation and methionine oxidation were chosen as the dynamic modifications. The sequence of *P. aeruginosa* LptA protein in a forward database was reversed to create a decoy database the same size as the forward database. Peptide sequences from both databases were cross-linked *in silico* in every possible combination and searched. Inter-link identifications were filtered by requiring 10 ppm mass accuracy, false discovery rate (FDR) <5%, *E*-value < 0.01, and ⩾3 spectral copies. Fragmentation spectra for each crosslinked peptide were validated using pLabel and manually inspected. Only those with 4 or more spectra are shown in Table 1.

2.7. Size exclusion chromatography/multi-angle light scattering (SEC/MALS) analysis of P. aeruginosa LptA

P. aeruginosa LptA was analyzed by SEC/MALS on an Agilent 1200 HPLC coupled to a Dawn HELEOS multi-angle light scattering detector and an in-line Optilab rEX differential refractometer (Wyatt, Goleta, CA) for measuring protein concentration. Protein was separated on a 7.8 × 300 mm WTC-030S5 SEC column (Wyatt) with mobile phase 25 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol and 5% (v/v) glycerol and flow rate 0.5 ml/min. Immediately before analysis, LptA was diluted in the mobile phase in the concentration range of 1–60 μM and 50 μl of sample was injected. The molar mass of LptA in the elution peak was calculated using Astra software version 5.3.2.16 (Wyatt).

2.8. Protein sequence alignment

P. aeruginosa and *E. coli* LptA amino acid sequences were aligned using Pairwise Align Protein of the Sequence Manipulation Suite at bioinformatics.org. The scoring matrix was BLOSUM62. The value for internal gaps was −6. The value for gaps preceding and following a sequence was zero. The alignment score was 193.

3. Results

LptA was shown to bind *P. aeruginosa* LPS using the published method [27] (Fig. 2). When excess LPS was mixed with His-tagged

Table 1
Identification of BS^3-crosslinked *P. aeruginosa* LptA peptides by LC–MS/MS.

Sequence[a]	Number of spectra	Calculated mass	Δmass	Δmass/calculated mass (ppm)	Crosslink
GLPSDREQPIR-KKAQ	4	1878.03229	−0.00120	−0.64	α-Amino to Lys-172
GLPSDR-KKAQ	11	1254.69319	0.00210	1.67	α-Amino to Lys-172
GLPSDREQPIR-KKAQ	1	1878.03229	−0.00192	−1.02	α-Amino to Lys-173
GLPSDR-KKAQ	3	1254.69319	0.00084	0.67	α-Amino to Lys-173

Residues involved in crosslinks are underlined.

[a] The sequence of the *P. aeruginosa* LptA construct after removal of the N-terminal His tag is: ^{23}GLPSDREQPIRVQADSAELDDKQGVAVYRGDVVVTQGSTKLTGNTV TLKQDKNGDIEVVTSVGKPAYYEQKPAPDKDVTKAYGLTIQYFVTQNRVVLIDQAKVIQEGNTFEGEKIVYDTQRQIVNAGRATGSQVTSPRPRIDMVIQPK^{172}K^{173}KAQ.

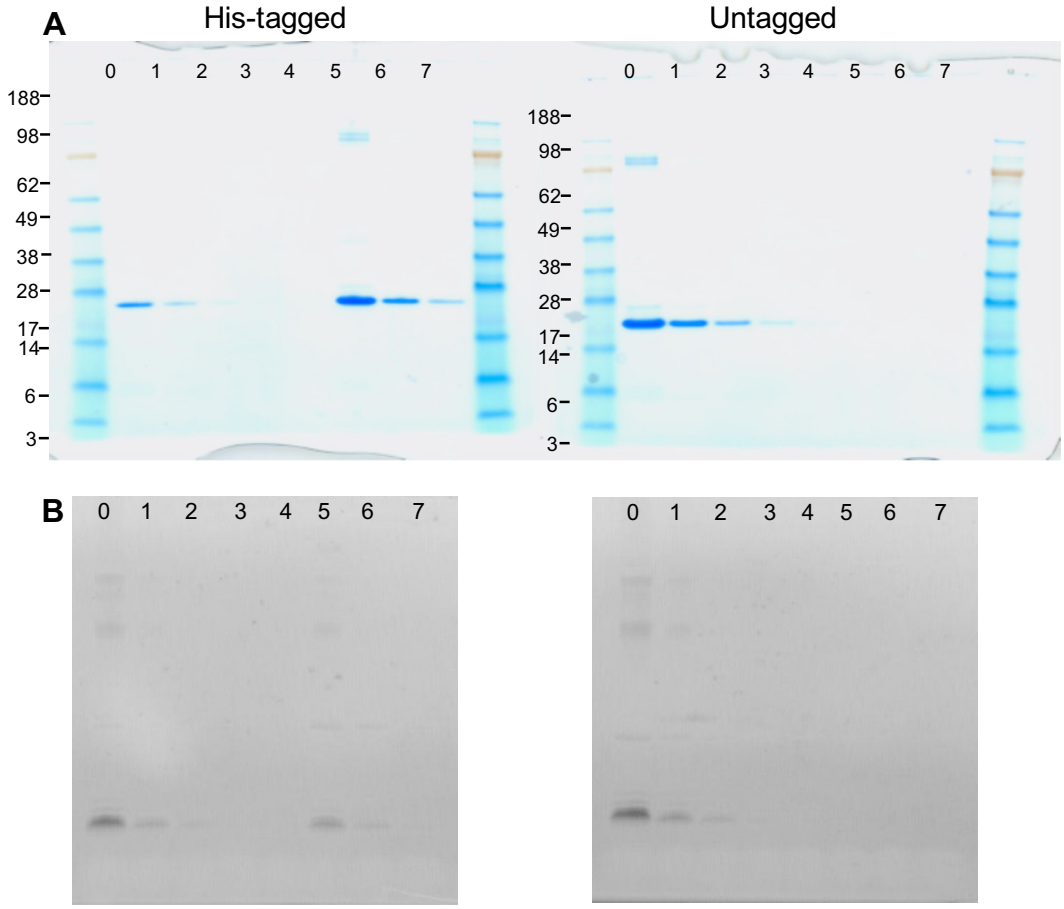

Fig. 2. LPS binding by *P. aeruginosa* LptA. (A) Coomassie Blue staining. (B) LPS staining. Fraction 0, supernatant; fractions 1–4, washes without imidazole; fractions 5–6, 0.3 M imidazole washes; fraction 7, 0.5 M imidazole wash.

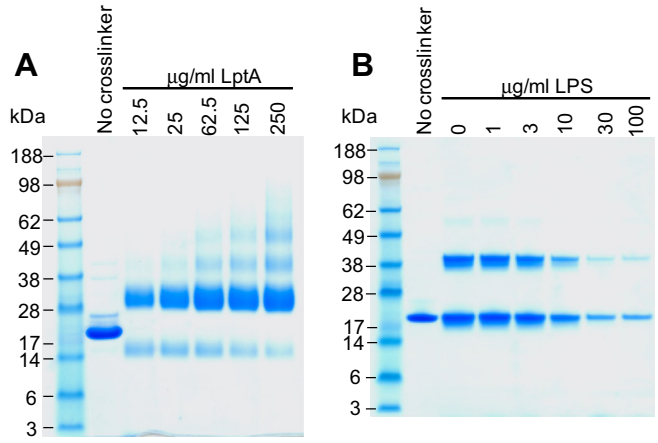

Fig. 3. *P. aeruginosa* LptA crosslinking with BS^3. (A) Crosslinking with 1 mM BS^3 at a range of LptA concentrations. (B) Crosslinking with 10 μM BS^3 at 8.8 μg/ml LptA and a range of *P. aeruginosa* LPS concentrations.

LptA and the mixture was combined with Ni^{2+} affinity resin, some LPS was unbound and eluted in the imidazole-free washes (fractions 0–4) and some eluted with the majority of the LptA when the resin was washed with imidazole (fractions 5–7). When untagged LptA was used, all the LPS and LptA eluted in the imidazole-free washes.

Purified *P. aeruginosa* LptA was readily crosslinked with BS^3, which crosslinks amino groups with a spacer arm of 11.4 Å. With 1 mM BS^3, 12.5 μg/ml (0.7 μM) LptA was found mainly as dimer, with some residual monomer (Fig. 3A). The apparent molecular masses on SDS–PAGE were lower than the nominal masses due to the extra negative charge imparted to the protein by modification of various lysine residues with one end of BS^3 followed by hydrolysis of the remaining sulfosuccinimide moiety. Band broadening resulted from heterogeneity in the number of such uncrosslinked modifications. Increasing the LptA concentration during crosslinking resulted in a reduction in the proportion of residual monomer and the appearance of small proportions of trimer and tetramer. However, the dimer was by far the predominant species at all LptA concentrations.

Reducing the concentration of BS^3 during the crosslinking reaction from 1 mM to 10 μM substantially reduced the apparent decrease in molecular mass of the protein while preserving the efficient formation of crosslinked dimer (Fig. 3B). At 8.8 μg/ml LptA (0.5 μM) and 10 μM BS^3, nearly half of the LptA was crosslinked dimer. The rest was monomer and a trace of trimer. Including *P. aeruginosa* LPS during the crosslinking reactions reduced the protein recovery from TCA/NaDOC precipitation, but had no noticeable effect on the proportions of monomer and dimer (Fig. 3B).

SEC-MALS was used to investigate the oligomerization of *P. aeruginosa* LptA without crosslinking. Over a range of LptA concentrations from 5 to 60 μM, only dimer was observed, with molecular mass 28 kDa. This result contrasts to with concentration-dependent formation of high-order oligomers of *E. coli* LptA [16–18].

To determine the LptA residues involved in crosslinking by BS^3, the dimer was cut from SDS–PAGE gels and digested with trypsin. The crosslinked peptides were identified by LC–MS/MS (Fig. 4 and

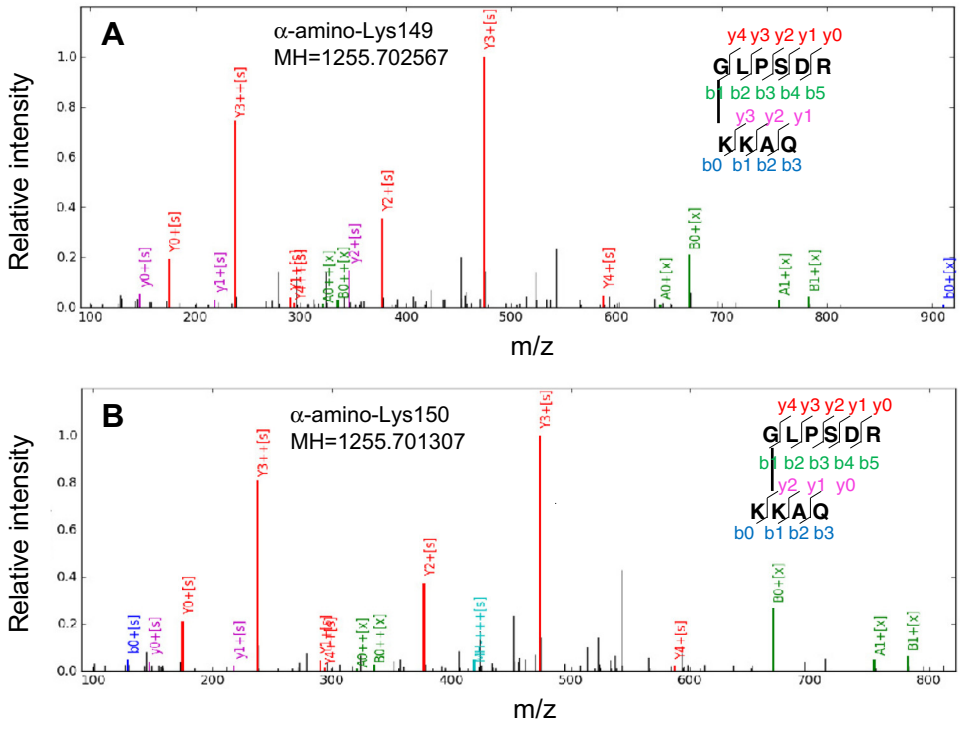

Fig. 4. LC–MS/MS sequence analysis of BS^3-crosslinked peptides of *P. aeruginosa* LptA. (A) α-Amino to Lys-172 crosslinked peptide. (B) α-Amino to Lys-173 crosslinked peptide. Observed peptide backbone cleavage is indicated and labeled for *b*- and *y*-ions in the spectra. Spectra were annotated using pLabel.

Table 1). Crosslinks were observed between the N-terminal α-amino group and the ε-amino groups of both Lys-172 and Lys-173, close to the C-terminus of the 175-residue protein. The residue numbering is based on the gene sequence, including the N-terminal export signal sequence. The N-terminal Gly-23 residue is a remnant of the expression vector after removal of the His-tag by TEV protease and is not native to LptA. The native sequence may begin at Leu-24 following removal of the export signal sequence by signal peptidase.

4. Discussion

From the above results, we conclude that the *P. aeruginosa* LptA dimers exist in a configuration in which the N-terminus of one monomer is close to the C-terminus of the other. The arrangement of the two monomers appears to preclude polymerization of oligomers, since this was not observed to a substantial extent. Therefore, a likely configuration is for each monomer to present the same surface to the other. This contrasts with the evidence that isolated *E. coli* LptA forms long head-to-tail oligomers [16].

Since the oligomerization of *P. aeruginosa* LptA was investigated for the isolated protein, it is possible that a different arrangement exists when LptA complexes with LptC and/or LptDE. *E. coli* LptA was dimeric in vivo based on UV photocrosslinking [28]. Dimerization occurred between residues near the N- and C-termini (His-37 and Val 163), consistent with our results with *P. aeruginosa* LptA. The same N- and C-terminal residues of *E. coli* LptA involved in LptA dimerization were also involved in crosslinking of LptA with LptC and LptD, respectively [28]. If the N-terminus of *P. aeruginosa* LptA binds more tightly to LptC than to the LptA C-terminus, and if the C-terminus of LptA binds more tightly to LptDE than to the LptA N-terminus, then LptA dimers could be prevented from forming for those LptA monomers with an LptC or LptDE partner. Bowyer et al. [14] measured the K_d of the *E. coli* LptA–LptC interaction to be about 0.5 μM by surface plasmon resonance. Schultz et al. [29] measured the K_d of the interaction between *E. coli* LptA and LptC to be 4 μM and the K_d for the interaction of *E. coli* LptA with itself to be 29 μM by electron paramagnetic resonance. The greater affinity of LptA for LptC than for LptA is consistent with the above hypothesis.

Alternatively, the *P. aeruginosa* LptA dimer observed with purified protein could function in vivo as a mobile LPS shuttle between the LptBFGC complex of the inner membrane and the LptDE complex of the outer membrane. Future work should investigate the effect of LptC and LptDE on oligomerization of *P. aeruginosa* LptA, and the oligomerization state of *P. aeruginosa* LptA in vivo.

Acknowledgments

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