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An induced folding process characterizes the partial-loss of function mutant LptAI36D in its interactions with ligands

Carlo Santambrogio, Paola Sperandeo, Francesca Barbieri, Alessandra M. Martorana, Alessandra Polissi *, Rita Grandori *

Dipartimento di Biotecnologie e Bioscienze, Piazza della Scienza 2, 20126 Milano, Italy

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ABSTRACT

Lipopolysaccharide (LPS) is an essential glycolipid of the outer membrane (OM) of Gram-negative bacteria with a tripartite structure: lipid A, oligosaccharide core and O antigen. Seven essential LPS-transport proteins (LptABCDEFG) move LPS to the cell surface. Lpt proteins are linked by structural homology, featuring a β-jellyroll domain that mediates protein–protein interactions and LPS binding. Analysis of LptA–LPS interaction by fluorescence spectroscopy is used here to evaluate the contribution of each LPS moiety in protein—ligand interactions, comparing the wild-type (wt) protein to the I36D mutant. In addition to a crucial role of lipid A, an unexpected contribution emerges for the core region in recognition and binding of Lpt proteins.

1. Introduction

The envelope of Gram-negative bacteria is composed of two lipid bilayers, termed inner and outer membrane (IM and OM), showing distinct composition and structural properties [1]. The two membranes delimit an aqueous compartment, the periplasm, containing a thin peptidoglycan layer. While the IM is a symmetric bilayer made of phospholipids, the OM is an unusual, asymmetric membrane composed of glycerophospholipids in the inner leaflet, and lipopolysaccharide (LPS) in the outer leaflet [1]. LPS is a complex glycolipid of variable length, composed of three elements: lipid A, which anchors LPS to the OM, the oligosaccharide core, and the so-called O-antigen (Fig. 1). The core can be further separated in inner (proximal to lipid A) and outer (proximal to O-antigen) regions, with the inner core containing always at least one residue of 3-deoxy-D-manno-oct-2ulosonic acid (Kdo) and several heptoses. The O-antigen is the less conserved portion of LPS and is composed of a variable number of repeating oligosaccharide units [2]. LPS molecules are assembled at the outer leaflet of the OM to form a barrier that prevents entry of many hydrophobic toxic compounds, including antibiotics, into the cell [3]. In *Escherichia coli*, LPS export to the cell surface is facilitated by seven essential Lpt proteins (LptABCDEFG) located in every cellular compartment (cytoplasm, IM, periplasm and OM) [4–8], which interact with each other forming a transenvelope complex [9]. The Lpt machinery may be divided in three subassemblies, LptBFGC at the IM [10], LptDE [11] at the OM and LptA, the periplasmic component that connects IM and OM complexes [12–14].

The crystal structure of LptA [15] shows 16 antiparallel β-strands designing a β-jellyroll with a hydrophobic binding cavity for LPS [15,16]. Interestingly, the same fold has been observed for the soluble periplasmic region of LptC [17], the N-terminal domain of LptD [18], and has been predicted in the periplasmic regions of LptF and LptG [13,19]. This structural conservation suggests that common mechanisms mediate protein–protein and protein–ligand interactions in this transport system. The number of LptA molecules that build the bridge connecting IM and OM is still not known. Previous crystallographic data obtained in the presence of LPS show formation of LptA fibers, in which subunits are arranged in a head-to-tail fashion designing a continuous cavity along the fiber axis [15]. In line with these observations, LptA oligomerizes in solution without a fixed stoichiometry, according to an n + 1 mechanism [20]. Oligomerization is coupled to a disorder-to-order transition involving the external strands of each monomer, which fold as they become engaged in a continuous, intermolecular β-sheet [15,20]. Such a mechanism is an example of the trade-off between specificity and covalency, generally observed in protein folding and binding [21]. Evidence of oligomerization to a predominantly dimeric state has been recently reported for the *Pseudomonas aeruginosa* LptA homologue [22].

Abbreviations: LPS, lipopolysaccharide; OM, outer membrane; IM, inner membrane; Kdo, 3-deoxy-D-manno-oct-2ulosonic acid; Lpt, lipopolysaccharide transport; Ra-LPS, core LPS; P-Etn, phosphoethanolamine; ANS, 1-anilino-8-naphthalene sulfonate; MIC, minimal inhibitory concentration.

* Corresponding authors.
E-mail addresses: alessandra.polissi@unimib.it (A. Polissi), rita.grandori@unimib.it (R. Grandori).

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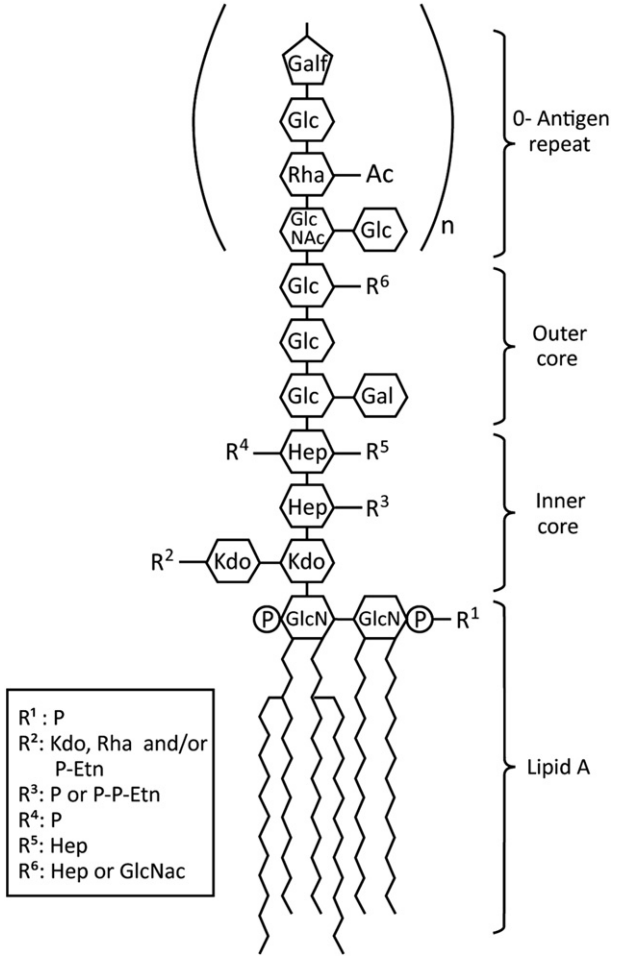

Fig. 1. Schematic structure of *E. coli* K-12 lipopolysaccharide (LPS). The three elements of LPS and the sugar composition of inner core, outer core, and O-specific chains are depicted. Glc, D-glucose; Gal, D-galactose; GlcN, D-glucosamine; Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, L-glycero-D-manno -heptose; GlcNAc, N-acetyl-D-glucosamine; Rha, L-rhamnose; Galf D-galactofuranose; P, phosphate; P-Etn, phosphoethanolamine; Ac, acetate. Possible, non stoichiometric substitutions are indicated in the inset. Ra-LPS refers to chemotypes containing Lipid A, inner core and outer core.

The details of LPS export to the cell surface are still unknown. *In vivo* photo-cross-linking [16] and *in vitro* pull-down experiments [17,23] have suggested that LPS directly binds to both LptA and LptC and transits across the periplasm, carried by LptA [16]. In particular, the I36 residue of LptA forms specific photo-cross-linkages to LPS [16]. Interestingly, this is a highly conserved residue [15], lying in an N-terminal cleft of LptA that, based on mutational, crystallographic and photo-cross-linking analyses, seems to modulate protein–protein (LptC–LptA or LptA–LptA) [13,24] and protein–LPS [16] interactions. Finally, binding parameters have been recently estimated for LptC interaction with Ra-LPS (LPS fragment missing the O-antigen) [25].

Here, fluorescence spectroscopy [26] is used to investigate the interaction of wild-type (wt) and I36D LptA with LPS derivatives (Ra-LPS and lipid A), in order to test the contribution of each ligand moiety and to investigate the mutant binding properties. Since LptA does not contain Trp residues, the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) is used, in competition experiments with the ligands.

2. Materials and methods

2.1. Protein expression and purification

Plasmid pET/LptA-H, expressing the wt protein fused to a C-terminal His_6 tag (LptA-H) has been described [15]. Vector pET/LptA^{I36D}-H, expressing the tagged mutant protein LptA^{I36D}, was generated by site-directed mutagenesis using oligonucleotides AP104 and AP105 [15] as primers and pET/LptA-H as a template [20].

Cultures of BL21(DE3) carrying pET/LptA-H or pET/LptA^{I36D}-H plasmid were grown overnight in 0,2% glucose M9 minimal medium, supplemented with ampicillin (100 $\mu g \cdot mL^{-1}$). LptA or LptA^{I36D} were purified as described [20] with the only difference that the fractions containing the purified protein (>90%) were pooled and concentrated using a Vivaspin 15R column (10,000 molecular weight cut-off; Sartorius Stedim Biotech GmbH, Goettingen, Germany). Concentrated fractions were desalted on PD-10 columns (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and eluted in 50 mM sodium phosphate, pH 8.0, 150 mM NaCl. Protein concentration was determined by a Coomassie (Bradford) assay kit (Thermo-Pierce, Rockford, IL, USA), using bovine serum albumin as a standard. Aliquots were snap frozen in liquid nitrogen and stored at −80 °C.

Ra-LPS was purified from *E. coli* K-12 MG1655 strain as previously described [25].

2.2. Fluorescence measurements

ANS (Sigma Aldrich, St. Louis, MO, USA) was used to perform titrations at fixed protein concentrations, in 50 mM sodium phosphate, 50 mM NaCl, pH 8. Fluoresce measurements were carried out on a Cary Eclipse instrument (Varian, Palo Alto, CA, USA) at 20 °C. The excitation wavelength was set at 374 nm to monitor ANS fluorescence in the emission range 400–600 nm and at 295 nm to monitor tryptophan and ANS fluorescence in the range 310–600 nm. After ANS addition, all the samples were incubated 10 min at 20 °C in the dark before measurements. The total volume added during the titrations was less than 10% of the initial volume. The spectra were corrected for the dilution factor.

LptA-ligand complexes were prepared in the same buffer, by mixing 25 μM protein with a 10-fold molar excess of Ra-LPS or lipid A (Sigma Aldrich, St. Louis, MO, USA) and incubating 1 h at room temperature on a rotary shaker. In the case of lipid A, the ligand was dissolved in 74:33:3 chloroform:methanol:water in an Eppendorf tube and the solvent was evaporated to obtain a film of lipid A molecules on the tube wall. The solution of LptA at the final desired volume and concentration was then added and incubated as described above.

2.3. Data fitting

Fluorescence datasets for LptA-ANS interaction have been analyzed according to a 1:1 binding model:

$$P + L \overset{K_d}{\leftrightarrow} PL$$

where P corresponds to LptA (wt or I36D), L to ANS, PL to the complex and K_d to the dissociation constant. Starting from the equation system

$$\begin{cases} [P]_t = [P] + [PL] \\ [L]_t = [L] + [PL] \\ K_d = \dfrac{[P] \cdot [L]}{[PL]} \end{cases} \quad (1)$$

the concentration of the complex $[PL]$ can be expressed as a function of the total concentrations $[P]_t$, $[L]_t$ and the K_d:

$$[PL] = \frac{[P]_t + [L]_t + K_d - \sqrt{\left([P]_t + [L]_t + K_d\right)^2 - 4 \cdot [P]_t \cdot [L]_t}}{2}. \quad (2)$$

Under the hypothesis that fluorescence emission is directly proportional to $[PL]$ ($F = c[PL]$), fluorescence data have been fitted by Eq. (2) using $[L]_t$ as independent variable and K_d and c as unknown parameters. The values for these parameters reported in Table 1 are those that