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Crystallization and preliminary X-ray analysis of LipL32 from *Leptospira interrogans* serovar Copenhageni

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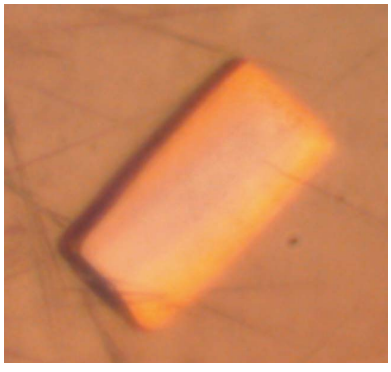

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LipL32 is a major surface protein that is expressed during infection by pathogenic *Leptospira*. Here, the crystallization of recombinant LipL32$_{21–272}$, which corresponds to the mature LipL32 protein minus its N-terminal lipid-anchored cysteine residue, is described. Selenomethionine-labelled LipL32$_{21–272}$ crystals diffracted to 2.25 Å resolution at a synchrotron source. The space group was $P3_121$ or $P3_221$ and the unit-cell parameters were $a = b = 126.7$, $c = 96.0$ Å.

1. Introduction

Leptospirosis is a widespread zoonosis caused by spirochetes of the genus *Leptospira* (Faine *et al.*, 1999; Levett, 2001). Pathogenic *Leptospira* species may cause clinical manifestations in the host such as nephritis, pulmonary haemorrhage and jaundice (Levett, 2001; Bharti *et al.*, 2003). The leptospiral outer membrane is composed of many lipoproteins, of which LipL32 is the most prominent (Haake *et al.*, 2000). It is expressed at high levels on the bacterial surface during both cultivation and natural infection and is the main antigen recognized during the humoral immune response to leptospirosis in humans (Cullen *et al.*, 2005; Haake *et al.*, 2000; Guerreiro *et al.*, 2001; Hauk *et al.*, 2008). After removal of its N-terminal signal peptide (residues 1–19), LipL32 is anchored to the leptospiral outer membrane *via* an N-terminal lipidated cysteine residue (Haake *et al.*, 2000; Haake, 2000; Setubal *et al.*, 2006). This lipoprotein is highly conserved among pathogenic *Leptospira* species (Haake *et al.*, 2000); no orthologues have yet been identified in the saprophytic *L. biflexa* (Picardeau *et al.*, 2008). Recently, a homologue was identified in the *Pseudoalteromonas tunicata* genome that presented functional similarities to LipL32 (Hoke *et al.*, 2008). The LipL32 C-terminus is recognized by the host immune system early in the course of infection and is the domain responsible for mediating interactions with extracellular matrix proteins such as plasma fibronectin, collagen type IV (Hauk *et al.*, 2008; Hoke *et al.*, 2008), collagen type I and laminin (Hoke *et al.*, 2008). LipL32 has been evaluated as an antigen for immunodiagnosis (Bomfim *et al.*, 2005; Dey *et al.*, 2004; Flannery *et al.*, 2001) and as a vaccine antigen, showing protection against *L. interrogans* challenge in animals immunized with LipL32 expressed by recombinant adenovirus (Branger *et al.*, 2001) or recombinant *Mycobacterium bovis* BCG (Seixas *et al.*, 2007) or presented as a DNA vaccine (Branger *et al.*, 2005). In the present work, we aimed to establish the conditions for growing LipL32 crystals in order to solve the tertiary structure of LipL32.

2. Cloning, expression and purification of LipL32

LipL32 was amplified by PCR from total *L. interrogans* serovar Copenhageni genomic DNA (strain Fiocruz L1-130) with the primers LipL32_F, 5′-*CTCGAG***CATATG**GGTGCTTTCGGTGGTCTG-3′, and LipL32_R, 5′-*AAGCTT*ACTTAGTCGCGTCACCTAATCCTCCA-3′. The database access codes for the protein (Q72SM7) and nucleotide (NC 005823) sequences were derived from the published sequence of the *L. interrogans* serovar Copenhageni genome (Nas-

cimento *et al.*, 2004). Nucleotides in italics indicate restriction sites (*Xho*I/*Hin*dIII) and nucleotides in bold represent an alternative restriction site (*Nde*I) in the LipL32_F primer. The amplified product (corresponding to amino acids 21–272 of LipL32) was cloned into pGEM-T Easy vector (Promega) and subcloned into the pAE expression vector (Ramos *et al.*, 2004) at the *Xho*I and *Hin*dIII sites. After digestion with *Nde*I and re-ligation, the final construct allowed the expression of LipL32 protein without an N-terminal polyhistidine tag, in which the initiation methionine is followed immediately by the codon for Gly21. This recombinant $LipL32_{21–272}$ therefore corresponds to the mature LipL32 protein minus its N-terminal lipid-anchored cysteine residue.

Unlabelled and selenomethionine-containing $LipL32_{21–272}$ were expressed in *Escherichia coli* strain BL21 SI (Bhandari & Gowrishankar, 1997). Selenomethionine-labelled protein was produced by growing a 1 l M9 culture medium lacking NaCl to an optical density (600 nm) of 0.8, at which point 100 mg l^{-1} lysine, 100 mg l^{-1} phenylalanine, 100 mg l^{-1} threonine, 50 mg l^{-1} isoleucine, 50 mg l^{-1} valine and 60 mg l^{-1} selenomethionine were added (adapted from Berne *et al.*, 1999). After 15 min, heterologous protein expression was induced by adding 300 m*M* NaCl and the cells were grown for an additonal 3 h. Cells were collected from the 1 l culture by centrifugation, resuspended in 100 ml 20 m*M* triethanolamine pH 7.8 and lysed in a French press (Thermo Spectronic). The soluble fraction was harvested by centrifugation at 8400*g* for 10 min. Purification of $LipL32_{21–272}$ proceeded in three steps as follows. The soluble fraction was applied onto a 5 ml Q-Sepharose Fast Flow column (GE Healthcare) previously equilibrated with 20 m*M* triethanolamine pH 7.8. The unbound flow-though fraction contained $LipL32_{21–272}$. This was then applied onto an SP-Sepharose Fast Flow column equilibrated in the above buffer, washed and bound protein was eluted with a step gradient consisting of 0.1, 0.3, 0.5, 0.7 and 1 *M* NaCl. The $LipL32_{21–272}$-containing fractions were pooled and NaCl was added to a final concentration of 3 *M*. This protein mixture was then bound to a Phenyl-Sepharose Fast Flow column (GE Healthcare) previously equilibrated with 20 m*M* triethanolamine pH 7.8, 3 *M* NaCl. $LipL32_{21–272}$ was eluted using a 2.5–0.1 *M* NaCl gradient followed by a wash in which NaCl was removed. Fractions containing $LipL32_{21–272}$ were pooled and dialyzed against 20 m*M* triethanolamine pH 7.8 followed by concentration using an Amicon system with a 10 kDa cutoff membrane. Purified unlabelled $LipL32_{21–272}$ was analyzed by MALDI–TOF mass spectrometry. The experimentally observed mass was 27 474 g mol^{-1}, which is close to the theoretical mass of $LipL32_{21–272}$ from which the initiation methionine has been removed (27 550g mol^{-1}).

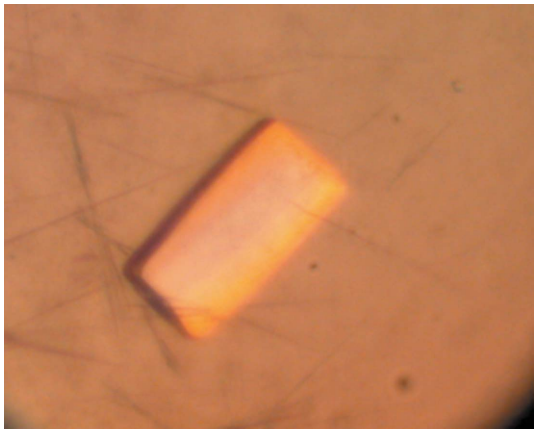

Figure 1
Crystal of selenomethionine-labelled LipL3221-272 from *L. interrogans* serovar Copenhageni. The crystal has dimensions of 0.1 × 0.1 × 0.3 mm.

Table 1
Crystal parameters and data-reduction statistics for recombinant SeMet-labelled LipL32.

Data were processed in space groups $P3_121$ and $P3_221$. Values in parentheses are for the highest resolution shell. For crystal 1, each member of a Friedel pair was counted as a separate reflection.

	Crystal 1		
	Peak	Inflection	Crystal 2
Unit-cell parameters (Å)			
a (Å)	126.2	126.3	126.7
b (Å)	126.2	126.3	126.7
c (Å)	95.9	95.9	96.0
Resolution range (Å)	40.00–2.93 (3.03–2.93)	40.00–3.00 (3.11–3.00)	40.00–2.25 (2.33–2.25)
No. of observed reflections	184445	175481	444816
No. of unique reflections	36676	34340	42419
$\langle I/\sigma(I)\rangle$	10.8 (2.0)	9.5 (1.8)	22.0 (1.9)
Multiplicity	5.0 (4.1)	5.1 (4.6)	10.5 (5.6)
Completeness (%)	99.6 (96.6)	100.0 (99.7)	99.4 (95.8)
R_{merge}† (%)	11.5 (47.7)	12.8 (59.5)	7.1 (46.6)
No. of images	160	160	204
Oscillation angle (°)	1	1	1
Wavelength (Å)	0.97814	0.97831	1.459
f'	−8.40	−11.4	
f''	6.65	3.80	

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Crystallization

Unlabelled (not shown) and selenomethionine-labelled $LipL32_{21–272}$ crystals (Fig. 1) were grown using the vapour-diffusion technique at 291 K using the Crystal Screen (Hampton Research) screening kit and 24-well sitting-drop plates. Optimization was then pursued by varying the precipitant concentration and the buffer pH. Suitable crystals for diffraction experiments were obtained by mixing equal volumes (1.5 µl) of 5 mg ml^{-1} protein in 20 m*M* triethanolamine pH 7.8 and reservoir solution consisting of 0.1 *M* HEPES pH 6.9–8.0 and 1.4 *M* trisodium citrate. Sitting drops were then equilibrated against 0.3 ml reservoir solution. Microseeding was used to improve the quality of the crystals. Mature-sized crystals appeared within one month (Fig. 1). The native and SeMet-labelled crystals had the same morphology (data not shown).

4. Data collection and preliminary structure analysis

MAD X-ray diffraction data was collected from selenomethionine-containing crystals on the protein crystallography W01B-MX2 beamline of Laboratório Nacional de Luz Síncrotron, Campinas, Brazil using a MAR Mosaic 225 CCD detector. SeMet-$LipL32_{21–272}$ crystals were maintained at 100 K in a nitrogen-gas stream during data acquisition (the crystallization solution was sufficient to cryoprotect the crystals). MAD data sets were collected using one crystal (crystal 1 in Table 1) at two wavelengths, 0.97814 and 0.978308 Å, corresponding to the peak and infection points of the fluorescence spectrum, respectively. The f' and f'' anomalous scattering factors in Table 1 were estimated from the fluorescence spectrum of the SeMet-labelled protein crystal using the program *CHOOCH* (Evans & Pettifer, 2001). The oscillation range for each image was 1° and images were collected from 0° to 80° and then from 180° to 260°. Another data set, using 1.459 Å radiation, was collected from a second SeMet-$LipL32_{21–272}$ crystal (crystal 2) that diffracted to 2.25 Å resolution. Analysis of systematic absences allowed us to define the

space group as either $P3_221$ or $P3_121$. The unit-cell parameters for crystal 2 were $a = b = 126.73$, $c = 96.00$ Å. Matthews coefficient analysis suggested that there were three or four protein molecules per asymmetric unit (Matthews coefficients of 2.02 and 2.69 Å^3 Da^{-1}, respectively). However, subsequent resolution of the structure using these data sets revealed that there were in fact only two molecules in the asymmetric unit (to be published elsewhere).

Diffraction-image data were indexed, integrated, scaled and merged using the *HKL*-2000 package (Otwinowski & Minor, 1997). Friedel mates were scaled separately during data processing of the crystal 1 MAD data set. Details of data-acquisition and data-processing statistics are shown in Table 1.

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