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Cloning, expression, purification and crystallization of an endotoxin-biosynthesis enzyme from *Neisseria meningitidis*

The enzyme phosphoethanolamine transferase A is involved in the addition of phosphoethanolamine moieties to lipid A in *Neisseria meningitidis*. The enzyme is composed of an N-terminal transmembrane domain and a C-terminal soluble domain that is present in the periplasm of the bacteria. A membrane-deletion construct of the enzyme was designed and expressed in *Escherichia coli*. Well ordered crystals that diffracted to 1.7 Å resolution were obtained by carrying out a limited trypsin digestion of the protein to remove a predicted N-terminal disordered portion. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 44.3$, $b = 71.6$, $c = 49.9$ Å, $\beta = 109.2^\circ$, and contained one molecule in the asymmetric unit.

1. Introduction

Neisseria meningitidis is an obligate human pathogen and is the causative agent of epidemic meningitis and septic shock (Stephens *et al.*, 2007). It possesses a classic Gram-negative envelope consisting of an inner phospholipid bilayer and an outer asymmetric membrane composed of an inner leaflet of phospholipids and an outer leaflet of lipooligosaccharide (LOS). Lipooligosaccharides, also called endotoxins, have multifaceted roles in protecting the pathogen from human cationic antimicrobial peptides (CAMPs; Tzeng *et al.*, 2005) and human complement (Ram *et al.*, 2003), aiding in the attachment of the bacteria to host cells (Virji *et al.*, 1995) and in mediating the pro-inflammatory response through an interaction with the host toll-like receptor 4–MD-2 complex (Brandtzaeg, 2003).

Meningococcal endotoxin consists of a short glycan attached to a lipid A moiety (Kahler & Stephens, 1998). The 1 and 4' headgroups of lipid A are variably decorated with phosphate, pyrophosphate and phosphoethanolamine (PEA). The decoration of lipid A with PEA has been shown to be the primary mediator of resistance to CAMPs (Tzeng *et al.*, 2005) and influences the cytokine response (Liu *et al.*, 2011) and attachment to host cells (Takahashi *et al.*, 2008). PEA groups are attached to the lipid A headgroups by LOS phosphoethanolamine transferase A (LptA or NmLptA; Cox *et al.*, 2003). This enzyme belongs to the YhjW/YjdB/YijP protein family within the alkaline phosphatase superfamily and is predicted to be an integral membrane protein consisting of a transmembrane domain and a soluble globular domain which is present in the periplasmic compartment. Although there has been very little work on the catalytic mechanisms of these enzymes, it has been proposed that LptA catalyses the transfer of PEA from a donor substrate, phosphatidylethanolamine, to the lipid A acceptor during transport of the LOS through the periplasm to the outer leaflet of the outer membrane (Bos *et al.*, 2007).

In order to understand the mechanism of substrate binding and catalysis by the enzyme, structural studies of the enzyme have been undertaken. Here, we report the cloning, expression, purification and crystallization of a recombinant soluble construct of LptA (sNmLptA) for further structural studies.

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2. Materials and methods

2.1. Clone of the soluble construct of LptA

The gene encoding wild-type NmLptA (*NmLptA*) was amplified by PCR from *N. meningitidis* strain NMB genomic DNA with the primer pair KAP70 (5'-ATGTCTGCCGACGTTTGAATGG-3') and KAP71 (5'-ATTGCCGTGATCGGGAACCTTGG-3') and was cloned into the *HincII* restriction site of the low-copy expression vector pHSG576 to form pCMK519. Splice overlap extension (SOE) PCR was used to construct soluble NmLptA (*sNmLptA*) from *NmLptA* on pCMK519. Two fragments from either side of the residues encoding the predicted transmembrane domain (residues 45–175) were amplified by PCR using two primer pairs, KAP70 with KAP205 (5'-GTAATCGGTGCCGTTAAAGGATGAAGCTCG-3') and KAP206 (5'-GGCACCGATTACGCCTCGTTTTTCCGC-3') with KAP71, to generate fragments encoding the signal sequence (residues 1–44) and globular domains (residues 176–544) of NmLptA, respectively. The PCR products from the first round of PCR were treated with *DpnI* to digest any chromosomal DNA before a second round of SOE PCR was conducted with the primer pair KAP64 (5'-CATGCCATGGTAGGAGGTCCAAATGATAAAACCGAACCTGAGGCCGAAGC-3') and KAP193 (5'-GGGAAGCTTTGCGCGGACGGCGGCAGGCTGCCAATATATC-3'), which were engineered to introduce an *NcoI* site upstream of the ATG start codon and a *HindIII* restriction site to replace the *NmLptA* stop codon, respectively. The PCR product containing *sNmLptA* was treated with T4 DNA polymerase, ligated into the *HincII* site of pHSG576 and transformed into *Escherichia coli* JM109. A clone that contained a 1.26 kb *sNmLptA* fragment was sequenced and named pCMK523. To create sNmLptA:His, pCMK523 was digested with *NcoI* and *HindIII* and ligated into the *NcoI/HindIII* site of pCMK133, an entry vector for the Gateway system (Invitrogen). The ligation was transformed into *E. coli* strain DH5 α , which is sensitive to the CcdB toxin, and plated onto LB agar containing kanamycin at 50 $\mu\text{g ml}^{-1}$. Kanamycin-resistant colonies were screened by colony PCR using primer pair KAP112 and KAP113 to amplify across the multiple cloning site of pCMK133 to detect an insert of 1.26 kb. Clones that were initially positive for this screen were restriction mapped to confirm the orientation and identity of the insert. Vectors that had the correct restriction-digest map were sequenced with KAP43 (5'-CTTAGCAGGAGACATTCCTTCCG-3') to ensure that the C-terminal hexahistidine tag fusion was in frame with the cloned gene. A clone that contained sNmLptA:His within pCMK133 was named pCMK525.

sNmLptA:His was expressed from the high-copy expression vector pTrc99A. *sNmLptA:His* was excised from pCMK525 by restriction digest with *NcoI* and *SmaI* and ligated into the *NcoI/SmaI* site of pTrc99A to form pCMK527.

2.2. Protein overproduction and purification

For the expression and purification of sNmLptA:His, pCMK527 was transformed into *E. coli* strain BL21(DE3)pLysS. Transformants were selected for by the inclusion of ampicillin (50 $\mu\text{g ml}^{-1}$) and chloramphenicol (34 $\mu\text{g ml}^{-1}$) on Luria Broth (LB)/agar plates. A single colony was used to inoculate 100 ml LB medium containing the above antibiotics at 310 K overnight as the starter culture. The starter culture was used to inoculate 1 l Terrific Broth, also containing the above antibiotics, such that the starting absorbance at 600 nm (OD_{600}) was approximately 0.1. Cells were grown in a shaking incubator at 310 K until the OD_{600} reached between 0.5 and 0.6. Protein expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.25 mM.

Incubation was continued at 298 K for 20 h, after which time the cells were harvested by centrifugation at 12 290g for 1 h at 277 K.

The harvested cells were resuspended in 50 mM sodium phosphate buffer pH 8.0 containing 300 mM sodium chloride, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF) at 277 K. Between 20 and 30 ml was used for each gram of pellet. Lysis was performed using an Emulsiflex C5 high-pressure homogenizer (Avestin). The lysate was centrifuged at 22 658g for 1 h at 277 K and the pellet discarded. All purification steps were carried out at 277 K unless otherwise stated. The supernatant was filtered using an 0.2 μm Acrodisc syringe filter (Pall Corporation) and applied onto a HisTrap crude extract chelating affinity column (GE Healthcare) equilibrated with binding buffer (50 mM sodium phosphate pH 8.0, 300 mM sodium chloride, 20 mM imidazole) using an ÄKTApurifier FPLC system (GE Healthcare). The unbound protein was washed with binding buffer until the absorbance at 280 nm reached a steady baseline value. The bound sNmLptA:His was eluted using an increasing gradient of elution buffer (50 mM sodium phosphate pH 8.0, 300 mM sodium chloride, 500 mM imidazole). A peak corresponding to sNmLptA:His eluted between 10% and 15% elution buffer. The collected eluent fractions were dialysed first into 50 mM sodium phosphate buffer pH 8.0, 50 mM NaCl to remove the imidazole using 3350 Da molecular-weight cutoff dialysis tubing (Cellu-Sep) and then into 50 mM HEPES pH 8.0, 50 mM NaCl.

2.3. Limited proteolysis

Limited proteolysis was carried out at 310 K overnight by digesting the dialysed protein with trypsin (an [sNmLptA:His]:[trypsin] ratio of 50:1 was used as determined by the Bradford assay). The tryptic digestion was quenched by the addition of 1 mM PMSF and the filtered digestion mixture was reappplied onto the HisTrap column equilibrated with 50 mM HEPES pH 8.0, 50 mM sodium chloride, 20 mM imidazole. A peak corresponding to sNmLptA eluted in the flowthrough. The digested protein was dialysed into 50 mM HEPES pH 8.0 containing 50 mM sodium chloride and was concentrated to 5–7 mg ml^{-1} , as determined by the Bradford assay, using an Amicon centrifugal filter unit (30 kDa cutoff).

The proteolysed sample was submitted for N-terminal sequencing by Edman degradation to identify the N-terminus and was analysed by MALDI-TOF/TOF mass spectrometry (Bruker Ultraflex II TOF/TOF) to establish its size.

2.4. Crystallization

A suitable protein concentration for crystallization of 5.2 mg ml^{-1} sNmLptA:His in 50 mM HEPES pH 8.0, 50 mM sodium chloride was determined using a PCT Pre-Crystallization Test (Hampton Research). Initial crystallization screens were carried out using protein that had not been treated with trypsin. The screens were set up using the Phoenix crystallization robot (Art Robbins Instruments) as sitting-drop vapour-diffusion experiments in 96-well Intelli-Plates (Hampton Research). The following crystallization screen kits from Hampton Research were used: Crystal Screen, Crystal Screen 2, Index, PEG/Ion and Crystal Screen Lite. The protein:precipitant ratios were varied as follows: 0.15 μl :0.15 μl , 0.15 μl :0.3 μl and 0.3 μl :0.15 μl . Granular precipitate was observed from conditions containing ammonium sulfate with various types of PEG (condition Nos. 20, 30, 31 and 32 from Crystal Screen). Optimization screening around the initial conditions, altering the concentrations of ammonium sulfate, PEG and buffers, was performed in hanging-drop VDX plates (Hampton Research) using 1 μl protein solution and 1 μl crystallization solution to give initial small crystals. Further optimi-

zations were achieved using the Additive Screen kit (Hampton Research) followed by the use of individually purchased detergents (Anatrace), protein that had undergone a limited trypsin digest (see above) and microseeding.

For microseeding, a 2 μ l drop containing small crystals was placed in an Eppendorf tube and 50 μ l of the mother-liquor solution was added. The crystals were crushed by vortexing the solution with Teflon seed beads (Hampton Research). This stock seed solution was diluted further as necessary to give 2–3 crystals per drop in the final crystallization drops. The drops in the final conditions used to obtain diffraction-quality crystals consisted of 1 μ l protein solution, 1 μ l crystallization solution (23–26% PEG 8K, 100 mM ammonium sulfate, 100 mM HEPES pH 7.5–8.0), 15 mM *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide (DDAO) and 0.2 μ l seed solution at the appropriate concentration. The drops were equilibrated at 293 K against 1 ml crystallization solution in the reservoir layered with 200 μ l Al's oil. Thin crystal plates appeared within 3–5 d of setup.

2.5. Data collection and processing

The crystal plates were looped from the crystallization drop using CryoLoops (Hampton Research), flash-cooled in liquid nitrogen and

shipped to the Australian Synchrotron (Melbourne). Initial diffraction tests and data collection were carried out on beamline MX2 equipped with an ADSC Quantum 315r detector. All diffraction tests were carried out on crystals maintained in a stream of nitrogen gas at 100 K. A full diffraction data set to 1.7 Å resolution was collected and processed with the *XDS* software (Kabsch, 2010). Data reduction was carried out using the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

3.1. Construct design and correct targeting

A deletion construct of neisserial lipopolysaccharide phosphoethanolamine transferase was produced by subcloning the C-terminal domain of the protein containing the predicted soluble portion of the enzyme into a bacterial expression vector, pTrc99A. In order to ensure that the protein was secreted into the periplasm, where the correct redox environment is found for disulfide-bond formation, a signal sequence (residues 1–44) was fused to the globular domain (amino-acid residues 176–544), resulting in deletion of the intervening transmembrane domain. Expression of sNmLptA:His and full cell lysis, maintaining an oxidizing environment (no reducing agent was added to the lysis buffers or to the purification protocol), gave adequate levels of soluble protein, which could be purified by Ni²⁺-affinity chromatography.

3.2. Limited proteolysis to improve crystal quality

Initial crystallization trials using the Ni²⁺-affinity purified protein resulted in crystals which were highly disordered in appearance and grew as inseparable stacked plates (Fig. 1*a*). The disorder in the crystals was also apparent in the diffraction pattern, in which multiple sets of diffraction lattices were visible; they were therefore unsuitable for further X-ray analysis. Bioinformatic analysis of the amino-acid sequence (not shown) of sNmLptA:His indicated that the N-terminal region of the soluble membrane-deletion protein was predicted to be disordered and thus it was thought that this region may have been the cause of the observed disorder in the crystals.

(a)

(b)

Figure 1

Crystals of sNmLptA obtained by vapour diffusion from 25% PEG 8K, 100 mM ammonium sulfate, 100 mM HEPES buffer pH 7.5. The protein drop consisted of 6.4 mg ml⁻¹ protein in 50 mM HEPES pH 7.5, 50 mM NaCl, 15 mM *N,N*-dimethyldodecylamine-*N*-oxide. (a) shows crystals obtained prior to a limited trypsin digest of the protein and (b) shows crystals obtained after performing a limited trypsin digest of the protein to remove an N-terminal fragment.

Figure 2

SDS polyacrylamide gel showing the limited proteolysis of sNmLptA using trypsin. The trypsin:LptA ratio is 1:50. Lane 2 contains untrypsinized protein. Lanes 3–6 are various loadings of trypsinized LptA. Lane 1 contains molecular-mass markers; selected markers are labelled in kDa.

Table 1

Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Resolution (Å)	19.68–1.70 (1.79–1.70)
No. of observations	216628 (11866)
No. of unique reflections	30870 (3300)
Completeness (%)	95.2 (70.6)
Multiplicity	7.0 (3.6)
R_{merge}	0.054 (0.344)
R_{meas}	0.059 (0.404)
$R_{\text{p.i.m.}}$	0.022 (0.203)
Average $I/\sigma(I)$	23.8 (3.5)

Previous work has shown that limited proteolysis can be used to remove regions of proteins that fail to contribute to a compact folded structure (Cohen *et al.*, 1995) and thus negatively impact on the formation of a well ordered crystal lattice. Such studies have shown a marked improvement in crystal quality and hence diffraction quality when limited proteolysis was carried out on the protein to remove the unstructured regions. An inspection of the sequence revealed a number of potential trypsin-cleavage sites near the start of the predicted ordered region of the protein. A limited proteolysis experiment using trypsin and subsequent analysis by SDS–PAGE revealed a decrease in the molecular weight of the protein (Fig. 2). When the trypsin-digested mixture was applied onto the Ni^{2+} -affinity chromatography column, the protein no longer bound to the Ni^{2+} resin. Further inspection of the sequence revealed a trypsin-cleavage site near to the C-terminus of the protein which would remove the hexahistidine tag, thus abrogating binding of the trypsin-digested protein to the column. To confirm the removal of the N-terminal predicted disordered region, an Edman-degradation N-terminal sequence was carried out (Australian Proteome Analysis Facility) on the undigested and the digested samples. The undigested sample gave a sequence at the N-terminus starting with Asn196 and the digested sample gave a sequence beginning with Ser210. This demonstrated that cleavage of the predicted soluble fragment consisting of residues 176–195 occurs during protein expression and that a limited trypsin digestion further cleaves the protein to remove residues 196–209, giving a start site of Ser210. This gives a soluble fragment of 335 residues and a calculated molecular weight of 37.8 kDa (not including the hexahistidine tag at the carboxyl-terminus of the enzyme). Mass spectrometry (MALDI-TOF/TOF) gave a molecular-ion peak of 37 800 (data not shown), matching the predicted mass of the protein from Ser210 to Arg544 and thus suggesting that the C-terminal hexahistidine tag was proteolytically removed.

Crystallization trials of the trypsin-digested fragment resulted in significant improvement in the crystal quality. Although the crystals still grew as clusters of plates, they were more separable relative to the crystals obtained prior to trypsin cleavage (Fig. 1*b*). Crystallization only occurred when a detergent additive was added to the

crystallization drop. Crystals have been obtained with a variety of detergents including *N,N*-dimethyldodecylamine-*N*-oxide (4–8 mM) and *N*-octyl- β -D-glucoside (17 mM). In addition, the crystallization process was slowed by the addition of a thin layer of Al's oil onto the well, which acts as a barrier decreasing the rate of drop equilibration.

3.3. Diffraction and space-group determination

Diffraction tests were carried out on the microfocus beamline (MX2) at the Australian Synchrotron. These tests were carried out on crystals that had not been cryoprotected prior to X-ray exposure; that is, the crystals were placed in a loop straight from the crystallization drop and flash-cooled in liquid nitrogen. Initial diffraction tests showed diffraction to 1.7 Å resolution. Analysis of the unit-cell parameters from the diffraction pattern indicated that the crystals could be indexed in a monoclinic lattice of space group $P2_1$, with unit-cell parameters $a = 44.3$, $b = 71.6$, $c = 49.9$ Å, $\beta = 109.2^\circ$. The data-collection and processing statistics are given in Table 1. The Matthews coefficient was computed to be $1.87 \text{ Å}^3 \text{ Da}^{-1}$, with a solvent content of 34.2%. This corresponds to one molecule per asymmetric unit (Matthews, 1968). Phasing of the crystals is currently in progress using a selenomethionine-mutant form of the protein, as seven methionine residues are present in the truncated version of the enzyme.

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