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Expression, crystallization and preliminary X-ray diffraction studies of recombinant *Bacillus anthracis* lethal factor

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The lethal factor (LF) produced by *Bacillus anthracis* is a Zn^{2+}-dependent endopeptidase which specifically cleaves the N-terminal tail of several MAP kinase kinases (MAPKKs). The recombinant expression, purification and crystallization of LF and of an inactive mutant consisting of a single amino-acid substitution in the conserved catalytic site are reported here. Both proteins crystallize in the cubic space group *I*432.

1. Introduction

Anthrax lethal toxin (LeTx) is a protein exotoxin secreted by virulent strains of *B. anthracis* which plays a major role in the pathogenesis of systemic anthrax. It is formed by the non-covalent association of the lethal factor (LF) with the protective antigen (PA; Leppla, 1999). Substantial insight into the function of PA has recently been gained by the determination of its crystallographic structure (Petosa *et al.*, 1997). PA binds to a ubiquitous plasma-membrane receptor and is than proteolytically processed to a 63 kDa form which oligomerizes and binds LF (Singh *et al.*, 1999). Endocytic uptake of LeTx triggers a conformational rearrangement in PA that results in the translocation of LF into the cytoplasm (Miller *et al.*, 1999).

The molecular basis of LF toxicity is poorly understood. Despite the ability of LF to reach the cytosol of virtually any cell, macrophages are the only cell type that acutely respond to toxin challenge. In macrophages, high doses of LeTx trigger production of reactive oxygen intermediates and induce cellular lysis shortly after toxin challenge (Hanna *et al.*, 1994). Sub-cytolytic doses mediate the release of cytokines from cultured macrophages (Hanna *et al.*, 1993).

LF is a 90 kDa protein containing a C-terminal HE*xx*H Zn^{2+}-binding site (amino acids 686–690 of mature LF) characteristic of most metallopeptidases. Permeable metallo-protease inhibitors block LeTx activity in macrophage cell lines, suggesting that LeTx cytotoxicity relies on LF metallopeptidase activity (Menard *et al.*, 1996). Consistent with this observation, site-directed mutagenesis of active-site residues His686, Glu687 and His690 partially or totally impairs LF activity (Hammond & Hanna, 1998). Analogous to the clostridial neurotoxins, LF has been proposed to target its substrates in a strictly selective manner (Hammond & Hanna, 1998; Kochi *et al.*, 1994). Using a combination of genetic and biochemical approaches, it was found that LF specifically recognizes and proteolyses several of the members of a family of dual specificity mitogen-activated protein kinase kinases (MAPKKs; Duesbery *et al.*, 1998; Vitale *et al.*, 1998). The latter are mid-components of three-kinase modular cascades that control cell responses to mitogenic and stress signals (Dhanasekaran & Premkumar Reddy, 1998).

As a first step toward understanding the structural basis of specific substrate recognition by LF, we have initiated a study aimed at determining the three-dimensional structure of this protein. Here, we describe the purification of LF from a recombinant bacterial system, the crystallization of the full-length molecule and of its Glu687Ala catalytic mutant (LF^{E687A}) and preliminary crystal characterization.

2. Experimental and results

2.1. Expression and purification

The LF and LF^{E687A} coding sequences (corresponding to residues 34–809 of the sequence with accession number P15917) were amplified by the polymerase chain reaction and subcloned in the *Bam*HI site of the pGEX-2TK vector (Amersham-Pharmacia) as previously described (Vitale *et al.*, 1998).

High-level soluble expression of the GST::LF and GST::LF^{E687A} 116 kDa fusion proteins was achieved in *Escherichia coli* strain BL21 DE3 pUBS 520 (Schenk *et al.*, 1995). The following protocol describes the purification of the wild-type protein, but applies equally well to the purification of the Glu687Ala mutant. Bacteria were grown in 2×YT medium (1.6% bacto tryptone, 1% yeast extract, 0.5% NaCl) to OD_{600} = 1 and induced for 4–5 h at 303 K with 0.1 m*M* IPTG (Fig. 1; lanes 1 and 2). Pellets from 4 l cultures were resuspended in

Table 1
Data-collection statistics.

The values in parentheses refer to the outer resolution shell.

X-ray source	BW7A at EMBL-DESY
Wavelength (Å)	0.8428
Detector	MAR345
Space group	$I432$ or $I4_132$
Unit-cell parameters (Å, °)	$a = b = c = 329.708$, $\alpha = \beta = \gamma = 90.000$
Total No. of reflections	22960
Resolution range (Å)	20.0–4.0
Redundancy	2.5
Completeness (%)	88.5 (85.3)
$I/\sigma(I)$	6.5 (2.4)
$R_{\rm merge}$ (%)	6.9 (32.3)

50 ml 50 m*M* Tris–HCl pH 8.3, 0.5 m*M* EDTA, 300 m*M* NaCl supplemented with five protease inhibitors tablets (Roche) and were lysed by passing the cell suspension twice through a French pressure cell at 8 MPa.

The supernatant obtained after high-speed centrifugation (Fig. 1; lane 3) was incubated with 4 ml of GSH–Sepharose 4B beads (Amersham-Pharmacia). After washing with 10 m*M* Tris–HCl pH 8.3, 0.5 m*M* EDTA in a step gradient of salt decreasing from 300 to 20 m*M* NaCl, the GSH–Sepharose beads were equilibrated in 50 m*M* Tris–HCl pH 8.3, 20 m*M* NaCl, 2.5 m*M* $CaCl_2$ (Fig. 1; lane 4). LF was subsequently cleaved from the GST tag with thrombin (Amersham-Pharmacia) for 1 h at a final concentration of 100 units per millilitre of beads (Fig. 1; lane 5). After cleavage, thrombin was removed by affinity chromatography with benzamidine Sepharose 6B (Amersham-Pharmacia). The protein was further purified by anion-exchange chromatography on a MonoQ column (Amersham-Pharmacia) and eluted using a linear gradient from 20 to 500 m*M* NaCl in 50 m*M* Tris–HCl pH 8.3, 0.5 m*M* EDTA; under these conditions, LF (and the LF^{E687A} mutant) eluted at 180 m*M* NaCl (Fig. 1; lane 6). This fraction was applied to size-exclusion chromatography on a Superdex 200 column (Amersham-Pharmacia) equilibrated with 10 m*M* Tris–HCl pH 8.3, 20 m*M* NaCl, 0.5 m*M* EDTA. As shown in the purification profile of wild-type LF (Fig. 1), the final product was more than 95% pure as judged by Coomassie blue staining after separation by SDS–PAGE (Fig. 1; lane 7). Identical results were obtained with the LF^{E687A} mutant (not shown). The overall yields were similar for the two proteins and were estimated at 3–4 mg of purified sample per litre of bacterial culture.

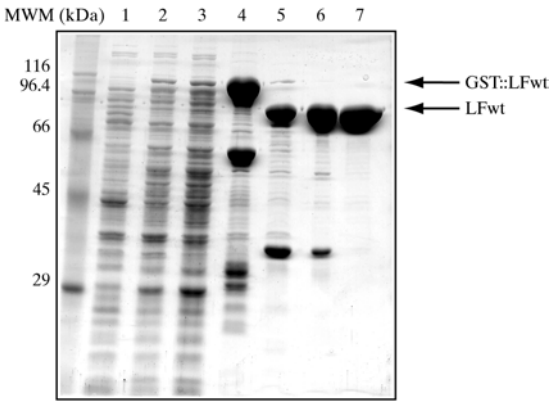

Figure 1
10% SDS–PAGE separation and Coomassie blue staining of samples obtained during expression/purification of the recombinant wild-type LF. Total lysate of BL21 DE3 pUBS 520 pGEX-2TK LF before (lane 1) and after (lane 2) IPTG induction; soluble fraction of the cell lysate (lane 3); GSH–Sepharose affinity purified GST::LF before (lane 4) and LF after (lane 5) thrombin cleavage; purified LF after anion-exchange (lane 6) and size-exclusion (lane 7) chromatography.

2.2. Crystallization

The purified proteins in 10 m*M* Tris pH 8.3, 20 m*M* NaCl, 0.5 m*M* EDTA were concentrated to 10 mg ml^{-1} using Centricon 30 concentrators (Amicon). Aliquots of the proteins were filtered through a Whatman Anodisc 13 0.1 µm filter and subjected to dynamic light-scattering (DLS) analysis in a Protein Solutions DynaPro 99 Instrument. Repeated experiments at room temperature and 277 K yielded consistent DLS spectra which could only be interpreted as bimodal molecular distributions consisting of a species with a hydrodynamic radius compatible with a monomer or a dimer (4.26 ± 0.86 nm) and high molecular-weight aggregates. Despite this discouraging preliminary result, both the wild-type and mutant proteins yielded diffraction-quality crystals without further sample purification or stabilization and without the addition of additives to decrease aggregation. The best crystals were obtained by the hanging- and sitting-drop techniques, mixing equal volumes of the 10 mg ml^{-1} protein stock solution with a reservoir solution containing 100 m*M* Tris pH 8.0, 1.9 *M* ammonium sulfate, and grew to their maximum size within a one-month period (Fig. 2). The wild-type and mutant proteins also behaved indistinguishably with respect to crystallization conditions.

2.3. X-ray crystallography

Preliminary analysis of X-ray diffraction data obtained in-house from both wild-type and LF^{E6987A} crystals with an R-AXIS IV imaging plate using Cu $K\alpha$ radiation generated by a Rigaku RU-300 rotating-anode generator (operated at 50 kV and 100 mA and focused using a double-mirror system; MSC, Yale) indicates that the wild-type and mutant proteins generate crystals of comparable diffraction quality (data not shown).

X-ray diffraction data from a crystal of the wild-type protein were collected on EMBL beamline BW7A at the DESY synchrotron facility in Hamburg, Germany, as detailed in Table 1. Crystals were flash-cooled in liquid nitrogen after a short incubation in a cryo-protectant buffer containing 100 m*M* Tris pH 8.0, 2.2 *M* ammonium sulfate, 25% glycerol. Crystals diffracted to at least 3.6 Å resolution with 0.5° oscillations and 10 min exposures. A complete data set at 4 Å resolution was collected in a single pass (Table 1). Diffraction data processing and reduction were carried out with the *DENZO*/*SCALEPACK* program suite (Otwinowski & Minor, 1997). Crystals belong to the cubic space group $I432$, with unit-cell parameters $a = b = c = 330.55$ Å, $\alpha = \beta = \gamma = 90.000°$. The Matthews coefficient (Matthews, 1985) is 3.1 $Å^3\ Da^{-1}$ if two molecules are assumed to be present in the asymmetric unit. Under the cryogenic conditions used, very limited radiation damage was observed and the overall mosaic spread was 0.232°.

3. Conclusions

While a high-resolution data set could not be collected owing to the limited availability of synchrotron time, our preliminary data-collection effort demonstrates that it is possible to collect diffraction data from LF crystals at a resolution compatible with structure determination. We will attempt to solve the phase problem using a combination of two complementary approaches. On one hand, we plan to create a selenomethionyl derivative of the protein in order to set up a multiwavelength anomalous dispersion (MAD) experiment. On the other hand, the availability of single-site derivatives may be very helpful in determining heavy-atom positions by Patterson methods in this high-symmetry space group. Thus, we plan to attempt the substitution of the Zn atom with Hg, following lines already investigated (Gomis-Rüth *et al.*, 1993). This experiment will be simplified by the fact that

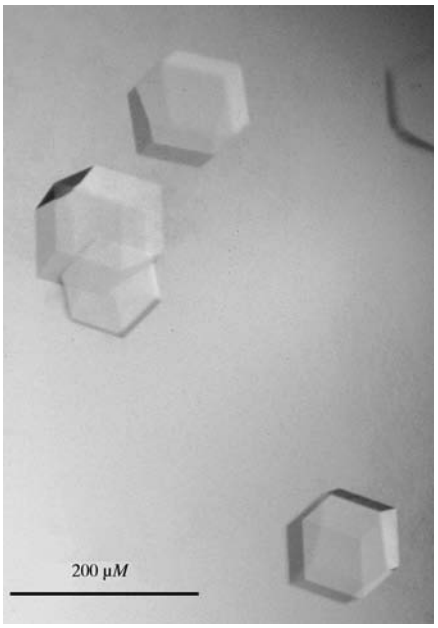

Figure 2
Crystals of recombinant wild-type anthrax lethal factor.

there are no competing thiol groups in LF. Finally, crystals are likely to contain two or three molecules in the asymmetric unit and phase extension may thus be achieved by non-crystallographic symmetry averaging.

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