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Crystallization and preliminary crystallographic analysis of SMase I, a sphingomyelinase from *Loxosceles laeta* spider venom

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SMase I, a 32 kDa sphingomyelinase found in *Loxosceles laeta* venom, is responsible for the major pathological effects of spider envenomation. This toxin has been cloned and functionally expressed as a fusion protein containing a 6×His tag at its N-terminus to yield a 33 kDa protein [Fernandes-Pedrosa *et al.* (2002), *Biochem. Biophys. Res. Commun.* **298**, 638–645]. The recombinant protein possesses all the biological properties ascribed to the whole *L. laeta* venom, including dermonecrotic and complement-dependent haemolytic activities. Dynamic light-scattering experiments conducted at 291 K demonstrate that the sample possesses a monomodal distribution, with a hydrodynamic radius of 3.57 nm. *L. laeta* SMase I was crystallized by the hanging-drop vapour-diffusion technique using the sparse-matrix method. Single crystals were obtained using a buffer solution consisting of 0.08 *M* HEPES and 0.9 *M* trisodium citrate, which was titrated to pH 7.5 using 0.25 *M* sodium hydroxide. Complete three-dimensional diffraction data were collected to 1.8 Å at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). The crystals belong to the hexagonal system (space group $P6_1$ or $P6_5$), with unit-cell parameters $a = b = 140.6$, $c = 113.6$ Å. A search for heavy-atom derivatives has been initiated and elucidation of the crystal structure is currently in progress.

1. Introduction

Loxoscelism is the clinical condition produced by the venom of spiders belonging to the genus *Loxosceles*, which can be observed as two well defined clinical variants: cutaneous loxoscelism and systemic or viscerocutaneous loxoscelism. We have recently identified, purified and characterized the toxins from *L. intermedia* venom that are responsible for all the local and systemic effects induced by whole venom (Tambourgi *et al.*, 1995; Tambourgi, Magnoli *et al.*, 1998). Two highly homologous proteins with molecular weights of 35 kDa were purified to homogeneity and shown to possess sphingomyelinase activity. Both these proteins are able to induce dermonecrosis in laboratory animals and rendered human erythrocytes (E) susceptible to lysis by complement (C) *in vitro*. In a mouse model of *Loxosceles* envenomation, the toxins also induced intravascular haemolysis and provoked a cytokine response similar to that observed in endotoxic shock (Tambourgi, Petricevich *et al.*, 1998).

Our research has focused on the effects of *Loxosceles* sphingomyelinases on erythrocytes and nucleated cells and our results indicate that the toxins induce the activation of membrane-bound metalloproteinases (Tambourgi *et al.*, 2000; van den Berg *et al.*, 2002). In the case of erythrocytes, this leads to increased susceptibility to activation of C *via* the classical pathway, possibly by inducing loss of membrane asymmetry (Tambourgi *et al.*, 2002), and *via* the alternative pathway because of metalloproteinase-induced cleavage of glycophorins (Tambourgi *et al.*, 2000). However, on nucleated cells the result is a decrease in C susceptibility (van den Berg *et al.*, 2002). The exact mechanisms of these events and their role in the pathology of loxoscelism remain to be elucidated.

The only proteins that display significant sequence homology to *Loxosceles* sphingomyelinases are bacterial toxins from *Corynebacterium pseudotuberculosis* (accession No. AAA99867; Bernheimer *et al.*, 1985) and *Arcanobacterium haemolyticum* (accession No. Q59121; Cuevas & Songer, 1993). These toxins are also sphingomyelinases but are generally referred to as phospholipases D (PLD). No significant sequence or structural homology was found with other phospholipases.

Phospholipases are frequently found as toxic components in animal venoms and bacterial toxins. Phospholipases promote the hydrolysis of ester bonds in phospholipids and are classified as phospholipases A_1, A_2, C and D depending on the position in the ester bond that is hydrolysed (van den Bosch, 1980). In contrast to most phospholipases, the *Loxosceles* and bacterial phospholipases display

unusual substrate specificity. Of the four major phospholipids in mammalian cell membranes, only sphingomyelin (SM) is hydrolysed by bacterial PLD and spider toxins, resulting in the formation of choline and ceramide-1-phosphate (Bernheimer *et al.*, 1985). We have recently demonstrated that SMase D from spiders and bacteria has intrinsic lysophospholipase D activity toward lysophosphatidyl choline (LPC). LPC hydrolysis yields the lipid mediator lysophosphatidic acid (LPA), a known inducer of platelet aggregation, endothelial hyperpermeability and pro-inflammatory responses (van Meeteren *et al.*, 2004).

The difficulty in obtaining large quantities of venom and purified venom components is the principal limiting factor in studying the mechanisms involved in loxoscelism. Recombinant expression of the sphingomyelinase is thus necessary for further functional and structural characterization of the toxin. We have cloned and expressed one of the sphingomyelinases from *L. laeta* venom, named SMase I (GenBank accession No. AY093599), which possessed all the biological properties ascribed to the whole venom, *e.g.* dermonecrotic and complement-dependent haemolytic activities and the ability to hydrolyse SM and LPC (Fernandes-Pedrosa *et al.*, 2002; van Meeteren *et al.*, 2004).

2. Methods

2.1. Recombinant protein expression and purification

L. laeta SMase I recombinant protein was produced as described previously (Fernandes-Pedrosa *et al.*, 2002). Briefly, pAE-*L. laeta* H17 cDNA-transformed *E. coli* BL21 (DE3) cells were inoculated in 50 ml of 2YT/amp and grown overnight at 310 K, induced with IPTG. Recombinant protein was harvested from the pellet using a French pressure cell and was purified on an Ni^{2+} Chelating Sepharose Fast Flow column (Pharmacia, Sweden; 64 × 10 mm). Homogeneity was determined by SDS–PAGE run under reducing and non-reducing conditions using 12% acrylamide gels (Laemmli, 1970). A single band of 33 kDa was observed in the gels. Samples were dialysed against PBS and stored at 253 K.

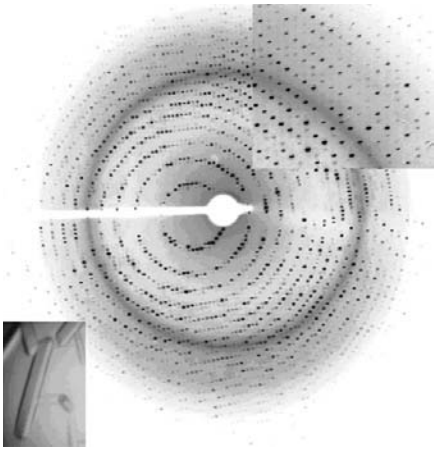

Figure 1
X-ray diffraction pattern of SMase I, a sphingomyelinase from *L. laeta* spider venom, obtained at the Laboratório Nacional de Luz Síncrotron using a MAR CCD detector. Top right inset, enlargement (×3) of part of the image; bottom left inset, photomicrograph of the crystals.

2.2. Dynamic light scattering

Dynamic light-scattering experiments were carried out using a DynaPro 810 (Protein Solutions) apparatus equipped with a temperature stabilizer. A protein solution of 1.0 mg ml^{-1} was prepared in 0.1 *M* imidazole pH 8.0. Standard curves of bovine serum albumin were used for calibration and the experiments were conducted at 291 K.

2.3. Crystallization

The sample was dialysed against 20 m*M* HEPES buffer pH 7.5 and concentrated to 10 mg ml^{-1}. Crystallization was performed by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates. Initial trials were carried out by the sparse-matrix method with some modifications (Jancarik & Kim, 1991). Typically, 1 µl drops of protein solution were mixed with an equal volume of screening solution and equilibrated over 0.8 ml of the latter in the reservoir solution. Large single crystals were obtained when a 1 µl protein droplet was mixed with an equal volume of reservoir solution consisting of 0.08 *M* HEPES buffer including 0.9 *M* trisodium citrate and was titrated to pH 7.5 with 0.25 *M* sodium hydroxide.

2.4. Data collection

The crystal was transferred to a cryoprotectant solution containing 20% glycerol and flash-frozen. The diffraction data were collected at the Laboratório Nacional de Luz Síncrotron (Campinas, Brazil), with the wavelength fixed at 1.423 Å. Diffraction intensities were measured using a MAR CCD detector. The data were indexed and scaled using the *DENZO* and *SCALEPACK* programs from the *HKL* package (Otwinowski & Minor, 1997).

Table 1
Data-collection results.

Values in parentheses are for the last resolution shell (1.86–1.80 Å).

Space group	$P6_1$ or $P6_5$
Unit-cell parameters (Å)	$a = b = 140.6$, $c = 113.6$
Maximum resolution (Å)	1.8
No. unique reflections	115146
$R_{\rm merge}$ (%)	4.5%(40.4)
Completeness (%)	97.8 (95.5)
$V_{\rm M}$ (Å^3 Da^{-1})	2.5
Solvent content (%)	49
No. molecules per AU	4
$I/\sigma(I)$	34.2 (4.1)

3. Results

Structural homogeneity in solution was observed by dynamic light scattering, which presented a monomodal distribution. Single crystals with dimensions 0.2 × 0.2 × 0.4 mm (inset in Fig. 1) were obtained and diffraction data were collected to 1.8 Å (Fig. 1) under cryogenic conditions (100 K). The diffraction data were indexed in space group *P*6, with unit-cell parameters $a = b = 140.6$, $c = 113.6$ Å. An examination of the systematic absences indicated that the crystals belonged to either space group $P6_1$ or to its enantiomorph $P6_5$. Processing of the 1 475 030 measured reflections to 1.8 Å led to 115 146 unique reflections with an $R_{\rm merge}$ of 4.5% (40.4% in the last shell, 1.86–1.80 Å) and a completeness of 97.8% (95.5% in the last shell). Data-processing statistics are presented in Table 1. Calculation of the self-rotation function assuming a molecular weight of 33 kDa per molecule resulted in a Matthews coefficient (Matthews, 1968) of 2.5 Å^3 Da^{-1} (49% solvent content) for four molecules in the asymmetric unit.

A search of the Protein Data Bank indicated that the highest sequence identity (26%) was observed between human Dj-1 (PDB code 1pdv) and the C-terminal region of SMase I. As a result of this low sequence identity with structures currently deposited in the Protein Data Bank, the phase problem will be solved using either multiple anomalous dispersion (MAD) or multiple isomorphous replacement methods (MIR). Knowledge of the three-dimensional structure will be important for understanding the steric requirements and mechanism of sphingomyelinases.

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