Crystallization and preliminary X-ray diffraction analysis of a novel Arg49 phospholipase A$_2$ homologue from *Zhaoermia mangshanensis* venom

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Zhaoermiatoxin, an Arg49 phospholipase A$_2$ homologue from *Zhaoermia mangshanensis* (formerly *Trimeresurus mangshanensis*, *Ermia mangshanensis*) venom is a novel member of the PLA$_2$-homologue family that possesses an arginine residue at position 49, probably arising from a secondary Lys49→Arg substitution that does not alter the catalytic inactivity towards phospholipids. Like other Lys49 PLA$_2$ homologues, zhaoermiatoxin induces oedema and strong myonecrosis without detectable PLA$_2$ catalytic activity. A single crystal with maximum dimensions of $0.2 \times 0.2 \times 0.5$ mm was used for X-ray diffraction data collection to a resolution of 2.05 Å using synchrotron radiation and the diffraction pattern was indexed in the hexagonal space group $P6_4$, with unit-cell parameters $a = 72.9$, $b = 72.9$, $c = 93.9$ Å.

1. Introduction

Lys49 phospholipase A$_2$ homologues (Lys49 PLA$_2$s) belong to a subfamily of snake-venom group II PLA$_2$s in which Asp49 is replaced by a lysine residue (reviewed by Ownby *et al.*, 1999; Murakami & Arni, 2003). These proteins are considered to be catalytically inactive owing to their inability to bind the cofactor (a calcium ion) and thus stabilize the tetrahedral intermediate observed in the calcium-dependent catalytic reaction promoted by Asp49 PLA$_2$s (van den Bergh *et al.*, 1989). Despite their catalytic inactivity, Lys49 PLA$_2$s are very potent in the induction of myonecrosis, a major and clinically highly challenging event in envenomation by most viperid snakes, based on one or more mechanisms that continue to be enigmatic to toxinologists. Consequently, a number of different strategies, such as structural analysis (Arni & Ward, 1996; Ownby *et al.*, 1999; Murakami & Arni, 2003), chemical modification (Andrião-Escarso *et al.*, 2000; Soares *et al.*, 2001), sequence-comparison analyses (Selistre de Araujo *et al.*, 1996; Ward *et al.*, 1998), charge-distribution analysis (Kini & Iwanaga, 1986; Kini & Evans, 1989), hydrophobicity profiles (Kini & Iwanaga, 1986), synthetic peptide studies (Lomonte *et al.*, 1994; Nuñez *et al.*, 2001) and site-directed mutagenesis (Ward *et al.*, 2002), have attempted to elucidate the structural determinants for the myotoxicity expressed by Lys49 PLA$_2$s. The three-dimensional structures of a large number of Lys49 PLA$_2$s in both native and complexed states have recently been elucidated (Arni *et al.*, 1995; Lee *et al.*, 2001; Ambrosio *et al.*, 2005; Murakami *et al.*, 2005, 2006, 2007) and have contributed to addressing the role of Lys122 in the myotoxicity of Lys49 PLA$_2$s (Ambrosio *et al.*, 2005; Watanabe *et al.*, 2005).

Compounds such as suramin, heparin, heparin-like glycosaminoglycans, related polyanions and polyethylene glycol derivatives have been shown to effectively inhibit the activity of myotoxic Lys49 PLA$_2$s both *in vitro* and *in vivo* (Murakami *et al.*, 2005, 2007). Recently, a new role has been proposed for Lys49 PLA$_2$s in the inhibition of the vascular endothelial growth factor and its receptor system, which plays a central role in angiogenic processes (Yamazaki *et al.*, 2005).

In this work, we report the crystallization and preliminary X-ray diffraction analysis of zhaoermiatoxin, isolated from the venom of the Chinese pit viper *Zhaoermia mangshanensis*, which contains a Lys49→Arg substitution and represents a novel member of the

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catalytically inactive PLA_2-homologue subfamily (Mebs *et al.*, 2006). Zhaoermiatoxin causes vasodilatation and oedema and also acute myonecrosis as part of a synergistic system which contributes to the overall oedematous and myotoxic response caused by the venom.

2. Materials and methods

2.1. Protein purification

Zhaoermiatoxin was purified from the crude venom of an adult male *Z. mangshanensis* following a two-step purification procedure which consisted of gel filtration on Sephadex G-100 followed by cation-exchange chromatography on a Resource S column as described previously (Mebs *et al.*, 2006). Prior to the crystallization trials, the zhaoermiatoxin sample was further purified by high-performance liquid chromatography (HPLC) on a C18 reversed-phase (RP) column. Standard SDS–PAGE performed according to Laemmli (1970) indicated the presence of zhaoermiatoxin as a single band with an apparent molecular weight of approximately 14 000 Da; the theoretical value obtained from the sequence (121 amino-acid residues, 14 of which are cysteines) was calculated to be 13 971.3 Da (Mebs *et al.*, 2006). Protein concentrations were measured by the Bradford method (Bradford, 1976).

2.2. Dynamic light scattering

Dynamic light-scattering experiments were carried out using a DynaPro 810 (Protein Solutions) equipped with a temperature stabilizer. A 1.0 mg ml^{-1} protein solution was prepared in 0.1 M HEPES pH 7.5. Standard curves of bovine serum albumin were used for calibration and the experiments were conducted at 291 K.

2.3. Crystallization

The sample was dissolved to a concentration of 30 mg ml^{-1} in 20 mM HEPES buffer pH 7.5. 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 using Crystal Screen HR2-110 and HR2-112 kits (Hampton Research). Typically, 1 µl protein solution was mixed with an equal volume of screening solution and equilibrated over 0.6 ml of the latter in the reservoir. Large single crystals were obtained when a 1.5 µl protein droplet was mixed with an equal volume of reservoir solution consisting of 0.2 M ammonium sulfate, 0.1 M sodium caco-dylate trihydrate pH 6.5 and 30%(w/v) polyethylene glycol 8000. The crystals were obtained after equilibrating the above solution for two months at room temperature.

2.4. X-ray diffraction data collection and analysis

X-ray diffraction data were collected at the DB03-MX1 beamline at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil; Polikarpov *et al.*, 1998). The crystal was transferred to a cryoprotectant solution containing 10% glycerol and flash-frozen in a gaseous nitrogen stream at 100 K. The wavelength of the radiation source was set to 1.427 Å and a MAR CCD detector was used to record the X-ray diffraction data. The crystal-to-detector distance was set to 95 mm. The data were indexed, integrated, reduced and scaled using the *DENZO* and *SCALEPACK* programs from the *HKL* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Structural homogeneity in solution was demonstrated by the dynamic light-scattering experiments, which presented a monomodal distribution. Single crystals with dimensions of $0.2 \times 0.2 \times 0.5$ mm (Fig. 1) were used for the X-ray diffraction experiments and the data were collected to 2.05 Å resolution (Fig. 2) under cryogenic conditions (100 K). The diffraction data were indexed in space group $P6$, with unit-cell parameters $a = 72.9$, $b = 72.9$, $c = 93.9$ Å. An examination of the systematic absences indicated that the crystals belonged to either space group $P6_2$ or its enantiomorph $P6_4$.

Processing of the 305 151 measured reflections to 2.05 Å resolution led to 17 842 unique reflections with an R_{merge} of 7.1% (33.3% in the last shell, 2.12–2.05 Å resolution) and a completeness of 99.9% (99.86% in the last shell). Taking into consideration the molecular weight of 13 972 Da (Mebs *et al.*, 2006), one dimer is present in the asymmetric unit, with a corresponding Matthews coefficient of
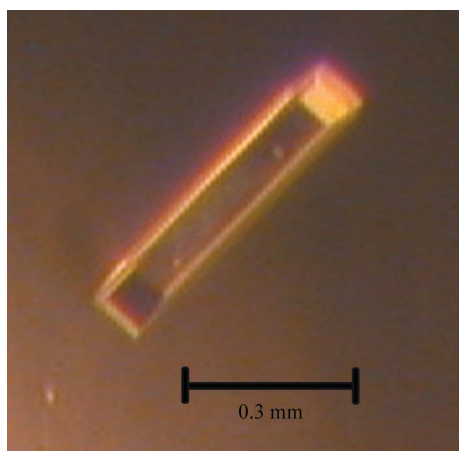

Figure 1
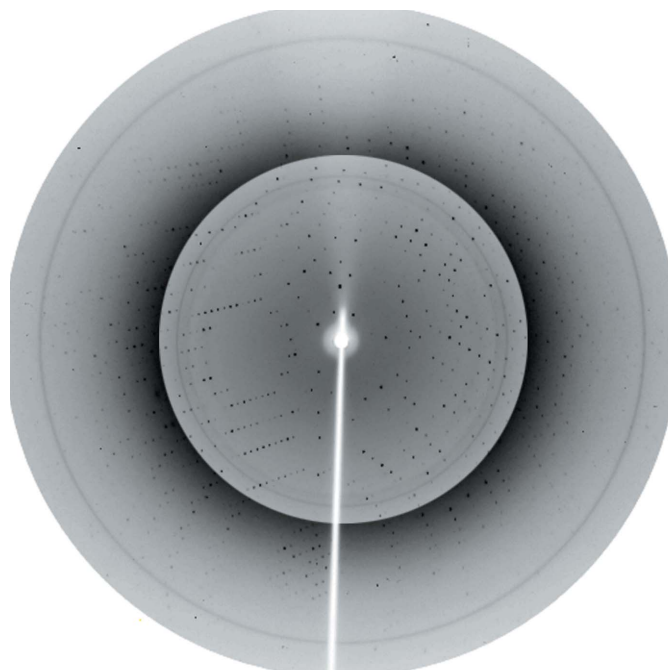Photomicrograph of a zhaoermiatoxin crystal.

Figure 2
X-ray diffraction pattern of zhaoermiatoxin crystals with a different contrast level at low and high resolution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Source	Synchrotron radiation
Temperature (K)	100
Detector	MAR CCD
Cryoprotectant	10% glycerol
Data-processing software	*DENZO/SCALEPACK*
Data collection and processing	
Wavelength (Å)	1.427
Space group	$P6_4$
Unit-cell parameters (Å)	$a = 72.9$, $b = 72.9$, $c = 93.9$
Matthews coefficient ($\text{Å}^3\,\text{Da}^{-1}$)	2.44
Solvent content (%)	49.25
No. of molecules in the asymmetric unit	2
No. of unique reflections	17842
No. of observed reflections	305151
Redundancy	12.5 (9.5)
Resolution range (Å)	25.0–2.05 (2.12–2.05)
Completeness (%)	99.9 (99.8)
R_{merge}† (%)	7.1 (33.3)
$\langle I/\sigma(I)\rangle$	33.4 (7)
Wilson B factor (Å^2)	39.1

† $R_{\text{merge}} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

2.44 $\text{Å}^3\,\text{Da}^{-1}$ and a solvent content of 49.25% (Matthews, 1968). Data-processing statistics are presented in Table 1.

The atomic coordinates of the Lys49 PLA_2 from *Bothrops asper* venom, which displays a sequence identity of 70%, were used to generate a search model (Murakami *et al.*, 2005; PDB code 1y4l) and molecular-replacement calculations were carried out using the program *AMoRe* in the resolution range 25.0–4.0 Å (Navaza, 1994). A clear solution was obtained for two molecules in the asymmetric unit in space group $P6_4$. *REFMAC*5 (Murshudov *et al.*, 1997) was used for rigid-body refinement of this solution in the resolution range 25.0–3.0 Å (excluding 5% of reflections for R_{free} calculations), resulting in a correlation coefficient of 60.3% and an R factor of 40.1% ($R_{\text{free}} = 48.3$%). Structure refinement and analysis are currently in progress.

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