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Crystallization and preliminary X-ray diffraction studies of piratoxin III, a D-49 phospholipase A_2 from the venom of *Bothrops pirajai*

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Piratoxin III (PrTX-III) is a phospholipase A_2 (PLA_2, E.C. 3.1.1.4, phosphatide *sn*-2 acylhydrolase) isolated from *Bothrops pirajai*. Crystals of PrTX-III were obtained using the vapour-diffusion technique and X-ray diffraction data have been collected to 2.7 Å resolution. The enzyme was crystallized in the space group *C*2 with unit-cell parameters $a = 60.88$, $b = 100.75$, $c = 48.19$ Å, $\beta = 123.89^\circ$. A molecular-replacement solution of the structure has been found using bothropstoxin I from the venom of *B. jararacussu* as a search model.

1. Introduction

The genus *Bothrops* comprises several species which are widely distributed in South and North America. Among the bioactive proteins from *Bothrops* venoms, phospholipases A_2 appear as a major component. Phospholipases A_2 (PLA_2, E.C. 3.1.1.4) are calcium-dependent enzymes which are responsible for the cleavage of the *sn*-2 ester bond of phospholipids (Deenen & de Haas, 1963). They are found in most animal tissues, mainly in the pancreatic juices of mammals and the venoms of snakes and insects. The PLA_2 enzymes are believed to participate in cellular functions and cell signalling (Kudo *et al.*, 1993), as well as in the formation of several important metabolic precursors (*e.g.* inflammatory response mediators) derived from cleavage of the phospholipids (Siraganian, 1988). These enzymes are classified into four groups, according to their extracellular or intracellular origin, primary structure and disulfide-bond pattern (Heinrikson, 1990; Dennis, 1994).

Class II PLA_2s constitute a large part of the venom of many species of snake, such as rattlesnakes and vipers (Mebs & Samejima, 1986). The enzymes belonging to this class can be subdivided into two distinct groups, according to enzymatic activity: the inactive group and the active group (Maraganore *et al.*, 1984). The enzymatically inactive PLA_2s (K-49 PLA_2, PLA_2-like myotoxins) are PLA_2s which exhibit low or no phospholipid cleavage activity. This is thought to arise from a substitution of the aspartate residue at position 49, the side chain of which is important in the binding of calcium ions (an essential cofactor), by a lysine residue (Holland *et al.*, 1990; Scott *et al.*, 1992; Arni *et al.*, 1995). However, despite the low or lack of enzymatic activity, K-49 PLA_2s exhibits several different pharmacological activities, such as post-synaptic neurotoxicity, oedema formation (Gutiérrez & Lomonte, 1995), myotoxicity (Lomonte *et al.*, 1994) and liposome and membrane disruption (Días *et al.*, 1991; Ruffini *et al.*, 1992). The enzymatically active PLA_2s (D-49 PLA_2s) hydrolyse the *sn*-2 ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides. The D-49 PLA_2s require calcium ion and are almost all at least 100 times more active when the substrate is condensed in micelles (Pieterson *et al.*, 1974) or lamellar aggregates such as monolayers, vesicles and membranes (Slotboom *et al.*, 1981).

In this work, we present the crystallization and preliminary diffraction data of piratoxin III (PrTX-III) myotoxin. PrTX-III is a D-49 PLA_2 with moderate PLA_2 activity, myotoxic and anticoagulant activity. It was isolated from *B. pirajai* snake venom (Toyama *et al.*, 1998) which was a kind gift from CEPIAC/CEPEC (Ministry of Agriculture, Bahia, Brazil). The enzymatically inactive K-49 PLA_2 piratoxin II (PrTX-II) from *B. pirajai* has previously been crystallized in our group and is currently in the process of structural refinement (Lee *et al.*, 1998). Comparison of two (active and inactive) phospholipases from the same organism will provide us with more information on the structural differences and shed light on the activity of these proteins.

2. Protein purification

PrTX-III was isolated and purified from the whole venom of *B. pirajai* by reverse-phase and cation-exchange HPLC. 20 mg of whole venom were dissolved in 250 µl of 0.1%(*v*/*v*) trifluoroacetic acid. The resulting sample was centrifuged and the supernatant was applied to a 0.78 × 30 cm u-Bondapack C-18 column (Waters 991 PDA system).

The purification of the venom was performed with a linear gradient of 0–66%(*v*/*v*) acetonitrile in 0.1%(*v*/*v*) trifluoroacetic acid at a flow rate of 2.0 ml min^{-1}

Table 1
Crystal data and data-collection statistics.

Crystal data	
Space group	*C*2
Unit-cell parameters (Å, °)	a = 60.88, b = 100.75, c = 48.19, β = 123.89
Data collection	
Resolution (Å)	2.70
Last resolution shell (Å)	2.79–2.70
Number of observations	15881
Number of unique reflections	5514
$R_{\rm merge}$ (%)	11.4
Last resolution shell (%)	26.4
Completeness (%)	84.5
Last resolution shell (%)	81.2

and was monitored at 280 nm. The PrTX-III fraction was lyophilized and dissolved in 0.05 *M* ammonium bicarbonate pH 7.4, centrifuged and applied to a 0.39 × 7.8 cm Protein-Pack SP 5PW cation-exchange column, which had previously been equilibrated with the same buffer. Elution of PrTX-III was performed using a 0.05–1.0 *M* ammonium bicarbonate (pH 7.4) linear gradient. The chromatographic run was performed at a flow rate of 1.0 ml min^{-1} and was monitored at 280 nm. The purified sample was lyophilized and used for crystallization trials.

3. Crystallization and data collection

Preliminary screening of the crystallization conditions was performed using a sparse-matrix screen at 291 K (Crystal Screens I and II, Hampton Research). Lyophilized PrTX-III was initially dissolved to a concentration of 10 mg ml^{-1} in water and used in the screening procedure. Small crystals were found in condition number 40 of the Crystal Screen I kit (20% 2-propanol, 20% PEG 4000, 0.1 *M* sodium citrate pH 5.6). A search for refined crystallization conditions was then performed. New crystals were grown at 291 K using the hanging-drop vapour-diffusion technique by mixing equal volumes (1 µl) of a protein solution concentrated to 5 mg ml^{-1} with a reservoir solution which contained 19% 2-propanol, 20% PEG 4000 and 0.1 *M* sodium citrate pH 5.5. Plate-like crystals measuring 0.1 × 0.1 × 0.02 mm appeared in 10–15 d.

X-ray diffraction data were collected at the protein crystallography beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) at the Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. The images were recorded using a MAR 345 image plate and synchrotron radiation of wavelength 1.38 Å. 110 oscillation images were collected corresponding to a total rotation of 132°. The collected images were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski, 1993). The crystals belong to the space group *C*2 with unit-cell parameters a = 60.88, b = 100.75, c = 48.19 Å, β = 123.89°. Data-set statistics are given in Table 1.

Calculations using the Matthews coefficient (Matthews, 1968) suggested the presence of two molecules per asymmetric unit (V_m = 2.84 Å^3 Da^{-1}). The crystal structure of PrTX-III was solved by the molecular-replacement method using the program *AMoRe* (Navaza, 1994). Several molecular-replacement search models were tested. The best solution was found with the most homologous PLA_2 available in the main databases (all non-redundant GenBank CDS translations, PDB, Swissprot, PIR and PRF), bothropstoxin I from *B. jararacussu*, which displayed 65% primary sequence identity with PrTX-III. The atomic coordinate file of bothropstoxin I was kindly provided by the authors (Da Silva-Giotto *et al.*, 1998). The two most significant rotation-search solutions [correlation coefficients (CC) of 23.5 and 21.9%] were used for the translation search using reflections in the resolution range 10–2.7 Å. The best solution of the translation search (CC = 43.1%; R factor = 49.2%) was subjected to ten cycles of rigid-body refinement against all data between 10 and 2.7 Å resolution (fitting function of *AMoRe*). The fitting yielded a solution with a correlation coefficient of 49.8% and an R factor of 48.1%. The crystal packing was inspected using the program *O* (Jones & Kjeldgaard, 1993) and did not show any crystallographic or non-crystallographic clashes. Initial refinement steps were performed using the maximum-likelihood method as implemented in the program *REFMAC* (Murshudov *et al.*, 1997). At present, the R factor of the model is 31.3% and $R_{\rm free}$ is 38.6%. Further model-building and refinement steps are under way.

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