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Preliminary X-ray crystallographic studies of BthTX-II, a myotoxic Asp49-phospholipase A_2 with low catalytic activity from *Bothrops jararacussu* venom

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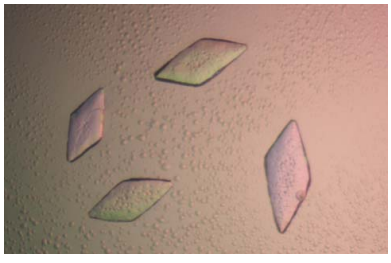

For the first time, a complete X-ray diffraction data set has been collected from a myotoxic Asp49-phospholipase A_2 (Asp49-PLA_2) with low catalytic activity (BthTX-II from *Bothrops jararacussu* venom) and a molecular-replacement solution has been obtained with a dimer in the asymmetric unit. The quaternary structure of BthTX-II resembles the myotoxin Asp49-PLA_2 PrTX-III (piratoxin III from *B. pirajai* venom) and all non-catalytic and myotoxic dimeric Lys49-PLA_2s. In contrast, the oligomeric structure of BthTX-II is different from the highly catalytic and non-myotoxic BthA-I (acidic PLA_2 from *B. jararacussu*). Thus, comparison between these structures should add insight into the catalytic and myotoxic activities of bothropic PLA_2s.

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) belong to a superfamily of proteins which hydrolyze the *sn*-2 acyl groups of membrane phospholipids to release fatty acids, arachidonic acid and lysophospholipids (van Deenen & de Haas, 1963). The coordination of the Ca^{2+} ion in the PLA_2 calcium-binding loop includes an Asp at position 49 which plays a crucial role in the stabilization of the tetrahedral transition-state intermediate in catalytically active PLA_2s (Scott *et al.*, 1992). In the genus *Bothrops*, PLA_2s are the main components of the venoms produced by species classified into this animal group. In addition to their primary catalytic role, snake-venom PLA_2s show other important toxic/pharmacological effects, including myonecrosis, neurotoxicity, cardiotoxicity and haemolytic, haemorrhagic, hypotensive, anticoagulant, platelet-aggregation inhibition and oedema-inducing activities (Gutiérrez & Lomonte, 1997; Ownby, 1998; Andrião-Escarso *et al.*, 2002). Some of these activities are correlated with the enzymatic activity, but others are completely independent (Kini & Evans, 1989; Soares & Giglio, 2004). It has been suggested that some specific sites of these molecules have biochemical properties that are responsible for the pharmacological and toxic actions, including the anticoagulant and platelet-inhibition activities (Kini & Evans, 1989). PLA_2s are also one of the enzymes involved in the production of eicosanoids. These molecules have physiological effects at very low concentrations; however, increases in their concentration can lead to inflammation (Needleman *et al.*, 1986). Thus, the study of specific PLA_2 inhibitors is important in the production of structure-based anti-inflammatory agents.

Many non-catalytic homologous PLA_2s (Lys49-PLA_2s) have been purified from *Bothrops* snake venoms and have been structurally and functionally characterized (Marchi-Salvador *et al.*, 2005, 2006; Watanabe *et al.*, 2005; Soares *et al.*, 2004; Magro *et al.*, 2003; Lee *et al.*, 2001; Arni *et al.*, 1995, 1999; da Silva-Giotto *et al.*, 1998). However, little is known about the bothropic catalytic PLA_2s (Asp49-PLA_2s; Magro *et al.*, 2004, 2005; Rigden *et al.*, 2003; Serrano *et al.*, 1999; Pereira *et al.*, 1998; Daniele *et al.*, 1995; Homsi-Brandeburgo *et al.*, 1988).

Despite the structures of a large number of PLA_2s having been solved by crystallography to date, many questions still need to be clarified. For example, there are PLA_2s with high, moderate and no catalytic activity (Magro *et al.*, 2004; Rigden *et al.*, 2003; da Silva-Giotto *et al.*, 1998). However, for all these 'classes' of PLA_2s the

majority of residues of the catalytic machinery are conserved. Similarly, toxic (*e.g.* myotoxity, cytotoxity) and pharmacological effects (*e.g.* anticoagulant, hypotensive and platelet-aggregation activities) are far from being completely understood.

An acidic catalytic PLA_2 (BthA-I) has been isolated from *B. jararacussu* venom and characterized (Andrião-Escarso *et al.*, 2002; Roberto *et al.*, 2004). BthA-I is three to four times more catalytically active than BthTX-II (bothropstoxin-II from *B. jararacussu*) and other basic Asp49-PLA_2s from *Bothrops* venoms, but is not myotoxic, cytotoxic or lethal (Andrião-Escarso *et al.*, 2002). Other activities demonstrated by this enzyme are time-independent oedema induction, hypotensive response in rats and platelet-aggregation inhibition (Andrião-Escarso *et al.*, 2002). The crystal structure of BthA-I has been recently described in two conformational states: monomeric and dimeric (Magro *et al.*, 2004). Additionally, Magro *et al.* (2005) solved the structure of BthA-I chemically modified with BPB (*p*-bromophenacyl bromide) and showed important tertiary and quaternary structural changes in this enzyme. This novel oligomeric structure is more energetically and conformationally stable than the native structure and the abolition of pharmacological activities (including anticoagulant, hypotensive effect and platelet-aggregation inhibition) by the ligand may be related to the oligomeric structural changes.

The isolation, biochemical/pharmacological characterization and amino-acid sequence of bothropstoxin II from *B. jararacussu* (BthTX-II) have been reported (Homsi-Brandeburgo *et al.*, 1988; Gutiérrez *et al.*, 1991; Pereira *et al.*, 1998). Protein sequencing indicated that BthTX-II is an Asp49-PLA_2 and consists of 120 amino acids (MW = 13 976 Da). The protein shows myotoxic, oedematogenic and haemolytic effects and low phospholipase activity (Homsi-Brandeburgo *et al.*, 1988; Gutiérrez *et al.*, 1991). Recently, it has been shown that BthTX-II induces platelet aggregation and secretion through multiple signal transduction pathways (Fuly *et al.*, 2004). Despite BthTX-II having been crystallized more than ten years ago (Bortoleto *et al.*, 1996), the structure has not been solved to date, probably owing to the low completeness of the data set (50–60% completeness). The crystals belonged to the tetragonal crystal system and preliminary analysis indicated the presence of three molecules in the asymmetric unit (Bortoleto *et al.*, 1996). However, a careful analysis of the Matthews coefficient indicated that a tetrameric conformation is also possible (V_M = 2.4 Å^3 Da^{-1}), which also occurs in the Lys49-PLA_2 M_jTX-I (myotoxin I from *B. moojeni* venom) structure formed of two Lys49-PLA_2 dimers (Marchi-Salvador *et al.*, 2005; personal communication).

In the present paper, we describe the crystallization of BthTX-II (bothropstoxin-II) from *B. jararacussu* venom in the monoclinic system, the collection of a complete X-ray diffraction data set and molecular-replacement solution. This study should improve the understanding of the relation of the myotoxic and low catalytic activity mechanisms to the structural features of this protein when compared with BthTX-I (Lys49-PLA_2 from *B. jararacussu* venom) and BthA-I, which possess no and high catalytic activity, respectively.

2. Experimental procedures

2.1. Purification

BthTX-II was isolated from *B. jararacussu* snake venom by gel-filtration and ion-exchange chromatography as previously described (Homsi-Brandeburgo *et al.*, 1988).

2.2. Crystallization

A lyophilized sample of BthTX-II was dissolved in ultrapure water at a concentration of 12 mg ml^{-1}. The sparse-matrix method (Jancarik & Kim, 1991) was used to perform initial screening of the crystallization conditions (Crystal Screens I and II; Hampton Research). Large crystals of BthTX-II were obtained by the conventional hanging-drop vapour-diffusion method (MacPherson, 1982), in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against 500 µl of the same precipitant solution. The BthTX-II was crystallized using a solution containing 20%(*v*/*v*) 2-propanol, 13%(*w*/*v*) polyethylene glycol 4000 and 0.1 *M* sodium citrate pH 5.6. The best crystals measured approximately 0.4 × 0.2 × 0.1 mm after two months at 291 K (Fig. 1).

2.3. X-ray data collection and processing

X-ray diffraction data from BthTX-II crystals were collected using a wavelength of 1.427 Å at a synchrotron-radiation source (Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) with a MAR CCD imaging-plate detector (MAR Research). A crystal was mounted in a nylon loop and flash-cooled in a stream of nitrogen at 100 K using no cryoprotectant. The crystal-to-detector distance was 100 mm and an oscillation range of 1° was used; 149 images were collected. The data were processed to 2.13 Å resolution using the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

The data-collection statistics are shown in Table 1. The data set is 96.1% complete at 2.13 Å resolution, with R_{merge} = 9.1%. The crystals belong to space group *C*2, with unit-cell parameters *a* = 58.9, *b* = 98.5, *c* = 46.7 Å, β = 125.9°.

Packing parameter calculations based on the protein molecular weight indicate the presence of a dimer in the asymmetric unit. This corresponds to a Matthews coefficient (Matthews, 1968) of 2.0 Å^3 Da^{-1} with a calculated solvent content of 37.4%, which are within the expected range for typical protein crystals (assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume).

The crystal structure was determined by molecular-replacement techniques implemented in the program *AMoRe* (Navaza, 1994) using the coordinates of a monomer of PrTX-III (PDB code 1gmz). The quaternary structure of BthTX-II is very similar to those of PrTX-III and all dimeric bothropic Lys49-PLA_2s (Rigden *et al.*, 2003; Soares *et al.*, 2004) and totally different from those of native dimeric

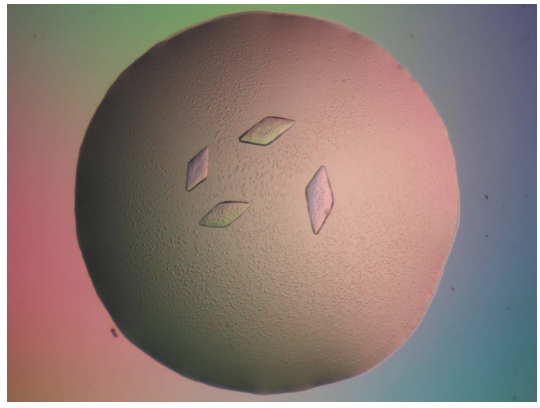

Figure 1
Crystals of BthTX-II from *B. jararacussu* venom

Table 1
X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Unit-cell parameters (Å, °)	a = 58.9, b = 98.5, c = 46.7, β = 125.9
Space group	$C2$
Resolution (Å)	40–2.13 (2.21–2.13)
Unique reflections	11560 (1127)
$R_{\rm merge}$† (%)	9.1 (26.4)
Completeness (%)	96.1 (94.2)
Radiation source	Synchrotron (LNLS-MX1)
Data-collection temperature (K)	100
$\sigma(I)$ cutoff for data processing‡	−3
Average $I/\sigma(I)$	10.6 (3.7)
Redundancy	3.0 (2.9)
Matthews coefficient $V_{\rm M}$ (Å^3 Da^{-1})	2.0
Molecules in the ASU	2
Solvent content (%)	37.4

† $R_{\rm merge} = \sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection. Calculated for $I > -3\sigma(I)$. ‡ Data processing used the *HKL* suite (Otwinowski & Minor, 1997).

BthA-I and BthA-I–bromophenacyl bromide and BthA-I–α-tocopherol complexes (Magro *et al.*, 2004, 2005; Takeda *et al.*, 2004).

In conclusion, a complete X-ray diffraction data set has been collected from a low catalytic activity Asp49-PLA$_2$ for the first time (to 2.13 Å) and a molecular-replacement structure solution has been obtained. The quaternary structure of BthTX-II resembles those of the myotoxin PrTX-III (which does not bind Ca^{2+} ions) and all non-catalytic and myotoxic dimeric Lys49-PLA$_2$s (Rigden *et al.*, 2003; Soares *et al.*, 2004). In contrast, the oligomeric structure of BthTX-II is different from that of the high catalytic activity and non-myotoxic BthA-I (Magro *et al.*, 2004). Thus, comparison between these structures should add insight into the catalytic and myotoxic activities of bothropic PLA$_2$s.

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