Crystallization and preliminary X-ray diffraction analysis of three myotoxic phospholipases A\(_2\) from Bothrops brazili venom

Two myotoxic and noncatalytic Lys49-phospholipases A\(_2\) (braziliantoxin-II and MT-II) and a myotoxic and catalytic phospholipase A\(_2\) (braziliantoxin-III) from the venom of the Amazonian snake Bothrops brazili were crystallized. The crystals diffracted to resolutions in the range 2.56–2.05 Å and belonged to space groups P\(_3\)\(_1\)2\(_1\) (braziliantoxin-II), P\(_6\)\(_{3}\)2\(_2\) (braziliantoxin-III) and P\(_2\)\(_1\) (MT-II). The structures were solved by molecular-replacement techniques. Both of the Lys49-phospholipases A\(_2\) (braziliantoxin-II and MT-II) contained a dimer in the asymmetric unit, while the Asp49-phospholipase A\(_2\) braziantoxin-III contained a monomer in its asymmetric unit. Analysis of the quaternary assemblies of the braziliantoxin-II and MT-II structures using the PISA program indicated that both models have a dimeric conformation in solution. The same analysis of the braziantoxin-III structure indicated that this protein does not dimerize in solution and probably acts as a monomer in vivo, similar to other snake-venom Asp49-phospholipases A\(_2\).

1. Introduction

Envenomation resulting from snakebites is an important public health problem in rural areas of Asia, Africa and Latin America. A recent study estimated that at least 421 000 envenomations and 20 000 deaths from ophidian accidents occur each year (Kasturiratne et al., 2008). However, owing to scarce statistical data on this topic, these numbers may be as high as 1 841 000 envenomations and 94 000 deaths (Kasturiratne et al., 2008). The mortality caused by snake bites is much greater than those caused by several neglected tropical diseases, including dengue haemorrhagic fever, cholera, leishmaniasis, schistosomiasis and Chagas disease (Williams et al., 2010). Only in recent years has this subject attracted massive attention from the scientific community, with the publication of important articles and reviews on the real impact of snakebites on health services (Gutiérrez et al., 2006; Kasturiratne et al., 2008; Williams et al., 2010). The World Health Organization (WHO) has recognized snakebites as a neglected tropical disease and the International Society on ToxinoLOGY has created The Global Snakebite Initiative (Williams et al., 2010). Both initiatives aim to reduce snakebite morbidity and mortality through a programme of sustainable approaches and outcome-oriented strategies. Although the majority of deaths owing to snakebite envenomation occur in south and south-east Asia and in sub-Saharan Africa (Kasturiratne et al., 2008), these accidents are also an important health problem in Latin America (Gutiérrez & Lomonte, 1995). They may result in drastic tissue damage and permanent disability (Gutiérrez & Lomonte, 1995), leading to economic and social problems. Snakes of the Bothrops genus (Viperidae family) are responsible for more than 85% of all reported ophidian accidents in Latin America (Fundação Nacional de Saúde, 2001; de Oliveira, 2009), and phospholipases A\(_2\) (PLA\(_2\)s) are one of the main components of their venoms (Fox & Serrano, 2008). These enzymes promote Ca\(^{2+}\)-dependent hydrolysis of the sn-2 acyl groups of membrane phospholipids, releasing fatty acids and lysophospholipids (Schaloske & Dennis, 2006). In addition to their catalytic activity, these proteins are involved in a wide spectrum of pharmacological activities, including neurotoxicity, myotoxicity and cardio-toxicity (Bon et al., 1979; Fletcher et al., 1981; Gutiérrez et al., 1991).
The catalytic site of these proteins is formed by a histidine at position 48 (according to the numbering system proposed by Renetseder et al., 1985) and the conserved residues Asp49, Tyr52 and Asp99. Two catalytic mechanisms of phospholipid hydrolysis by PLAs have been proposed: the single-water mechanism (Scott, Otwinowski et al., 1990; Scott, White et al., 1990) and the assisting-water mechanism (Rogers et al., 1996; Yu et al., 1998). Briefly, the single-water mechanism proposes that after phospholipid binding His48 N(ι) abstracts a proton from a structurally conserved water, initiating nucleophilic attack on the sn-2 position of the substrate and forming an intermediate tetrahedral oxyanion which is stabilized by the Ca(2+) cofactor (Scott, Otwinowski et al., 1990; Scott, White et al., 1990). The assisting-water mechanism suggests that two different water molecules are involved in the formation and breakdown of the tetrahedral intermediate. In this model, a water molecule coordinated by the Ca(2+) ion performs the nucleophilic attack. This catalytic water is also stabilized by a second water molecule that is hydrogen bonded to His48 and Asp99 (Rogers et al., 1996; Yu et al., 1998).

In snakes of the Viperidae family, an important subgroup of PLAs, the Lys49-PLAs, are found that exhibit natural replacements of the Tyr28 and Asp49 residues by Asn28 and Lys49, respectively (Holland et al., 1990; Fernandes et al., 2010). These substitutions hinder binding of the Ca(2+) ion, the essential cofactor for PLA2 catalysis, resulting in an inability to promote phospholipid hydrolysis (Arni & Ward, 1996; Fernandes et al., 2010). Despite their catalytic inactivity, Lys49-PLAs play an important role in ophic accidents, inducing drastic local myonecrosis by a Ca(2+)-independent mechanism which is not efficiently neutralized by conventional serum therapy, the action of which is related to systemic mechanisms (Gutiérrez & Lomonte, 1995). Synthetic peptides and site-directed mutagenesis experiments have shown that the 115–129 segment of the C-terminal region is responsible for this myotoxic activity (Ward et al., 2002; Lomonte et al., 2003; Chioato et al., 2007). Recently, a myotoxic site in Lys49-PLAs that is specific to venoms from snakes of the Bothrops genus has been proposed that contains three residues: Lys115, Arg118 (C-terminus) and Lys20 (N-terminus) (dos Santos, Soares et al., 2009).

B. brazili is a snake that lives in the Amazonian region, being found in Brazil, Colombia, Ecuador, Guyana, Peru, Suriname and French Guiana (Campbell & Lamar, 2004). Access to antivenoms in the remote areas of this region is very limited owing to natural geographic barriers and the vast territory. Moreover, the commercial therapeutic anti-bothropic serum produced by the Butantan Institute (Brazil) has a low efficacy against Amazonian snakes (Muniz et al., 2000). Despite the large amount of protein structural data available for snakes of the Bothrops genus (Magro et al., 2004; Murakami et al., 2007; dos Santos, Fernandes et al., 2009; Fernandes et al., 2010; dos Santos et al., 2011), no structural studies related to Amazonian snakes have been reported to date.

In this work, we report the crystallization, X-ray diffraction data collection and molecular-replacement solution of three myotoxic phospholipases A2 (the Asp49-PLA2 braziliantoxin-III and the Lys49-PLA2 braziliantoxin-II and MT-II) from the venom of the Amazonian snake B. brazili.

2. Materials and methods

2.1. Protein purification and crystallization

Braziliantoxin II (BbTX-II) and braziliantoxin-III (BbTX-III) were isolated from B. brazili venom by single-step reverse-phase HPLC as described previously (Huancahuire-Vega et al., 2009). MT-II was obtained by the fractionation of B. brazili venom on a CM-Sepharose column (2 x 20 cm) as described previously (Costa et al., 2008). Lyophilized samples of BbTX-III and MT-II were dissolved in ultrapure water to a concentration of 12 mg ml⁻¹. A lyophilized sample of BbTX-II was dissolved in 300 mM phosphate buffer to the same concentration. The sparse-matrix method (Jancarik & Kim, 2003), in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against the following reservoir solutions (500 µl): 30%(w/v) polyethylene glycol 4000, 0.25 M lithium sulfate, 0.1 M Tris–HCl pH 8.5 for BbTX-II, 2%(w/v) polyethylene glycol 400, 2.0 M ammonium sulfate, 0.1 M Na HEPES.
Table 1
X-ray diffraction data-collection and processing statistics.
Values in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
<th></th>
<th>BbTX-II</th>
<th>BbTX-III</th>
<th>MT-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit-cell parameters (Å, °)</td>
<td>a = b = 56.43, c = 129.08</td>
<td>a = b = 70.818, c = 105.83</td>
<td>a = b = 71.41, c = 44.42</td>
</tr>
<tr>
<td>Space group</td>
<td>P3121</td>
<td>P622</td>
<td>P21</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>40–2.11</td>
<td>50–2.70</td>
<td>20–2.08</td>
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<tr>
<td>Unique reflections</td>
<td>13825 (1381)</td>
<td>4562 (443)</td>
<td>13821 (1396)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>95.9 (98.7)</td>
<td>97.2 (99.1)</td>
<td>96.4 (97.5)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>6.3 (49.0)</td>
<td>13.4 (48.4)</td>
<td>12.9 (35.3)</td>
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<tr>
<td>Radiation source</td>
<td>MX1 station, LNLS</td>
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<td></td>
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<tr>
<td>Data-collection temperature (K)</td>
<td>100</td>
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<td></td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>95.9 (98.7)</td>
<td>97.2 (99.1)</td>
<td>96.4 (97.5)</td>
</tr>
<tr>
<td>Matthews coefficient (Å³ Da⁻¹)</td>
<td>0.74 cm³ g⁻¹</td>
<td></td>
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<tr>
<td>pH</td>
<td>7.5 for BbTX-III and 30%w/v polyethylene glycol 8000, 0.25 M ammonium sulfate, 0.1 M sodium cacodylate pH 6.5 for MT-II. Crystals were grown at 291 K for approximately three weeks for MT-II and BbTX-II and for three months for BbTX-III (Fig. 1).</td>
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2.2. X-ray data collection and processing
X-ray diffraction data from all crystals were collected at a wavelength of 1.435 Å using a synchrotron-radiation source (MX1 station, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) and a MAR CCD imaging-plate detector (MAR Research). The crystals were mounted in a nylon loop and flash-cooled in a stream of nitrogen gas at 100 K without using a cryoprotectant. The data were processed using the HKL program package (Otwinowski & Minor, 1997).

3. Results and discussion
Data-collection statistics are shown in Table 1. The crystals diffracted to resolutions in the range 2.56–2.05 Å and belonged to space groups P3121 (BbTX-II), P622 (BbTX-III) and P21 (MT-II). The crystal structures were determined by molecular-replacement techniques as implemented in the program MOLREP (Vagin & Teplyakov, 2010) using the coordinates of piratoxin-I (PrTX-I; PDB entry 2q2j; dos Eaker & Eklund, 1998), MT-II and BbTX-II and for three months for BbTX-III (Fig. 1).

Calculations based on the protein molecular weight indicated the presence of one molecule of BbTX-III and two molecules of BbTX-II and MT-II in the asymmetric unit, corresponding to Matthews coefficients (Vₘ; Matthews, 1968) of 2.12 Å³ Da⁻¹ for BbTX-II, 2.74 Å³ Da⁻¹ for BbTX-III and 2.16 Å³ Da⁻¹ for MT-II. These values are within the range for typical protein crystals, assuming a value of 0.74 cm³ g⁻¹ for the protein partial specific volume. Analysis of the quaternary assemblies of the BbTX-II and MT-II crystallographic models using the PISA program (Krissinel & Henrick, 2007) showed a complexation significance score of 1.0, indicating that both models present a dimeric conformation in solution. The analysis of the BbTX-III structure indicated that this protein does not dimerize in solution and probably acts as a monomer in vivo, similar to other snake-venom Asp49-PLA₂s (Carredano et al., 1998; Xu et al., 2003; Murakami et al., 2006).

In conclusion, BbTX-II, BbTX-III and MT-II from B. brazili were crystallized and X-ray diffraction data were collected. The structures of the Lys49-PLA₂s BbTX-II and MT-II showed a dimeric conformation, while the Asp49-PLA₂, BbTX-III presented a monomeric conformation. Elucidation of the native structures and of structures of possible complexes with different ligands may be useful for the development of effective inhibitors that can be used as supplemental treatments to serum therapy and as important models for synthesis of new drugs.

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References


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