Crystallization and Preliminary X-Ray Crystallographic Studies of a Myotoxic Lys49-phospholipase A_2 from *Bothrops jararacussu* Venom Complexed with α-Tocopherol Inhibitor

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Abstract: Bothropstoxin I (BthTX-I), a non-catalytic and myotoxic Lys49-PLA_2 from *Bothrops jararacussu* venom, has been crystallized alone and complexed with α-tocopherol inhibitor. These crystals have been shown to diffract X-rays between 2.17 and 1.83 Å resolution. The BthTX-I/α-tocopherol complex crystals are not isomorphous with those of the native protein. This suggests the inhibitor binding has lead to changes in the quaternary structure and a different conformation may have been obtained.

Keywords: Crystallization, X-ray crystallography, Lys49-phospholipase A_2, *Bothrops jararacussu* venom, myotoxity, α-tocopherol, vitamin E.

INTRODUCTION

Among Latin America venomous snakes, those of the *Bothrops* genus are responsible for approximately 90% of ophidian accidents (Ferreira T 1992)[1] (Ribeiro RAMB 1998) [2]. These envenomations are characterized by prominent local tissue damage due to myonecrosis, hemorrhage, and edema (Rosenberg HT 1990) [3]. Phospholipases A_2 (PLA_2s, EC 3.1.1.4) are the main components of these venoms and, in addition to their catalytic role; they show a wide variety of pharmacological activities, such as neurotoxicity, myotoxicity and cardiotoxicity. They also affect coagulation cascade, platelet aggregation and the inflammatory response (Kini PACMPP 1997) [4] (Andrião-Escarso BP 2002) [5]. Some of these activities are correlated to the enzymatic activity and others are completely independent (Kini T 1989) [6] (Soares T 2004) [7].

Myotoxic PLA2s are widely distributed among venomous snakes and they can be subdivided into, at least, three subclasses: (a) the Asp49 enzymes with high catalytic activity, (b) the Ser49 enzymes with lower catalytic activity and (c) the Lys49 enzymes with very limited or not measurable catalytic activity (Shimohigashi JB 1995) [8] (Ownby T 1999) [9]. The most abundant protein in Viperidae venoms is a natural mutant where the Asp49 was changed to Lys (subclass c). This natural mutation prevents calcium binding and the protein lacks catalytic activity. However, this Lys49-PLA_2s are capable of destabilizing the integrity of membranes and provoke release from liposomes (Rufini B 1992) [10].

Several crystal structures of myotoxic Lys49-PLA_2s from *Bothrops* genus have been solved (Arni AC 1995) [11] (de Azevedo T 1998) [12] (da Silva-Giotto PSFG 1998) [13] (Arni ABB 1999) [14] (Lee B 2001) [15] (Magro BBRC 2003) [16] (Watanabe B 2005) [17] (Marchi-Salvador ACF 2005) [18] (Marchi-Salvador ACF 2006) [19], however the structural bases of toxicological effects seems to be difficult to interpret just by analysis of native Lys49-PLA_2s structures. Recently, MjTX-II has been co-crystallized with stearic acid and the crystal structure was solved demonstrating that the Lys122 is fundamental to keep the fatty acid in the active site (Watanabe B 2005) [17]. It has been observed by site-direct mutagenesis in the Lys49-PLA_2 BthTX-I that the Lys122→Ala mutation alters both myotoxic and Ca^+ independent membrane damage activities (Chioato BJ 2002) [20]. Therefore, detailed studies with other Lys49-PLA_2s and different ligands seems to be fundamental for understanding the role of C-terminal region, particularly involving the Lys122 residue.

PLA_2s are also one of the enzymes involved in the production of eicosanoids. These molecules have physiological effects at very low concentrations; however at high concentrations they can lead to the inflammatory state (Needleman RB 1986) [21]. Then, the study of specific PLA_2s inhibitors can be important in the production of structure-based anti-inflammatory agents. The α-tocopherol (vitamin E) is involved in the regulation of the production of eicosanoids by inhibition of both PLA_2 (Pentland BC 1992) [22] and cyclooxygenase activities (Abate FRBM 2000) [23]. Vitamin E has been used to slow the progression of Alzheimer´s and Parkinson´s diseases (Ebadi PN 1996) [24] (Sano NEJM 1997) [25]. It has been considered that vitamin E acts by inhibiting PLA_2 activity and stabilizing neural membranes and synapses. Then, it is very important to know the exact way the interaction of this inhibitor with PLA_2s occurs. The first attempt with this goal was the structural study of the complex formed by a dimeric PLA_2 from *Daboia pulchella* and α-tocopherol (Chandra JMB 2002) [26]. In this structure it was observed α-tocopherol bound to the catalytic site for

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Fig. (1). (**A**) Crystal of BthTX-I from *B. jararacussu* (**B**) Crystal of BthTX-I/α-tocopherol complex from *B. jararacussu.*

only one monomer while the other was inaccessible due the conformation of Trp31 residue. Kinetics and inhibition studies showed to be in agreement with the crystallographic results (Chandra JMB 2002) [26]. Recently, we performed studies with the BthTX-I and the Lys49-PLA_2 PrTX-I from *Bothrops pirajai* in the presence of α-tocopherol and observed myotoxic activity was also reduced to approximately 50 % (data not shown).

The crystal structure of native Bothropstoxin I (BthTX-I) has been solved (da Silva-Giotto PSFG 1998) [13], however, the coordinates are not available in the RCSB Protein Data Bank in order to perform structural comparative studies with different complexes.

In the present paper, we describe the crystallization and X-ray diffraction data collection of BthTX-I from *Bothrops jararacussu* venom in the native form and complexed with α-tocopherol (αT) obtained in the same crystallization conditions. This study can add insights into the myotoxic, cytotoxic and other pharmacological activities related to the structural changes of this protein, particularly involving the C-terminal and "active site" regions.

MATERIAL AND METHODS

Crystallization

BthTX-I was isolated from *Bothrops jararacussu* snake venom as previously described (Jancarik JAC 1991) [28]. The lyophilized sample of BthTX-I was dissolved in ultrapure water at the concentration of 12 mg/ml. Initial screening of the crystallization conditions used the sparse matrix method (McPherson IMC 2002) [29] (Crystal Screens I ,Hampton Research). Native and α-tocopherol complexed crystals were obtained by hanging or sitting drop vapor diffusion method (Otwinowski ME 1997) [30] where 1-2 µl of protein and 1-2 µl reservoir drop were mixed and equilibrated against 0.5 ml reservoir solution. Crystals for native and complexed form were obtained with a reservoir solution containing 0.12-0.18 M Lithium sulfate, 30% (w/v) polyethylene glycol 4000 and 0.1 M Tris-HCl pH 8.5. Additionally, for BthTX-I-αT complex, 2µl of α-tocopherol was added to the protein/reservoir drop. The best crystals for BthTX-I (Fig. **1A**) and BthTX-I-αT complex (Fig. **1B**) were obtained at 291 K after two to three weeks.

X-Ray Data Collection and Processing

X-ray diffraction data of all crystals were collected at a wavelength of 1.431 Å (at 100 K) 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 steam of nitrogen at 100 K using no cryoprotectant. The data were processed using HKL program package (Matthews JMB 1968) [31].

RESULTS AND DISCUSSION

The data collection statistics are shown in Table **1**. Both data sets are approximately 96 % complete at 2.17 and 1.83 Å resolution, respectively for native and complexed structures. The complex crystals are not isomorphous to the native protein, and belong to the space group $P2_1$, while native BthTX-I crystals belong to the $P3_121$.

Packing parameter calculations based on a protein molecular weight indicate the presence of a dimer in the asymmetric unit for all crystals. This corresponds to a Matthews coefficient (Navaza AC 1994) [32] of approximately 2.1 $Å^3$/Da for both native and complexed crystals, within the expected range for typical protein crystals (assuming a value of 0.74 cm^3/g for the protein partial specific volume). The crystal structures were determined by molecular replacement techniques implemented in the program AMoRe (Jones CMMMD 1990) [33] using the coordinates of a monomer of native PrTX-II (Lee B 2001) [15]. An electron density for α-tocopherol was observed in the hydrophobic channel for both monomers of BthTX-I-αT complex (Fig. **2**).

In conclusion, native BthTX-I was crystallized, and X-ray diffraction data were collected at 2.17 Å. BthTX-I was co-crystallized with α-tocopherol under the same physicochemical conditions as the native protein. X-ray diffraction data were collected at 1.83 Å, the molecular replacement solution was obtained and, a well-ordered electron density for α-tocopherol in the hydrophobic substrate-binding

Table 1. X-Ray Diffraction Data Collection and Processing Statistics

	BthTX-I	**BthTX-I/ αT**
Unit Cell (Å)	a=–b=55.9; c=127.8; γ=120°	a=38.4 b=70.1 c=43.8 β=102.2°
Space Group	$P3_121$	$P2_1$
Resolution (Å)	40-2.17 (2.25-2.17)[a]	40-1.83 (1.90-1.83)[a]
Unique reflections	12383 (1198)[a]	19435 (1824)[a]
Completeness (%)	96.3 (96.9)[a]	96.2 (92.0)[a]
R_{merge}[b] (%)	6.6 (65.6)[a]	6.8 (33.5)[a]
Radiation source	Synchrotron (LNLS – MX1)	
Data collection temperature (K)	100	
I/σ(I)	16.73 (2.15)[a]	22.76 (2.36)[a]
Redundancy	3.8 (3.8)[a]	3.5 (3.3)[a]
Matthews coefficient V_M (Å^3/Dalton)	2.132	2.1
Molecules in the asymmetric unit	2	
Solvent content (%)	42.4	41.6

[a]Numbers in parenthesis are for the highest resolution shell. [b] $R_{merge} = \Sigma_{hkl}(\Sigma_i(|I_{hkl,i}-\langle I_{hkl}\rangle|))/\Sigma_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices h, k and l, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection. Calculated for **$I > -3\sigma(I)$**. Data were processed using the HKL suite [30].

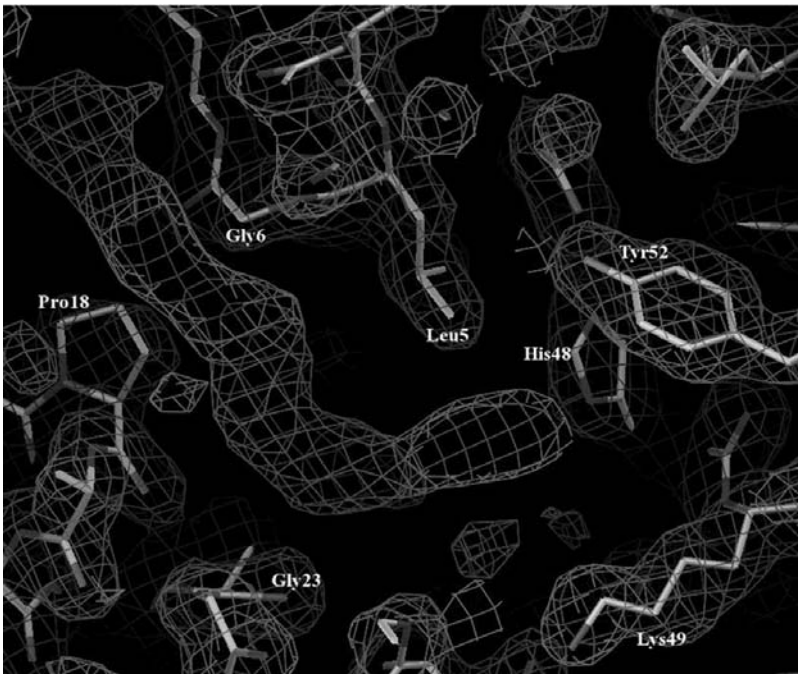

Fig. (2). $|F_{obs}|-|F_c|$ electron density map contoured at 1.2 standard deviations in the "active site" region of monomer A of the BthTX-I/α-tocopherol complex.

channel was observed. The complex crystal is not isomorphous to the native protein and belongs to the space group $P2_1$ while native BthTX-I crystals belong to the $P3_121$. This suggests that the binding of the inhibitor led to changes in the quaternary structure resulting in an alternative conformation for the protein. Intriguingly, the myotoxic and cytotoxic activities of non-catalytic BthTX-I and other Lys49-PLA_2s decrease dramatically when ligands are bound at the "active

site" (Marchi-Salvador ACF 2006) [19] (Soares T 1989) [7]. Possible explanations for this fact are that the α-tocopherol binding may result in conformational changes of C-terminal residues (e.g. Lys122) which may be indirectly interacting with the "active site" affecting the toxic mechanisms. Then, detailed studies with this complex might add insights into the myotoxic and cytotoxic mechanisms of Lys49-PLA_2s and, eventually, clarify the role of C-terminal region.

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ABBREVIATIONS

PLA_2 = Phospholipase A_2

BthTX-I = Bothropstoxin-I

BthTX-I-αT = Complex BthTX-I and α-tocopherol

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