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Structural evidence for a fatty acid-independent myotoxic mechanism for a phospholipase A_2-like toxin

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ABSTRACT

The myotoxic mechanism for PLA_2-like toxins has been proposed recently to be initiated by an allosteric change induced by a fatty acid binding to the protein, leading to the alignment of the membrane docking site (MDoS) and membrane disrupting site (MDiS). Previous structural studies performed by us demonstrated that MjTX-II, a PLA_2-like toxin isolated from *Bothrops moojeni*, presents a different mode of ligand-interaction caused by natural amino acid substitutions and an insertion. Herein, we present four crystal structures of MjTX-II, in its *apo* state and complexed with fatty acids of different lengths. Analyses of these structures revealed slightly different oligomeric conformations but with both MDoSs in an arrangement that resembles an active-state PLA_2-like structure. To explore the structural transitions between *apo* protein and fatty-acid complexes, we performed Normal Mode Molecular Dynamics simulations, revealing that oligomeric conformations of MjTX-II/fatty acid complexes may be reached in solution by the *apo* structure. Similar simulations with typical PLA_2-like structures demonstrated that this transition is not possible without the presence of fatty acids. Thus, we hypothesize that MjTX-II does not require fatty acids to be active, although these ligands may eventually help in its stabilization by the formation of hydrogen bonds. Therefore, these results complement previous findings for MjTX-II and help us understand its particular ligand-binding properties and, more importantly, its particular mechanism of action, with a possible impact on the design of structure-based inhibitors for PLA_2-like toxins in general.

1. Introduction

Accidents caused by members of the *Bothrops* genus represent approximately 85% of snakebites in Latin America [1–3]. *Bothrops moojeni* are found in South America, especially Argentina, Bolivia, Brazil and Paraguay [4]. In Brazil, this species is distributed in the central and southeastern regions of the country in the ecosystems called “cerrado” and “araucaria forests” [5]. Due to their large size, aggressive behavior and adaptability to environmental changes, a high number of accidents involving *B. moojeni* are observed highlights its clinical and scientific importance [4].

The principal effect of bothropic accidents is local myonecrosis, mainly caused by snake venom metalloproteinases (SVMPs), which can act in microvessels and extracellular matrices; and by myotoxic phospholipases A_2 (PLA_2s) and PLA_2-like proteins, which act directly on muscle fibers and lymphatic vessels [6,7]. The rapid action of these toxins makes an efficient neutralization by antivenom administration difficult, causing permanent tissue loss and disability that in some cases may require the amputation of the victim's affected limb [6,8,9].

PLA_2-like proteins are catalytically inactive due to the natural mutations at positions 49 and 28 that disable calcium coordination [10–12]. The Asp49Lys substitution is the most common of this class and the mutants are simply known as Lys49-PLA_2s. These proteins conserve the tertiary structures of PLA_2s but present different oligomeric structures and their myotoxic activity is associated with oligomeric conformational changes and with different functional sites [13–16]. It was suggested that the myotoxic activity of PLA_2-like proteins emerges from two functional sites: the membrane docking site (MDoS) and the membrane disrupting site (MDiS) [13,14]. Regarding the primary sequences of PLA_2-like toxins from *Bothrops* genus, the conserved residues Lys20, Lys115 and Arg118 result in a cationic MDoS, and Leu122 and Phe125 result in a hydrophobic MDiS [14,15]. Recently, the mechanism for the myotoxicity of PLA_2-like proteins was updated [17] suggesting five basic steps: *i*) entry of fatty acids into hydrophobic channels; *ii*) allosteric activation of toxin with alignment of the functional sites (MDoS and MDiS); *iii*) toxin binding at the membrane by the MDoS; *iv*) membrane rupture by the MDiS and *v*) cell death.

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Table 1
List of toxins used in structural comparison to MjTX-II/fatty acids crystal structures.

Toxin	Organism	PDB id
MjTX-II/PEG4K [18]	*B. moojeni*	4KF3
MjTX-II/stearic acid (or MjTX-II/FA18) [20]	*B. moojeni*	1XXS
MjTX-II/suramin [16]	*B. moojeni*	4YV5
apo-BthTX-I [12]	*B. jararacussu*	3I3H
apo-BthTX-I [12]	*B. jararacussu*	3HZD
BthTX-I/PEG4K [12]	*B. jararacussu*	3IQ3
BthTX-I/α-tocopherol [13]	*B. jararacussu*	3CXI
BthTX-I/BPB [12]	*B. jararacussu*	3HZW
BthTX-I/PEG [35]	*B. jararacussu*	2H8I
BthTX-I/Zn^{+2} [17]	*B. jararacussu*	4WTB
MTX-II [14]	*B. brazilis*	4K09
BbTX-II/PEG [14]	*B. brazilis*	4K06
apo-PrTX-I [13]	*B. pirajai*	2Q2J
PrTX-I/α-tocopherol [13]	*B. pirajai*	3CYL
PrTX-I/BPB [12]	*B. pirajai*	2OK9
PrTX-I/aristolochic acid [36]	*B. pirajai*	4YZ7
PrTX-I/rosmarinic acid [37]	*B. pirajai*	3QNL
PrTX-II [38]	*B. pirajai*	1QLL
BnIV [39]	*B. neuwiedi*	3MLM
BnSP-7 [40]	*B. pauloensis*	1PA0
apo-BaspTX-II [41]	*B. asper*	1CLP
BaspTX-II/suramin [42]	*B. asper*	1Y4L

One of the main toxins found in *B. moojeni* venom is the PLA_2-like myotoxin II (MjTX-II), which has a molecular weight of 13.9 kDa and 122 residues. *In vitro* myographic studies demonstrate that this toxin can produce an irreversible and time-dependent blockage of directly and indirectly evoked twitches in isolated mouse diaphragm muscle preparations [16,18]. First elucidated in 1997 [19], the MjTX-II crystal structure has been also determined as a complex with different ligands (stearic acid, polyethylene glycol 4000 and suramin) [16,18,20] (Table 1), although no *apo* model is available in the Protein Data Bank. Therefore, according to recent proposals [13,15], only the "active form" of MjTX-II is known.

The present work reveals the crystal structure of *apo*-MjTX-II and MjTX-II complexed to fatty acids with three different lengths (caproic, caprylic and myristic acids, with 6, 8 and 14 carbons, respectively).The structural studies performed here contribute to the discussion regarding the uniqueness of MjTX-II compared to other PLA_2s-like toxins, culminating with evidence of a particular myotoxic mechanism. These results suggest that MjTX-II is a PLA_2-like toxin that it does not require allosteric changes (usually induced by fatty acid binding to PLA_2-like toxins) to attain an "active form".

2. Experimental procedures

2.1. Toxin isolation and fatty acid characteristics

Crude *B. moojeni* venom was subjected to two chromatographic steps. The first step consisted of ion-exchange chromatography using a CM *fast flow* column (5 mL - *GE healthcare*) and a gradient of 0.05–0.5 M ammonium bicarbonate at pH 8.0, as described previously [21]. To improve the purity of the sample, the fractions obtained from ion-exchange that correspond to the MjTX-II peak were subjected to reverse phase chromatography using a gradient of 0–70% (v/v) acetonitrile (diluted in 0.1% trifluoroacetic acid) on a C18 column (CLC-ODS *Shimadzu*).

Caproic acid (a 6-carbon fatty acid; FA6), caprylic acid (an 8-carbon fatty acid; FA8) and myristic acid (a 14-carbon fatty acid; FA14) were purchased from Sigma-Aldrich, USA.

2.2. Crystallization and X-ray data collection

Samples of the purified fractions of MjTX-II were concentrated to 10 $mg{\cdot}mL^{-1}$ (calculated by measuring absorbance at 280 nm, and using the protein molecular weight and extinction molar coefficient) and used in the crystallization trials. Co-crystallization trials were set up to obtain 1:8 molar ratios (protein:fatty acids). Crystals were obtained by the vapor-diffusion method [22], setting up hanging drops composed of 1 μL of protein or protein-complex solution and 1 μL reservoir solution that were equilibrated against a reservoir containing 500 μL solution [Polyethylene glycol (PEG) 4000, Tris HCl (pH 8.5), lithium sulfate], at 291 K. Crystals were mounted in a *nylon* loop and flash-cooled in a stream of nitrogen at 100 K, without cryoprotectant. X-ray diffraction data were collected using a synchrotron radiation source (MX2 station, LNLS, Brazil) and a CCD-imaging plate detector (MAR Research) at a wavelength of 1.425 Å.

Table 2
X-ray data-collection and refinement statistics.

	Apo-MjTX-II	MjTX-II/FA6	MjTX-II/FA8	MjTX-II/FA14
Unit-cell parameters (Å, °)	a = b = 63.8; c = 126.4	a = 51.2; b = 63.0; c = 88.1	a = 51.1; b = 61.7; c = 54.0; β = 118.9	a = 50.5; b = 62.8; c = 86.8
Space group	$P3_221$	$P2_12_12_1$	$P2_1$	$P2_12_12_1$
Resolution (Å)	27.34–1.99 (2.061–1.99)[a]	26.83–1.70 (1.78–1.70)[a]	26.67–1.75 (1.81–1.75)[a]	35.86–1.94 (2.01–1.94)[a]
Unique reflections	20,857 (2018)[a]	31,375 (3872)[a]	28,286 (2895)[a]	20,107 (1913)[a]
Completeness (%)	99.7 (98.1)[a]	97.5 (98.2)[a]	96.1 (98.6)[a]	96.6 (92.7)[a]
I/σ (I)	9.0 (3.9)[a]	25.7 (3.8)[a]	8.4 (3.2)[a]	7.8 (2.0)[a]
Multiplicity	7.5 (7.1)[a]	4.8 (4.6)[a]	3.0 (3.0)[a]	5.3 (5.3)[a]
R_{merge}[b] (%)	12.4 (87.6)[a]	5.1 (39.6)[a]	10.7 (55.7)[a]	17.2 (67.8)[a]
R_{cryst}	18.9	18.9	17.3	17.0
R_{free}	22.90	20.51	20.16	19.83
$CC_{1/2}$	–	0.87	0.90	0.94
Number of molecules/averaged B-factor				
Protein	2/32.8	2/36.6	2/18.7	2/21.2
Water	222/39.2	304/41.1	374/31.2	313/30.8
Fatty acid	–	1/47.4	2/23.2	2/25.7
Polyethylene glycol	–	–	–	1/31.7
Sulfate ions	6/71.2	7/75.0	6/32.0	6/35.8
Wilson B-factor ($Å^2$)/MolProbity score	27.68/2.0	24.84/1.6	17.09/1.7	21.76/2.2
Ramachandran plot (%)[c]				
Favored	97.0	95.0	96.0	95.0
Outliers	0.0	0.8	0.5	0.5

[a] Numbers in parenthesis are for the highest resolution shell.

[b] $R_{merge} = \Sigma_{hkl}[\Sigma_i(I_{hkl,i} - \langle I_{hkl}\rangle|)] / \Sigma_{hkl}, \langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices h, k and l, and $\langle I_{hkl}\rangle$s the mean intensity of that reflection. Calculated for $I > -3\sigma(I)$.

[c] Calculated with MolProbity program [43].