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

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Abstract

The phospholipases A_2 (PLA_2, E.C. 3.1.1.4, phosphatide *sn*2 acylhydrolases) are the major components of the venom of several snakes. They are responsible for several important pharmacological effects observed in ophidian incidents. PLA_2 piratoxin II from *Bothrops pirajai* has been crystallized by the vapour-diffusion technique. X-ray diffraction data have been collected to 2.04 Å resolution (90.2% complete, $R_{\rm merge} = 0.070$). The space group is $P2_1$ and the cell parameters are $a = 46.19$, $b = 60.36$, $c = 58.74$ Å and $\beta = 96.05^\circ$. The structure has been solved by molecular replacement using the crystallographic structure of PLA_2 from *Bothrops asper* (PDB code 1CLP) as a search model.

1. Introduction

The phospholipases A_2 (PLA_2, E.C. 3.1.1.4, phosphatide *sn*2 acylhydrolases) are present in the venom of several snake species (Mebs & Samejima, 1986). These enzymes specifically hydrolyse the *sn*2 ester bond of phospholipids (van Deenen & de Haas, 1963), having an enhanced activity towards phospholipids in a micellar or lamellar aggregate rather than freely diffusable single phospholipid molecules (Slotboom *et al.*, 1981).

A large number of different PLA_2s have been described in several different organisms in nature, and they have been traditionally classified as intracellular and extracellular (Dennis, 1994). The extracellular PLA_2s are small proteins of 119–143 amino acids, with molecular weights varying from 12 to 15 kDa. They have been separated into three different classes according to amino-acid sequence and disulfide-bonding pattern (Heinrikson *et al.*, 1977; Renetseder *et al.*, 1985). Class I enzymes are present in *Elapidae* and *Hydrophidae* snake venoms and mammalian pancreatic juice. Class II enzymes have been isolated from snake venoms of *Crotalidae* and *Viparidae* species and, more recently, they have been detected in mammalian non-pancreatic tissues (Johansen *et al.*, 1992; Kudo *et al.*, 1993). Finally, there are the class III enzymes, which have been isolated from lizard and bee venoms. Crystallographic experiments showed that the distinct classes have different three-dimensional structures. Classes I and II have a similar overall folding pattern, wheras class III PLA_2s adopt a different folding pattern, where the only conserved structural motif between this class and class I/II is the calcium-binding site (White *et al.*, 1990)

The class II PLA_2s are of special importance since they constitute a large part of the venoms of the most dangerous species of snake, such as rattlesnakes and vipers (Mebs & Samejima, 1986). The enzymes belonging to this class have been subdivided into two subgroups: (i) enzymatically active D49 PLA_2s and (ii) K49 PLA_2s, which have little or no enzymatic activity (Maraganore *et al.*, 1984). The difference in enzymatic activity is due to the substitution of the residue responsible for the binding of a calcium ion, which is an essential cofactor. Crystal structures have revealed that the calcium ion-binding site that is in D49 is occupied by Nζ in K49, hence resulting in a loss of catalytic activity (Holland *et al.*, 1990; Scott *et al.*, 1992; Arni *et al.*, 1995). Despite the low or non-existent enzymatic activity, K49 PLA_2s are capable of disrupting liposomes (Díaz *et al.*, 1991; Rufini *et al.*, 1992). They are also responsible for muscular necrosis and oedema formation (Lomonte *et al.*, 1994). All these effects are independent of catalytic activity, suggesting the presence of an additional motif in the enzyme, additional to the active site, that interacts with some substrates (Gutiérrez & Lomonte, 1995).

Although there are 20 PLA_2 crystallographic structures deposited in the Protein Data Bank (as of March, 1998), the reason for calcium-independent activity is still unknown. New PLA_2 structures might help to shed some light on the reason for this activity. Piratoxin II (PrTX-II) is a PLA_2 isolated from the venom of *Bothrops pirajai*, a *Crotalidae* snake geographically restricted to the southern region of Bahia state in Brazil. Although highly homologous to the abundant piratoxin I (PrTX-I) present in the same venom, PrTX-II could be separated and analysed (Toyama *et al.*, 1995). Under physiological conditions, it exists as a ~26 kDa homodimer and is present in the venom as a minor fraction (less than 10%) when compared to PrTX-I (Toyama *et al.*, 1995).

Here we report the crystallization conditions and preliminary data on the crystals of PrTx-II, and its molecular-replacement solution.

2. Methods and results

Isolation of PrTx-II from the *Bothrops pirajai* venom by semi-preparative reverse-phase HPLC has been described previously (Toyama *et al.*, 1995).

Initial screening of the crystallization conditions has been performed using a sparse-matrix screen at room temperature (301 K) (Crystal Screen I and II, Hampton Research). The hanging-drop vapour-diffusion technique was used for all conditions. Drops containing 1 µl of precipitant solution and 1 µl protein solution were prepared on siliconized glass coverslips and suspended over a reservoir containing 500 µl of the same precipitant solution. The first attempts, performed

Table 1. *Data-collection statistics*

Crystal data

Space group	$P2_1$
Cell parameters	a = 46.19, b = 60.36, c = 58.74 Å; β = 96.05°

Data collection

	Crystal 1	Crystal 2
Resolution (Å)	2.65	2.04
Last resolution shell	2.74–2.65 Å	2.11–2.04 Å
Number of observations	24255	23809
Number of unique reflections	8531	6934
$R_{\rm merge}$ (%)	10.8	7.0
$R_{\rm merge}$, last resolution shell (%)	31.4	26.0
Completeness (%)	90.2	90.2
Completeness, last resolution shell (%)	93.4	72.5

with a protein dissolved in water at a concentration of 10 mg ml^{-1}, showed no crystal formation in any of 96 tested conditions. New trials were performed with protein solution concentrated to 20 mg ml^{-1}, using the same conditions at 277 K. After approximately 20 d, small crystals (~0.05 mm) appeared at condition 17 of the Crystal Screen I kit (30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* lithium sulfate), but their morphology suggested that they were twinned. Slightly modified crystallization conditions resulted in better crystals. They grew within 40 d at 277 K in 28% PEG 3350, 0.25 *M* lithium sulfate and 0.1 *M* Tris–HCl pH 8.5. These were flat crystal plates with approximate dimensions 0.1 × 0.1 × 0.02 mm.

Crystals were mounted in quartz capillaries and data collection was performed at the Protein Crystallographic (PCr) beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) at the Laboratório Nacional de Luz Síncrotron (LNLS) located in Campinas, Brazil. The synchrotron radiation wavelength was set to 1.38 Å and all diffraction images were collected on a MAR 345 image plate. The image plate was operated in the 300 mm scanning mode and the crystal-to-detector distance was set to 200 mm. Two data sets were collected. The first data set was collected from a single crystal to 2.65 Å at 277 K. A total of 78 scanning-oscillation images were recorded (steps of 1.8°, total of 133.2° rotation) and processed with *DENZO* and *SCALEPACK* (*HKL* program suite; Otwinowski, 1993). The second data collection resulted in a higher resolution data set. 58 images were recorded in steps of 1.5°, totalling 87° rotation. This data set was collected from another crystal which diffracted to 2.04 Å resolution. During data collection this crystal was also chilled to 277 K. Images were processed with *DENZO* and *SCALEPACK*. Statistics for both data sets are given in Table 1.

Assuming a molecular mass of 13 kDa, we estimated the number of molecules in the asymmetric unit using the Matthews method (Matthews, 1968). There were two possible solutions: one (V_m = 2.11 Å^3 Da^{-1}) indicating three molecules per asymmetric unit and the other (V_m = 3.17 Å^3 Da^{-1}) suggesting two molecules per asymmetric unit. With this information, we solved the crystal structure by molecular-replacement methods using the *AMoRe* program (Navaza, 1994). We used as a search model a monomer of the PLA_2 dimer of *Bothrops asper* (PDB code 1CLP), including all amino-acid residues and their side chains (Arni *et al.*, 1995). The two most significant unique rotation-search solutions have correlation coefficients of 23.6 and 21.6%, whereas all other solutions were below 11.1%. These two solutions were used in the translation-function search and the best solution was subjected to ten cycles of rigid-body refinement against data between 10 and 3.3 Å resolution (fitting function of *AMoRe*). The fitting yielded a solution with a correlation coefficient of 63.4% and an R factor of 38.9%. The high V_m for the two-molecule solution can, therefore, be explained by the relatively high solvent content (~61%) of the crystal. The crystal packing was inspected using the program *O* (Jones & Kjeldgaard, 1993). Using the same PLA_2 from *Bothrops asper* (1CLP) as search model, but now as a dimer, we also performed self-rotation, translation and rigid-body refinement functions (*AMoRe*) in the resolution range 3.3–10 Å, to verify if the monomeric PrTX-II crystals have similar interactions to the 1CLP dimeric model. After rigid-body refinement, the final correlation coefficient was 45.6 and the final R factor was 46.1%, suggesting that the interaction of the monomers in the PrTX-II crystal are different to those described by Arni *et al.* (1995). This was subsequently confirmed by inspection of the monomer–monomer interactions using the program *O* (Jones & Kjeldgaard, 1993). Further steps of model building and refinement are in progress.

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