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Crystallization and preliminary X-ray crystallographic analysis of the heterodimeric crotoxin complex and the isolated subunits crotapotin and phospholipase A_2

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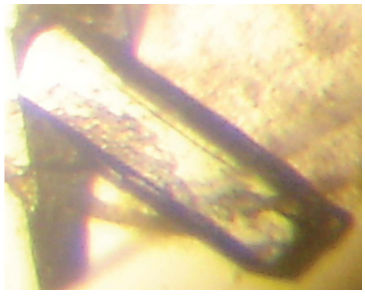


Crotoxin, a potent neurotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus*, exists as a heterodimer formed between a phospholipase A_2 and a catalytically inactive acidic phospholipase A_2 analogue (crotapotin). Large single crystals of the crotoxin complex and of the isolated subunits have been obtained. The crotoxin complex crystal belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 38.2, b = 68.7, c = 84.2 Å, and diffracted to 1.75 Å resolution. The crystal of the phospholipase A_2 domain belongs to the hexagonal space group $P6_122$ (or its enantiomorph $P6_522$), with unit-cell parameters $a = b$ = 38.7, c = 286.7 Å, and diffracted to 2.6 Å resolution. The crotapotin crystal diffracted to 2.3 Å resolution; however, the highly diffuse diffraction pattern did not permit unambiguous assignment of the unit-cell parameters.

1. Introduction

Crotoxin, the major protein present in the venom of the Brazilian rattlesnake (*Crotalus durissus terrificus*), is a very potent myotoxic neurotoxin (Slotta & Fraenkel-Conrat, 1938; Ponce-Soto *et al.*, 2007) that blocks the transmission of neuromuscular signals (Bon *et al.*, 1989; Gopalakrishnakone *et al.*, 1984). Crotoxin is a heterodimeric complex composed of two distinct subunits: a basic component (component B; MW = 14 350 Da, pI $\simeq$ 8.2) which possesses phospholipase activity and an acidic nontoxic catalytically inactive protein, crotapotin (component A; MW = 9490 Da, pI $\simeq$ 3.4) (Breithaupt *et al.*, 1971; Hendon & Fraenkel-Conrat, 1971; Rubsamen *et al.*, 1971; Dorandeu *et al.*, 2002). Whilst the isolated subunits are either nontoxic or are only mildly toxic, they recombine to form a complex which exhibits the same toxicity as the original sample (Hendon & Fraenkel-Conrat, 1971; Habermann *et al.*, 1972; Dorandeu *et al.*, 2002). Crotoxin has received attention owing to its cytotoxic activity against a variety of murine (Corin *et al.*, 1993) and human tumour cell lines *in vitro* (Rudd *et al.*, 1994). Antitumour efficacy *in vivo*, using daily administration of crotoxin, has been demonstrated on Lewis lung carcinoma (83% growth inhibition; Newman *et al.*, 1993) and MX-1 human mammary carcinoma (69% growth inhibition). A lower activity (44% growth inhibition) was observed with HL-60 leukaemia cells, suggesting that crotoxin may possess specificity towards solid tumours (Cura *et al.*, 2002). Recently, several groups have described other pharmacological activities of crotoxin, such as opiate and acetylcholine-independent analgesic actions (Zhang *et al.*, 2006), inhibition of the activity of small GTPases in rat macrophages (Sampaio *et al.*, 2006) and immunosuppresive effects (Rangel-Santos, Lima *et al.*, 2004).

Crotapotin consists of three polypeptide chains linked together by disulfide bridges and acts as an inhibitory 'chaperone' subunit that prevents promiscuous interactions of the phospholipase with phospholipids on membrane surfaces other than its target membrane. The currently accepted model suggests that when the crotoxin complex reaches the target membrane, the complex dissociates and the enzymatically active phospholipase subunit binds, whilst crotapotin, the inactive subunit, is released in solution (Hawgood & Smith, 1977; Jeng *et al.*, 1978; Bon *et al.*, 1979). Although crotapotin is enzymati-

cally inactive (Habermann & Breithaupt, 1978; Bon *et al.*, 1979; Verheij *et al.*, 1980; Gopalakrishnakone *et al.*, 1984), it increases the toxicity of phospholipase A_2 by approximately tenfold (Bon, 1982) and also seems to play a role in oligomeric assembly, protein transport, DNA replication and mRNA turnover (Ellis, 1990). It has been reported that crotapotin has an inhibitory effect on mouse paw oedema, myonecrosis and liposome membrane disruption induced by both catalytically active Asp49 and naturally occurring inactive Lys49 mutant (Arni *et al.*, 1995; Murakami & Arni, 2003) phospholipases A_2 from various snake venoms (Landucci *et al.*, 2000; Cecchini *et al.*, 2004).

The single-chain phospholipase A_2 subunit (PLA_2; EC 3.1.1.4), intramolecularly cross-linked by eight disulfide bridges, catalyses the hydrolysis of the acyl ester bond at the *sn*-2 position of phospholipids (Baek *et al.*, 2000) based on a catalytic mechanism similar to that of mammalian $sPLA_2$s (Gelb *et al.*, 1995). This PLA_2 subunit induces neurotoxicity (Harris, 1991; Faure *et al.*, 2000) and myotoxicity (Gopalakrishnakone *et al.*, 1984; Kouyoumdjian *et al.*, 1986; Mebs & Ownby, 1990; Rangel-Santos, Dos-Santos *et al.*, 2004) *via* selective membrane-binding sites that direct it to specific cellular targets (Kini & Evans, 1989) and activates specific cytokines (IL-1β and TNF-α) which can lead to the release of arachidonic acid and the subsequent production of other pro-inflammatory mediators (prostaglandins, leukotrienes and platelet-activating factors; Crowl *et al.*, 1991; Kudo *et al.*, 1993; Vadas *et al.*, 1993).

Crystallization and data collection of crotoxin (Achari *et al.*, 1985) and the homologous heterodimeric Mojave toxin from *C. viridis viridis* (Ownby *et al.*, 1997) have been reported. Although these share high sequence homology with other phospholipases A_2 (>80%) for which structures have been determined, molecular replacement has been unsuccessful in determining the structures of these heterodimeric proteins. Thus, in order to clarify the molecular basis for the pharmacokinetic properties of crotoxin, we have successfully crystallized crotoxin in the absence of amines such as pyridine and collected X-ray diffraction data. We have also crystallized the isolated subunits of the crotoxin complex.

2. Materials and methods

2.1. Purification of the crotoxin complex and its subunits

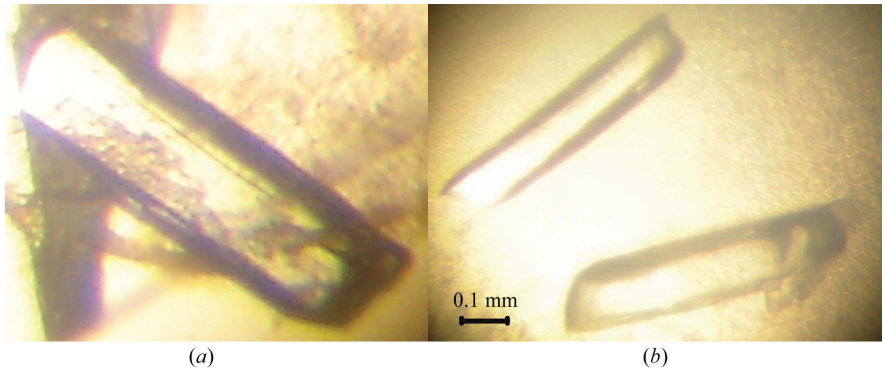
The crude venom of *C. durissus terrificus* was obtained from a local serpentarium (Ilha Solteira, Brazil) and the crotoxin complex was isolated by gel filtration using Sephadex G-75 (Amersham Biosciences) followed by anion-exchange chromatography on a Resource Q column (Amersham Biosciences). Crotoxin dissociation and isolation of subunits was performed following the procedure of Hendon & Fraenkel-Conrat (1971). The purity and homogeneity were checked with SDS–PAGE (purity >98%; Laemmli, 1970) and the protein concentration was measured by the Bradford method (Bradford, 1976).

2.2. Crystallization

The crotoxin complex and both subunits were concentrated using microconcentrators (Centripep, Amicon) to 10 mg ml^{-1} and stored in 20 m*M* Tris–HCl buffer pH 8.0. Crystallization trials were carried out by the hanging-drop vapour-diffusion method in 24-well tissue-culture plates using Crystal Screen HR2-110 and HR2-112 (Hampton Research). Typically, 1 µl drops of protein solution were mixed with an equal volume of screening solution and equilibrated over a reservoir containing 1 ml of the latter. Once initial crystallization conditions had been determined, they were optimized for the three samples at 291 K. Crystals of the PLA_2 subunit were obtained from a reservoir solution containing 0.1 *M* Tris–HCl pH 8.5 and 8%(*w*/*v*) PEG 8000; they grew to dimensions of 0.6 × 0.1 × 0.1 mm within 17 d (Fig. 1*a*). Suitable crystals of the crotapotin subunit were obtained from a reservoir solution containing 0.05 *M* cadmium sulfate, 0.1 *M* HEPES pH 7.5 and 1 *M* sodium acetate and grew to dimensions of 0.8 × 0.2 × 0.2 mm within 30 d (Fig. 1*b*). Crotoxin crystals with maximum dimensions of 0.2 × 0.2 × 0.03 mm were obtained from a solution containing 28%(*v*/*v*) PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* calcium chloride dehydrate and 3–9%(*v*/*v*) glycerol.

2.3. X-ray data collection and analysis

X-ray diffraction data were collected at the DB03-MX1 beamline at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas-Brazil; Polikarpov *et al.*, 1998). A single crystal was soaked in reservoir solution that additionally contained 20% glycerol and was flash-frozen in a gaseous nitrogen stream at 100 K. The wavelength of the radiation source was set to 1.43 Å and a MAR CCD detector was used to record the X-ray diffraction data for the crotapotin and PLA_2 subunits. For data collection from the crotoxin complex crystals, the wavelength of the radiation source was set to 1.54 Å and a MAR345 image-plate detector was used. The raw intensities were indexed, integrated, reduced and scaled using the *DENZO* and *SCALEPACK* programs from the *HKL* suite (Otwinowski & Minor, 1997).



(*a*) (*b*)

Figure 1
Photomicrographs of (*a*) PLA_2 and (*b*) crotapotin crystals.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	Crotoxin complex	PLA_2 subunit
Data collection		
Source	Synchrotron radiation	Synchrotron radiation
Temperature (K)	100	100
Detector	MAR345	MAR CCD
Cryoprotectant	20% glycerol	20% glycerol
Data-processing software	*DENZO*/*SCALEPACK*	*DENZO*/*SCALEPACK*
Diffraction data		
Wavelength† (Å)	1.547	1.427
Space group	$P2_12_12$	$P6_122$ or $P6_522$
Unit-cell parameters (Å)	a = 38.16, b = 68.74, c = 84.17	$a = b$ = 38.7, c = 286.7
Matthews coefficient (Å^3 Da^{-1})	2.0	2.2
Solvent content (%)	37.2	42.6
No. of molecules in the ASU	1 crotapotin + 1 PLA_2	1 PLA_2
No. of unique reflections	22937	4588
Redundancy	8.2 (3.6)	19.5 (12.3)
Resolution range (Å)	30.0–1.75 (1.79–1.75)	30.0–2.6 (2.69–2.60)
Completeness (%)	99.4 (96.6)	99.3 (99.1)
R_{merge}‡ (%)	7.0 (57.9)	8.8 (14.0)
$\langle I/\sigma(I)\rangle$	27.69 (2.42)	23.73 (21.93)

† Wavelengths were those present at the time of data collection. ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

3. Results and discussion

3.1. Crotoxin complex

A single crystal with maximum dimensions of 0.2 × 0.2 × 0.03 mm was used for X-ray diffraction data collection to 1.75 Å resolution under cryogenic conditions (100 K). The intensities were indexed in the orthorhombic space group $P2_12_12$ based on the systematic absences, with unit-cell parameters a = 38.2, b = 68.7, c = 84.2 Å. Processing of the 417 235 measured reflections led to an R_{merge} of 7.0% (57.9% in the last shell, 1.79–1.75 Å resolution) and a completeness of 99.4% (99.6% in the last shell). Taking into consideration the molecular weight of 23 840 Da, one heterodimer is present in the asymmetric unit with a corresponding Matthews coefficient of 2.0 Å^3 Da^{-1} and a solvent content of 37.2% (Matthews, 1968). Data-processing statistics are presented in Table 1.

3.2. PLA_2 subunit

Single crystals with dimensions of 0.6 × 0.1 × 0.1 mm were used for X-ray diffraction data collection to 2.6 Å resolution under cryogenic conditions (100 K). The intensities were indexed in the hexagonal space group $P622$, with unit-cell parameters $a = b$ = 38.7, c = 286.7 Å. Examination of the systematic absences indicated that the crystals belonged to either space group $P6_122$ or to its enantiomorph $P6_522$. Processing of the 331 117 measured reflections led to a data set to 2.6 Å resolution with an R_{merge} of 8.8% (14.0% in the last shell, 2.69–2.60 Å resolution) and a completeness of 99.3% (99.1% in the last shell). Assuming a molecular weight of 14 350 Da, the solvent-content calculations (Matthews, 1968) indicate the presence of a single molecule in the asymmetric unit with a corresponding Matthews coefficient of 2.2 Å^3 Da^{-1} and a solvent content of 42.6%. The crystal parameters and data-processing statistics are summarized in Table 1.

3.3. Crotapotin subunit

Single crystals with dimensions 0.8 × 0.2 × 0.2 mm were used for X-ray diffraction experiments and the data were collected to 2.3 Å resolution under cryogenic conditions (100 K). The X-ray diffraction pattern was characterized by diffuse elongated irregular spots. Various algorithms implemented in *DENZO* (Otwinowski & Minor, 1997), the *XDS* package (Kabsch, 1993), *MOSFLM* (Leslie, 1992) and *LABELIT* (Sauter *et al.*, 2004) were used in an attempt to index the reflections. However, no clear solution was obtained.

3.4. Concluding remarks

The structures of a large number of phospholipases A_2 have been deposited with the Protein Data Bank and based on the high sequence homology, a number of search models have been generated. However, no clear solution has been obtained by molecular replacement to date for either the heterodimer or the isolated subunits. To overcome this, the 'quick cryo-soaking' technique will be used (Dauter *et al.*, 2000).

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