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Crystallization and preliminary crystallographic studies of a phospholipase A_2 from the venom of the Brazilian snake *Bothrops moojeni*

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A phospholipase A_2 purified from the venom of the snake *Bothrops moojeni* has been crystallized by vapour-diffusion techniques in hanging drops at 291 K. The crystals, which were grown in the absence of Ca^{2+}, belong to the cubic system, space group $P432$, with unit-cell parameters $a = b = c = 91.86$ Å, and contain one molecule in the asymmetric unit (V_M = 2.71 Å^3 Da^{-1}). X-ray diffraction experiments provide data to 2.35 Å resolution collected on a rotating-anode home source at cryogenic temperatures. The structure has been solved *via* molecular-replacement techniques using a single monomer of the crystallographic structure of the phospholipase from the Western diamondback rattlesnake (*Crotalus atrox*) as a search model.

1. Introduction

Phospholipases A_2 (PLA_2s; E.C. 3.1.1.4; Dennis, 1983) are calcium-dependent enzymes that catalyse the hydrolysis of the *sn*-2 fatty acyl bond of phospholipids, liberating free fatty acids and lysophospholipids. They are found to occur in a large variety of snake venoms and mammalian exocrine glands. Although showing high sequential conservation, PLA_2s may exhibit major functional differences in their biological actions and play central roles in diverse cellular processes including phospholipidic digestion and metabolism, host defence and signal transduction (Mukherjee *et al.*, 1994).

Interest in the action of PLA_2 also derives from its implication in mediating the inflammatory response through the release of arachidonic acid from the *sn*-2 position of phospholipids in the plasma membrane. Arachidonate is the precursor of the eicosanoid mediators of inflammation, including leukotrienes, thromboxanes and prostaglandins (Irvine, 1982; Chang *et al.*, 1987), and the non-pancreatic secretary PLA_2 structure has therefore served as the basis for the rational design of potent inhibitors which have potential application in the treatment of chronic inflammatory disease (Schevitz *et al.*, 1995).

PLA_2s obtained from various snake and bee venoms and from mammalian pancreas have been very well characterized, mechanistically elucidated and structurally defined (Souza *et al.*, 2000). They are highly conserved and are characterized by several structural features: an N-terminal 12-residue α-helix, a relatively long loop segment containing the calcium-binding site, a pair of large nearly parallel α-helices and a short antiparallel double-stranded β-sheet (Arni & Ward, 1996).

Despite secondary and tertiary structural similarities observed between PLA_2s, members of this class of enzymes have been described as occurring in different oligomerization states in a concentration-dependent and pH-dependent manner. From the biochemical and crystallographic studies it has been found that PLA_2 may occur in a monomeric, a dimeric and even a trimeric form (Fremont *et al.*, 1993). Considerable attention has been devoted to understanding the physiological relevance of the oligomerization state of phospholipases A_2 and has raised an interesting theme for discussion.

Some authors believe that most phospholipases A_2 function as dimers even if they exist as monomers in diluted solution. The second phospholipid-binding site could have an allosteric function during catalysis (Roberts *et al.*, 1979) in a cooperative interaction between monomers in response to the binding of the substrate and Ca^{2+}. On the other hand, the unique dimeric structure obtained in the crystallographic study of a typical PLA_2, the phospholipase A_2 of the Western diamondback rattlesnake *C. atrox* (Keith *et al.*, 1981; Brunie *et al.*, 1985), has no obvious pathway by which a phospholipid head group could gain access to the active site. Furthermore, some of the catalytic residues and part of the Ca^{2+}-binding loop participate in dimer formation, therefore rendering this structure catalytically inactive.

In this study, we report the crystallization, preliminary X-ray diffraction analysis and structure determination of a typical D49 phospholipase A_2, BM-PLA_2, isolated from the venom of the snake *B. moojeni* (Caissaca). BM-PLA_2 is an acidic protein of pI 4.6 which

Table 1
Data-collection statistics for BM-PLA_2 crystals.

Resolution (Å)	Independent reflections	R_{sym}† (%)	Completeness (%)	$I > 3\sigma(I)$ (%)
20.00–5.04	675	4.9	99.6	97.6
5.04–4.01	612	6.1	99.8	97.7
4.01–3.51	601	8.1	99.5	98.8
3.51–3.19	586	10.5	99.7	93.5
3.19–2.96	572	14.6	99.1	91.3
2.96–2.79	580	20.7	99.5	81.4
2.79–2.65	573	27.5	98.5	69.5
2.65–2.53	565	40.0	97.2	57.7
2.53–2.43	541	51.0	95.4	45.1
2.43–2.35	534	61.2	94.0	36.7
Total	5839	9.1	98.3	78.0

† $R_{sym} = \sum_{hkl} \sum_{refl} |I(hkl, j) - \overline{I}(hkl)| / \sum_{hkl} \sum_{refl} I(hkl)$.

presents a high phospholipase activity associated with high blood-clotting, high proteolytic and low haemorrhagic activities.

2. Materials and methods

The enzyme was initially purified as described by Reichl *et al.* (1989). For the success of the crystallization experiments, the addition of a new step in the purification protocol proved to be necessary and consisted of gel-filtration chromatography on Superdex-75, eluted at a flow rate of 1 ml min^{-1} using phosphate-buffered saline (PBS; 0.15 *M* NaCl, 0.15 *M* $Na_2HPO_4.12H_2O$ pH 7.4). The enzyme activity was determined by indirect haemolytic activity according to the method described by Gutierrez *et al.* (1984). The enzyme was extensively dialysed against milli-Q water and concentrated to 10 mg ml^{-1} based on its extinction coefficient at 280 nm ($E^{1\%}_{1\,cm} = 13.96$).

Crystals were obtained using the vapour-diffusion technique in hanging drops (McPherson, 1982) at 291 K. The drops contained equal amounts (2.5 µl) of reservoir and protein solution. BM-PLA_2 crystals grow from 20–28% solution of a mixture of 10% saturated monobasic dihydrogen phosphate (NaH_2PO_4) and 90% saturated dipotassium phosphate (K_2HPO_4) in milli-Q water. Cubic shaped crystals appeared after four weeks and have been shown to be reproducible (Fig. 1).

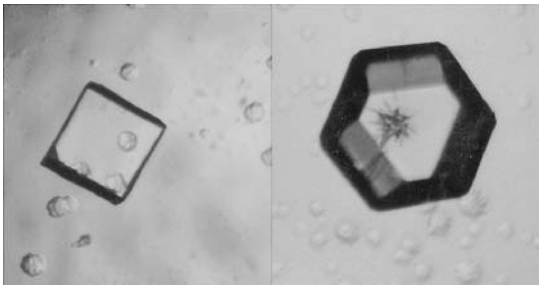

Figure 1
BM-PLA_2 crystals. Approximate dimensions are 0.4 × 0.4 × 0.4 mm.

Data were collected on a Rigaku R-AXIS II image-plate area-detector system using Cu $K\alpha$ radiation from an RU-200C rotating-anode generator operating at 50 kV and 100 mA. Owing to the high sensitivity to both X-radiation and mechanical stress exhibited by the crystals, data collection at low temperature proved to be essential for obtaining a data set of acceptable quality. For cryoexperiments the crystals were soaked in the reservoir solution containing 35% glycerol prior to flash-freezing in a nitrogen stream at 100 K using an Oxford Instruments cryocooling system.

The structure was solved by molecular replacement as implemented in the *AMoRe* package (Navaza, 1997), using a single monomer of the crystal structure of the Western diamondback rattlesnake *C. atrox* (Brunie *et al.*, 1985) as a search model and using data in the resolution range 15–3.5 Å.

3. Results and discussion

BM-PLA_2 crystals belong to the cubic system, space group *P*432, with a unit-cell parameter $a = 91.86$ Å. Data were processed and scaled with the *DENZO* package (Otwinowski & Minor, 1996). A total of 42 083 collected reflections were merged to 5839 independent reflections with a multiplicity of 7.2 to 2.35 Å resolution. Data-processing statistics are shown in Table 1.

Assuming a molecular mass of 11 900 Da (estimated by the sedimentation–diffusion ratio and by SDS–polyacrylamide gel electrophoresis; Reichl *et al.*, 1989), the 24 symmetry operations inherent to the space group *P*432 and a protein partial specific volume of 0.74 $cm^3\,g^{-1}$, we estimate one monomer per asymmetric unit, giving physicochemical parameters which lie within the range commonly observed for protein crystals. The crystal density is calculated as 1.16 $g\,cm^{-3}$ and is associated with a solvent content of 54.7% by volume, with a calculated V_M value of 2.71 $Å^3\,Da^{-1}$ (Matthews, 1968).

The first solution found by *AMoRe* after both rotation and translation functions (Navaza, 1993; Navaza & Vernoslova, 1995) followed by rigid-body refinement (Castellano *et al.*, 1992) shows a correlation coefficient and an R_{factor} of 53.3 and 44.5%, respectively (rotation 25.0, 10.0, 341.9° and translation 0.2506, 0.1485, 0.5796). The correlation coefficient and *R* factor for the second solution are 37.8 and 50.0%, respectively, indicating a good contrast between the first two potential molecular-replacement solutions, thereby eliminating possible ambiguity. Analysis of the molecular packing within the unit cell was performed using the program *O* (Kleywegt & Jones, 1994) and shows no steric hindrance for the best solution.

A preliminary rigid-body refinement followed by positional and *B*-factor refinements using the search-model amino-acid sequence were performed showing *R* factor and R_{free} values of 38.9 and 45%, respectively. Further refinement steps will be performed as soon as the primary structure of BM-PLA_2 becomes available.

Inspection of the monomer–monomer interactions derived from the molecular-replacement solution indicates the formation of a dimeric structure, generated by a crystallographic twofold axis, which appears to be effectively identical to that observed in PLA_2 from *C. atrox* (Keith *et al.*, 1981). What seems remarkable about such a dimer is the fact that the interface between monomers results in occlusion of the phospholipase active site; consequently, the physiological relevance of such oligomerization has been questioned (Arni *et al.*, 1995).

Evidence from sedimentation–diffusion experiments also points to the presence of a dimer in solution (Reichl *et al.*, 1989). For concentrations above 1.8 mg ml^{-1} the estimated molecular weight for BM-PLA2 was found to be 19 kDa, compatible with a dimer.

In addition, the analysis of the subunit interface between monomers, a consequence of dimer formation, is that a total of more than 1250 $Å^2$ of surface area per monomer becomes buried within the interface in the case of *C. atrox*, leading to a shape-complementary index (S_c; Lawrence & Colman, 1993) of 0.68. These values indicate that the dimer interface is both large and complementary. By way of comparison, the mean value for the buried surface area of dimeric proteins of 12 kDa molecular weight is estimated to be only 720 $Å^2$ (Jones & Thornton, 1995), the much greater area observed for *C. atrox* and *B. moojeni* phospholipases A_2 being indicative of considerable stability (Janin, 1995). Typically, S_c values fall within the range 0.70–0.76 for

stable oligomeric complexes and between 0.64 and 0.68 for antibody–antigen complexes, for example. The value of 0.68, slightly below the range for a stable dimer, suggests that dissociation into two monomers might be expected under adequate physiological conditions.

As seen in the crystal structure of the bovine enzyme (Dijkstra *et al.*, 1981) and its complexes, as well as those of the PLA_2 from bee venom and that from the Taiwanese cobra *Naja naja atra* (Chang *et al.*, 1996), the Ca^{2+} ion is an obligatory requirement for activity. In the absence of substrate, the calcium ion is coordinated to three backbone carbonyls and the side-chain carboxylate of Asp49 as well as two exchangeable water molecules which are excluded on substrate binding. In our case, as in *C. atrox*, there is no evidence for a calcium ion in the crystal structure, as no electron density is found in this region of the map.

When comparing the calcium-free dimer of *C. atrox*, now also observed in *B. moojeni*, with other crystal structures where the calcium ion is present in the active site, it becomes evident that the conformation of the calcium-binding elements is distorted to stabilize the dimer formation. The binding of calcium would disrupt the dimer by competing for the same binding sites.

Partial amino-acid sequencing of the N-terminal portion of BM-PLA_2 (data not shown) shows greater than 70% sequence identity to *C. atrox* and the similarity of the two interfaces suggests that this may not be merely the consequence of crystal packing. Rather, the observation of the same inactive dimeric structure in two independent venom phospholipases suggests that this may represent a storage form of the enzyme. Activation of this dimer would be expected to occur by dissociation into two monomers in the presence of Ca^{2+} ions through their binding to the calcium-binding loop. This process would expose the active site and complete its formation *via* the incorporation of Ca^{2+}, which is essential for the stabilization of the tetrahedral intermediate of the reaction. Such a mechanism was first suggested by Brunie *et al.* (1985), but its acceptance has been hampered by the lack of other structures present in the form of an inactive dimer. BM-PLA_2 provides some addition support for this idea.

Amino-acid sequencing (which is currently in progress), together with full crystallographic refinement of the structure of BM-PLA_2, should help to provide a more definitive answer to this question. In parallel, a search for crystals of the Ca^{2+}-saturated form of BM-PLA_2 is also in progress.

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