Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction studies of phospholipase D from *Streptomyces antibioticus*

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Received 8 May 1998
Accepted 5 August 1998

Phospholipase D (E.C. 3.1.4.4) from *Streptomyces antibioticus* has been crystallized in six crystal forms using the hanging-drop vapour-diffusion method. The type III and V crystals belong to monoclinic and hexagonal systems, respectively. All of the other crystal forms, types I, II, IV and VI, belong to orthorhombic space group $P2_12_12_1$. Of these four types, the type VI crystals are suitable for X-ray structure determination. Crystal data for type VI crystals are: $a = 50.1$, $b = 98.7$, $c = 107.6$ Å, $V = 532\,100$ Å^3, $Z = 4$ and $V_m = 2.47$ Å^3 Da^{-1}. Type VI crystals diffract to at least 2.3 Å resolution. A total of 11 295 independent reflections to 3 Å resolution have been collected from a type VI crystal using a conventional X-ray source, and its structural analysis is currently being conducted using isomorphous replacement methods.

1. Introduction

Phospholipase D (PLD, E.C. 3.1.4.4) hydrolyzes phospholipids with the formation of phosphatidic acid and alcohols. PLD also catalyzes transphosphatidylation, which is a kind of transesterification (Yang *et al.*, 1966).

In mammalian cells, PLD plays an important role in signal transduction (Exton, 1994), and some genes of animal PLD have been cloned (Hammond *et al.*, 1995; Colley *et al.*, 1997). Elucidating the structure–function relationship requires determination of the three-dimensional structure of PLD. However, the tertiary structure is not yet known, partly because most animal PLDs are membrane bound and relatively large (consisting of more than 1000 amino acids) and partly because producing animal PLD in large quantities is difficult.

The Laboratory of Molecular Biotechnology group have been studying PLD from *S. antibioticus* (Shimbo *et al.*, 1993; Iwasaki *et al.*, 1994, 1995; Mishima *et al.*, 1997). This enzyme is useful for industrial applications owing to its strong transphosphatidylation activity, by which various phospholipids can be synthesized (Juneja *et al.*, 1988). In addition, the bacterial PLD is a soluble protein consisting of 509 amino-acid residues and having a relatively small molecular mass (molecular weight 53 972). It is interesting that the bacterial PLD contains the 'HKD motif', a highly conserved sequence of H*x*K*xxxx*D found in other eukaryotic PLDs (Hammond *et al.*, 1995; Ponting & Kerr, 1996; Sung *et al.*, 1997). This suggests that the bacterial enzyme uses a catalytic mechanism similar to those of other eukaryotic PLDs.

The present paper reports the crystallization and preliminary X-ray studies of the bacterial PLD in order to clarify its catalytic function.

2. Experimental

2.1. Preparation and purification of PLD

A recombinant strain of *Escherichia coli* BL21 (DE3) carrying a plasmid pPELB-PLD3, which contains the PLD gene of *S. antibioticus* (Mishima *et al.*, 1997), was cultivated with 2 l of synthetic medium (Iwasaki *et al.*, 1995) in a jar fermentor using a fed-batch operation (Yamane, 1993) to achieve high cell density. When the cell density reached about 20 g dry-cell weight per litre of broth (after about 30 h), 100 g of solid lactose was added to induce the expression of the PLD gene and the cells were cultivated for further 20 h.

Culture supernatant recovered *via* centrifugation was used as a starting material for the purification of PLD and was precipitated with 30–60% saturated ammonium sulfate. During purification, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) was added to each buffer in order to avoid proteolytic degradation. The precipitate was dissolved in 50 m*M* sodium acetate buffer (pH 4.5) and dialyzed against the same buffer. After removing insoluble materials by centrifugation, the supernatant was incubated at 323 K for 2 h in order to aggregate the impurities. The aggregated materials were again removed *via* centrifugation, and PLD was purified from the supernatant through a series of column chromatographies including CM-Toyopearl, DEAE-Toyopearl, Resource Q and Sephacryl

S-200. The purified PLD was extensively dialyzed against 10 m*M* tris(hydroxymethyl)aminomethane–HCl (Tris–HCl) buffer (pH 8.0) in order to remove PMSF before crystallization.

2.2. Crystallization and X-ray analysis

Conventional precipitants, such as ammonium sulfate, polyethlene glycol (PEG) or 2-methyl-2,4-pentanediol (MPD) were tried at different concentrations over a broad pH range using the hanging-drop vapour-diffusion method at 277, 291 and 303 K. 3 µl of the protein solution was mixed with an equal volume of a reservoir solution. The volume of the reservoir solution was 1 ml in all trials. Type I, II and III crystals were grown at 291 K and types IV, V and VI were obtained at 303 K (Table 1). Under the best conditions of the reservoir solution, *i.e.* 20%(*w*/*v*) PEG 6000 in 0.1 *M* Tris buffer (pH 8.0), type VI crystals were obtained.

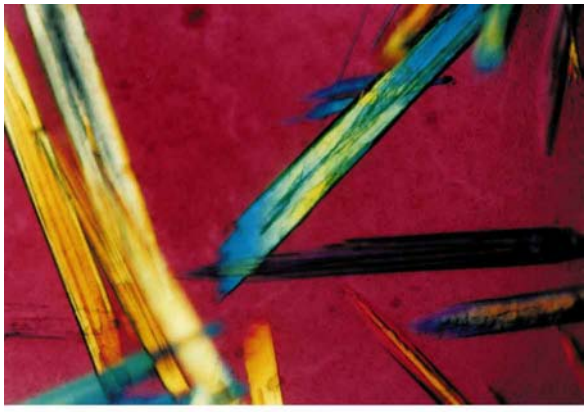

Figure 1
(*a*) Type I and (*b*) type VI crystals of PLD. The sizes of the crystals are approximately 0.15 × 0.10 × 1.5 mm (type I) and 0.15 × 0.12 × 0.5 mm (type VI)

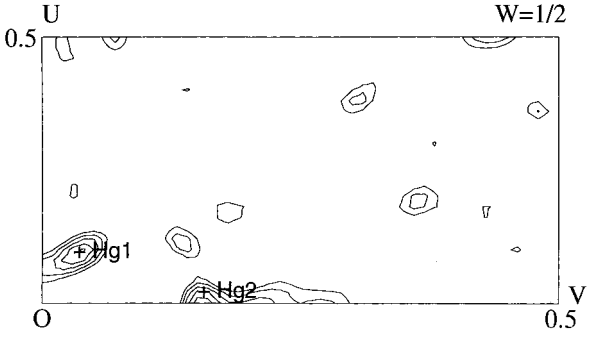

Figure 2
A Harker section (w = 0.5) of the difference Patterson map for the Hg derivative at 4.5 Å resolution. Two heavy-atom sites, Hg1 and Hg2, are shown. Contours are drawn every σ, starting from the 3σ level.

Isomorphous derivatives were prepared by soaking type VI crystals in heavy-atom solutions. Several platinum and mercury compounds were tried; however, these compounds almost cracked the crystals. The only heavy-atom reagent found to be suitable was $Hg(CH_3COO)_2$. The optimal soaking period and concentration of the heavy-atom reagent are 3 d and 1 m*M*, respectively.

Characterization and data collection of type I, III, V and VI native crystals were performed using the oscillation method on a DIP-100 imaging-plate detector system (MAC Science Co., Japan) with focusing-mirror optics mounted on an RU-300 Rigaku rotating-anode generator. The derivative data set from a type VI crystal [named Hg(VI)] was also obtained using the same equipment. Oscillation images were processed using the *DENZO* and *SCALEPACK* programs (Otwinowski, 1993; Minor, 1993). For type II and IV crystals, native data were collected using the Weissenberg method at the BL41XU station, SPring-8, Hyogo, Japan. Weissenberg images were processed using the *WEIS* program (Higashi, 1989).

Scaling between native and derivative data and calculation of difference Patterson maps were performed using the *PROTEIN* program system (Steigemann, 1993).

3. Results and discussion

Two different crystal morphologies were grown within 5–7 d under the same conditions at 291 K. Furthermore, the different crystal morphologies even coexisted in the same drop of 0.1 *M* 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer (pH 6.0). These were rectangular pillar-shaped and rhombic pillar-shaped crystals (type I and type II, respectively). Distinguishing between type I and type II crystals by visual observation is difficult (Fig. 1*a*). Thin blade-like crystals (type III) were grown in 0.1 *M* piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) at 291 K.

At 291 K, these crystals appear only in bundles. Attempts were made to improve the crystal habit by changing the buffer and varying the precipitant concentration, the pH and the temperature. None of these variations led to significant improvement of the crystal habit, except for the change in temperature. At 277 K, only needle-like crystals which were unsuitable for X-ray analysis could be grown. By increasing the temperature, individual crystals could be obtained. The optimal temperature is 303 K. Rhombic plate-like crystals (type IV) having sharp edges of up to 0.075 mm in size were grown in 0.1 *M* MES buffer (pH 6.5); however, type IV crystals were rarely observed. Hexagonal bipyramidal crystals (type V) were grown from a drop containing 46 mg ml^{-1} protein. Finally, rectangular pillar-shaped crystals (type VI) were grown in 0.1 *M* Tris buffer (pH 8.0) with good reproducibility. Type VI crystals up to 0.15 × 0.12 × 0.5 mm in size were grown within one week (Fig. 1*b*). Crystallization conditions and cell parameters are summarized in Table z1.

A summary of the data collection and processing is given in Table 2. All orthorhombic crystals (types I, II, IV and VI) belong to space group $P2_12_12_1$. Each crystal probably contains four molecules in the unit cell, taking the V_m (Matthews, 1968) value of the crystal into consideration. The monoclinic unit cell of type III suggests that this cell contains four molecules, leading to V_m = 1.95 Å^3 Da^{-1}. Type V crystals belong to a hexagonal system, giving V_m = 2.01 Å^3 Da^{-1} if Z = 18. This suggests that three molecules are contained in the asymmetric unit in type V crystals.

In order to solve the structure of the type VI crystals, difference Patterson maps for the native and Hg(VI) crystals were calcu-

Table 1
Crystallization conditions and cell parameters.

Crystal type	Type I	Type II	Type III	Type IV	Type V	Type VI
Precipitant	20% PEG 6000	15% PEG 6000	55% MPD	20% PEG 6000	20% PEG 6000	20% PEG 6000
Protein concentration (%)	0.5	0.5	0.5	0.5	2.3	0.5
Buffer	0.1 *M* MES	0.1 *M* MES	0.1 *M* PIPES	0.1 *M* MES	0.1 *M* MES	0.1 *M* Tris
pH	6.0	6.0	7.0	6.5	6.6	8.0
Temperature (K)	291	291	291	303	303	303
Crystal shape	Rectangular pillar	Rhombic pillar	Thin blade	Rhombic plate	Hexagonal bipyramid	Rectangular pillar
Crystal system	Orthorhombic	Orthorhombic	Monoclinic	Orthorhombic	Hexagonal	Orthorhombic
Cell parameters						
a (Å)	61.2	62.7	96.5	60.7	99.0	50.1
b (Å)	77.8	85.3	81.3	87.6	—	98.7
c (Å)	99.2	100.5	56.3	91.9	230.3	107.6
β (°)	—	—	107.5	—	—	—
V (Å^3)	4.72×10^5	5.38×10^5	4.21×10^5	4.89×10^5	1.95×10^6	5.32×10^5

Table 2
Summary of data collection and processing.

Crystal type	Type I	Type II	Type IV	Type V	Hg(VI)
Method	OSC†	WEIS‡	WEIS	OSC	OSC
Wavelength (Å)	1.54	1.00	1.00	1.54	1.54
Crystal-to-IP distance (mm)	120	560	560	120	170
IP number	55	20	19	110	50
Oscillation range (°)	2.0	5.0	5.0	1.0	2.0
Number of oscillations	40	1	1	32	40
Oscillation speed (° s^{-1})	16	5	2	8	8
Exposure time per IP (s)	300.0	1.0	2.5	480.0	600.0
Coupling constant (° mm^{-1})	—	1.0	1.0	—	—
Resolution (Å)	3.0	2.8	3.5	3.0	4.5
Total reflections	31200	48514	25888	53361	12916
Independent reflections	7844	12595	6440	11295	3438
with $I > 3\sigma(I)$	6224	10627	5228	10613	2242
$R_{\rm merge}$§	0.106	0.041	0.072	0.086	0.102
Completeness (%)	77.8	90.7	97.3	96.9	82.9
with $I > 3\sigma(I)$	61.8	76.5	79.0	85.9	65.2
of final shell (%)	73.6	88.2	98.6	93.5	82.5
with $I > 3\sigma(I)$	45.7	56.8	68.4	68.8	63.7
$R_{\rm iso}$¶ [type VI *versus* Hg(VI)]	—	—	—	—	0.194

† OSC: DIP-100 diffractometer and RU-300 X-ray generator. ‡ WEIS: Weissenberg camera and synchrotron source. § $R_{\rm merge} = \sum|I_{\rm obs} - \langle I\rangle|/\sum\langle I\rangle$, where $I_{\rm obs}$ is an observed intensity and $\langle I\rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections. ¶ $R_{\rm iso} = \sum|F_{\rm PH} - F_{\rm P}|/\sum F_{\rm P}$, where $F_{\rm P}$ is the structural amplitude from the native data and $F_{\rm PH}$ is the structural amplitude of the corresponding reflection from the derivative data.

lated. A Harker section of the difference Patterson map calculated at 4.5 Å resolution is shown in Fig. 2. The positions of the heavy-atom sites Hg1 and Hg2 were determined. At present, the conditions for Hg-derivative preparation of type VI is being refined, and additional derivatives are currently being sought.

We would like to thank Drs Nobuo Kamiya, Yoshiaki Kawano and Masahide Kawamoto from the RIKEN Institute of Structural Biology at SPring-8, Harima, Japan, for help with the data collection. The synchrotron-radiation experiments were performed at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (Proposal No. 1997B0097-NL-np).

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