Crystal structure of the complex formed between a group I Phospholipase A₂ and a naturally occurring fatty acid at 2.7 Å resolution

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Abstract

This is the first evidence of a naturally bound fatty acid to a group I Phospholipase A₂ (PLA₂) and also to a PLA₂ with Asp 49. The fatty acid identified as *n*-tridecanoic acid is observed at the substrate recognition site of PLA₂ hydrophobic channel. The complex was isolated from the venom of *Bungarus caeruleus* (Common Indian Krait). The primary sequence of the PLA₂ was determined using the cDNA method. Three-dimensional structure has been solved by the molecular replacement method and refined using the CNS package to a final R factor of 19.8% for the data in the resolution range from 20.0 to 2.7 Å. The final refined model is comprised of 912 protein atoms, one sodium ion, one molecule of *n*-tridecanoic acid, and 60 water molecules. The sodium ion is located in the calcium-binding loop with a sevenfold coordination. A characteristic extra electron density was observed in the hydrophobic channel of the enzyme, into which a molecule of *n*-tridecanoic acid was clearly fitted. The MALDI-TOF measurements of the crystals had earlier indicated an increase in the molecular mass of PLA₂ by 212 Da over the native PLA₂. A major part of the ligand fits well in the binding pocket and interacts directly with His 48 and Asp 49. Although the overall structure of PLA₂ in the present complex is similar to the native structure reported earlier, it differs significantly in the folding of its calcium-binding loop.

Keywords: *n*-tridecanoic acid; PLA₂ complex; crystal structure; regulation

Phospholipases A₂s (PLA₂s) are widely distributed in the animal world. They are mainly divided into two classes, called groups I and II, on the basis of their chain lengths and S-S bridge structures (Heinrikson et al. 1977; Davidson and Dennis 1990). Group I contains enzymes from mammalian pancreatic species and venom of *Elapidae* (kraits and cobras) and *Hydrophiidae* (sea snakes), whereas PLA₂s from *Crotalidae* (rattle snakes and pit vipers), *Viperidae* (old

world vipers), and human synovial fluid belong to group II. The PLA₂s from human and snake venom sources may show variations in their primary and quaternary structures, and often possess different pharmacological properties; but, the catalytic functions of both human and snake venom PLA₂s are essentially similar. They have grossly similar binding sites with identical catalytic residues, and the mode of inhibitor bindings are also similar (Verheij et al. 1980; Thunnissen et al. 1990). It is well known that PLA₂ converts phospholipids into arachidonic acid, leading to the final products that are commonly defined as eicosanoids (van Deenen et al. 1963). A low concentration of eicosanoids is required to maintain a number of physiological processes (van Deenen and de Haas 1963). An excess production of some of these compounds is related to pathological conditions characterized by inflammation, rheumatism, arthritis, and edema (Dennis 1987). In order to restrict the excess

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Abbreviations: PLA₂, Phospholipase A₂; PDB, Protein Data Bank; rms, root-mean-square; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; CNS, Crystallography & NMR systems; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

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production of eicosanoids, the catalytic actions of PLA₂ and other enzymes involved in the different steps of the cascade reaction need to be regulated. In order to control such chronic inflammatory diseases, specific potent inhibitors of PLA₂ and other enzymes are required to be developed. Several crystal structures of PLA₂ with its various inhibitors have been reported (Cha et al. 1996; Sekar et al. 1997; Chandra et al. 2002a,b,c,d; Hansford et al. 2003; Singh et al. 2003). The crystal structures of complexes with transition-state analogs (Scott et al. 1990, 1991; White et al. 1990; Sekar et al. 1998) have provided insights into catalytic mechanism. The present structure reveals the presence of a fatty acid in the hydrophobic channel. This is the first naturally occurring PLA₂ with a fatty acid at the binding site, despite the presence of Asp 49. There are three earlier reports of PLA₂s having *n*-tridecanoic acid (Lee et al. 2001), palmitic acid (de Azevedo et al. 1999), and lauric acid (PDB: 1S8G), with all of them having Lys at the 49th position, where its presence was attributed as a product of incomplete hydrolysis due to Lys 49. Recently, the crystal structure of the complex of PLA₂ with elaidoylamide has been reported at 2.13 Å resolution (Georgieva et al. 2004). We present here a new observation on formation of a complex of PLA₂ with a fatty acid naturally present in the venom that might be a product of hydrolysis of phospholipids.

Results and Discussion

MALDI-TOF profile

The observed MALDI-TOF profile using crystal samples showed two peaks corresponding to 13198.1 and 13412.1 Da. The first peak matched exactly with the molecular mass

of PLA₂. The second peak, with an excess molecular mass of 214, was presumably of a complex of PLA₂ with an unknown ligand. An additional peak was also observed in the low molecular mass range corresponding to 208.6, presumably of dissociated ligand. It showed that the small molecular mass ligand was partly dissociated under MALDI-TOF conditions. Since the crystals were washed properly before using them on the MALDI-TOF, the presence of a ligand peak on the MALDI-TOF clearly indicated that the additional nonprotein ligand was part of the crystals.

Sequence of PLA₂

There are 411 base pairs that correspond to 137 amino acids (GenBank accession no. AY455754). The first 19 amino acids belong to a signal peptide. The mature protein has 118 amino acid residues. So far, the sequences of only four isoforms of PLA₂ from *Bungarus caeruleus* have been determined (Fig. 1). The sequence identities of these four isoforms varied from 73% to 85%. The present sequence has a higher pI value, and hence, it is more basic than other isoforms. The most significant sequence variations are observed in the β -wing region (residues 75–84) and the C-terminal part (residues 113–120) of these isoforms. The notable feature of the sequences of Krait venom PLA₂s is the presence of four glycines of seven residues between Cys 29 and Pro 37. It showed that the loop Cys 29–Pro 37 in Krait venom PLA₂s is indeed very flexible with anchors at Cys 29 and Pro 37. A strikingly notable feature in the sequence of the present PLA₂ is also related to the strong presence of several basic residues such as Lys 56, Lys 83, Lys 85, Arg 88, Lys 103, and Arg 114. The corresponding residues in other isoforms of Krait PLA₂ are nonbasic amino acids (Fig. 1). Also, the presence of Leu 5 in place of conserved Phe 5 is unique to this PLA₂. Only in Lys 49 group II PLA₂s, such

Figure 1. Multiple sequence alignment of present isoform of PLA₂ (A) (1TC8, AY455754) with its other three isoforms from *Bungarus caeruleus* venom: monomer PLA₂ (B) (1FE5, AF297663), dimer PLA₂ (C) (1U4J, AY455755), and trimer PLA₂ (D) (1G2X, AY455756), as well as with two fatty acid-binding Lys 49 PLA₂s; Piratoxin from *Bothrops pirajai* (PLA₂ E, 1QLL) and PLA₂ from *Agkistrodon contortrix laticinctus* (PLA₂ F, 1S8G). The identical residues are shown in blue, cysteines in yellow, and the important differences in red. The numbering scheme followed here is of PLA₂ (A).

as Piratoxin II from *Bothrops pirajai* (Lee et al. 2001), a Lys 49 PLA₂ from *Agkistrodon contortrix laticinctus* (PDB: 1S8G; Fig. 1), and Myotoxin I from *Bothrops nummifer* (de Azevedo et al. 1999), Leu is present at this position.

Crystal structure of the PLA₂ complex

The final electron density map [2Fo–F_c] represents a continuous and well-defined density for the backbone as well as for the side chains. The final R and R_{free} factors in the resolution range 20.0–2.7 Å were 0.20 and 0.24, respectively. The overall B factor for the structure was 34.2 Å². The final model of PLA₂ is of good stereochemical quality with rms deviations from ideal values being 0.009 Å and 1.3° for bond lengths and angles, respectively. A Ramachandran plot of the main-chain torsion angles (φ,ψ) (Ramachandran and Sasisekharan 1968), calculated using PROCHECK (Laskowski et al. 1993), showed that 87.6% of the residues were found in the most allowed regions.

The overall molecular framework of PLA₂ conserves all main features of the PLA₂ type of folding, although N-terminal helix (H1) is slightly shorter (residues 2–11) than that observed in earlier isoforms (Singh et al. 2001). The helix 2 (H2) extends from residues 40 to 55, while helix 3 (H3) from residues 90 to 108. The structure also contains a double-stranded antiparallel β-sheet designated as β-wing (residues 75–78 and 81–84). There are two helical turns involving residues 19–22 (SH4) and 113–115 (SH5) (Fig. 2).

The superimposition of the present structure on another isoform of PLA₂ from Krait venom (Singh et al. 2001)

Figure 2. The overall structure of PLA₂. The labels of α-helices and β-sheets are shown. The side chains of active site residues have been indicated. The ligand *n*-tridecanoic acid is also shown (magenta). The dotted lines indicate hydrogen bonds between PLA₂ and *n*-tridecanoic acid. The figure was produced using the programs MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt 1994).

Figure 3. Difference electron density [Fo–Fc] map at 2.5 σ cut off, into which the molecule of *n*-tridecanoic acid was modeled. The map was computed using all of the data to 2.7 Å resolution, and atoms of fatty acid were not present in the phase calculations. The numbering scheme of this ligand is also shown. The figure was produced using the programs BOBSCRIPT (Esnouf 1997) and RASTER3D (Merritt 1994).

showed an rms shift of 0.8 Å for their C^α positions. The notable variations were observed in the calcium-binding loops and for the C-terminal segments that are generally the most flexible regions of group I PLA₂s. It may be mentioned here that both of these isoforms have sodium ion in their calcium-binding loops. Also, there is a considerable change in the conformation of the segment from Trp 19 to Asn 24, as compared with earlier reported monomers (Singh et al. 2001). The side chain of Trp 19 in the present structure is significantly twisted, as compared with the earlier unliganded structure of Krait PLA₂ (Singh et al. 2001). This change in the orientation of Trp 19 may be the result of complex formation with a fatty acid.

Interactions between PLA₂ and *n*-tridecanoic acid

This is the first group I PLA₂ in which a fatty acid has been observed in the hydrophobic substrate-binding site. This is also the first PLA₂ with a normal catalytic composition with Asp 49, where a fatty acid has been found anchored at the substrate recognition site. The only other structures that have been observed with a fatty acid are myotoxin I from *Bothrops nummifer* (de Azevedo et al. 1999), piratoxin II from *Bothrops pirajai* (Lee et al. 2001), and a recently deposited structure of a Lys 49 PLA₂ from *Agkistrodon contortrix laticinctus* (PDB: 1S8G). All of these belong to group II and have a lysine residue at position 49. A similar electron density was also observed at the identical site in the Lys 49 PLA₂ from *Agkistrodon piscivorus piscivorus* (Holland et al. 1990). As seen from Figure 3, the *n*-tridecanoic acid is anchored to PLA₂ through the most commonly observed mode of binding, in which both His 48 and Asp 49 participate. The carboxyl oxygen atom O1 of *n*-tridecanoic acid forms a hydrogen bond with

water molecule OW7, which in turn interacts with both His 48 and Asp 49 through well-formed hydrogen bonds (Table 1). O1 atom also forms a hydrogen bond with His 48 N^{δ1}. The second oxygen atom of the carboxyl group forms a hydrogen bond with Gly 30 N. It may be noted that in a number of PLA₂-inhibitor complexes, the water molecule was found displaced by the ligand (Chandra et al. 2002a,b,c,d). In the present case, however, the binding of *n*-tridecanoic acid to PLA₂ occurs through a water molecule that has also been observed in several PLA₂-inhibitor complexes (Schevitz et al. 1995). The hydrocarbon chain of *n*-tridecanoic acid extends outwardly through the hydrophobic channel. The *n*-tridecanoic acid is almost completely buried in the hydrophobic channel of the enzyme. The buried surface areas of *n*-tridecanoic acid (324 Å²) and PLA₂ (228 Å²) show that the two molecules are indeed in a close fit. Also, the residues that get buried in the PLA₂ upon complex formation with fatty acid are Leu 5, Trp 19, Tyr 22, Gly 30, Lys 31, and Phe 101, as shown by a considerable decrease in their accessible surface areas from 76 Å² to 22 Å² for Trp 19; from 35 Å² to 5 Å² for Gly 30, and from 195 Å² to 174 Å² for Lys 31. It indicates that the fatty acid is inaccessible to solvent with its buried surface area amounting to 71% of the buried surface of PLA₂. It compares well with that of palmitic acid (76%) in the myotoxin I, a Lys 49 PLA₂ from *Bothrops nummifer* (de Azevedo et al. 1999). The solvent accessible surface areas were calculated using the CCP4 program Areaimol (Collaborative Computational Project 4 1994).

It was reportedly believed that fatty acids can be observed in a bound state in the active sites of those PLA₂s that had Asp 49 mutated to Lys 49 or if it is an inactive enzyme due to other alterations in the protein structure. However, the present studies and a recently reported complex of PLA₂ with elaidoylamide (Georgieva et al. 2004) indicate that the mutation of Asp 49 to Lys 49 is not the only condition. Despite considerable structural differences between

the present group I isoform and group II piratoxin, the nature of binding and the placement of the fatty acid are also nearly similar. Although the requirement of inhibition of PLA₂ in the venom is understandable, but as to why only particular isoforms are to be inhibited may have something to do with the basic environment in the molecule and not necessarily that Asp 49 has to be mutated to Lys 49. Therefore, if fatty acids are present in the venoms, they are likely to be complexed with all of those isoforms of PLA₂ that have a desirable basic environment, as in the present case. Thus, these studies provide important insights into the binding mechanism of PLA₂ with fatty acids. Similar observations were made in the complex of PLA₂ with elaidoylamide (Georgieva et al. 2004).

Materials and methods

Purification of the complex of PLA₂

Lyophilized *B. caeruleus* venom was obtained from the Irula cooperative snake farm, Tamil nadu, India, and purified as reported earlier (Singh et al. 2001). An amount (1 g) of venom was dissolved in deionized water to a concentration of 50 mg/mL. This was centrifuged at 8000g for 15 min to remove insoluble material. The supernatant was loaded onto a cation exchanger column, CM-Sephadex C-25 (60×1 cm), that had been equilibrated with 0.05 M ammonium acetate (pH 5.0) at 277 K. Proteins absorbed were eluted with a linear gradient from 0.05 M (pH 5.0) to 0.5 M (pH 7.0) of ammonium acetate. The flow rate was maintained at 6 mL/hr and 5-mL fractions were collected. A total of 11 major peaks corresponding to different isoforms of Krait PLA₂ were observed. The fractions corresponding to peak number VII were picked up and further fractionated on a Sephadex G-75 column (100×1 cm) with 0.1 M ammonium acetate (pH 6.5) at 277 K. The flow rate was maintained at 6 mL/hr and 5-mL fractions were collected. The major fraction was pooled and lyophilized. This contained homogenous preparation. The purity was checked on SDS-PAGE, which showed a single band with a molecular mass of ~13 kDa.

MALDI-TOF

The mass spectrometric analysis was performed on the washed protein crystals of krait PLA₂ complex using the MALDI-TOF instrument (KRATOS analytical). Crystals of PLA₂ were dissolved in distilled water (~10 mg/mL). Prior to the acquisition of spectra, 100 μL of PLA₂ solution was mixed with 100 μL of 0.2% aqueous trifluoroacetic acid. A total of 1 μL of the acidified solution was then spotted onto a stainless-steel sample slide, followed by 1 mL of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix solution (10 mg/mL in 50:50 ethanol/water containing 0.1% trifluoroacetic acid).

Sequence determination

The complete cDNA sequence determination of the purified samples was undertaken. The venom glands of Krait (*B. caeruleus*) were obtained from Irula Snake Catchers Cooperative Society,

Table 1. Interactions between PLA₂ and TDA

PLA ₂	Tridecanoic acid	Distances (Å)
Trp19 C ^{ε2}	D1 C12	3.25
Trp19 C ^{δ1}	D1 C12	3.41
Trp19 C ^{δ1}	D1 C13	3.58
Gly30 N	D1 O1	3.08
Gly30 C ^α	D1 C3	3.79
Gly30 C ^α	D1 C6	3.49
Gly30 C	D1 C6	3.40
Lys31 C ^α	D1 C6	3.85
His48 N ^{δ1}	D1 O2	3.07
His48 N ^{δ1}	OW 7	2.86
OW7	D1 O2	3.01
As49 O ^{δ1}	OW 7	2.96
OW	D1 O2	3.01

Table 2. Data collection and processing statistics

Space group	P4 ₁ 2 ₁ 2
Cell dimensions (Å)	
a = b	53.8
c	82.5
Vm (Å ³ /Da)	2.3
Solvent content (%)	46
Z	8
Resolution range (Å)	20.0–2.7
Number of reflections	
Measured	72,784
Unique	3,380
Redundancy	6.3
Completeness (%)	
Overall	90.1
Last shell (2.8–2.7 Å)	90.2
R _{merge} (%)	16.5
R _{merge} last shell (2.8–2.7 Å) (%)	40.7
Average I/σ(I)	5.3
I/σ(I) Last shell (2.8–2.7 Å)	2.4

Chennai, with the permission of the Government of Tamil Nadu. The glands were collected 2 d after devenomization of the animals. The minced glands were stored in guanidine isothiocyanate (4 M) solution at –70°C prior to use. The tissues were homogenized using polytron (Kinematica) homogenizer. The total RNA was extracted with an equal volume of phenolchloroform (1:1) mixture. Quantification of RNA was done spectrophotometrically. A total of 10 µg of total RNA was used for cDNA synthesis using Revert Aid M-MuLV reverse transcriptase (MBI) and oligo (dT)₁₈ primer. Conserved nucleotide sequences of group I PLA₂s were used for construction of primers. The oligonucleotides 5'-AAATGTATC CTGCTCACCTTCT-3' and 5'-GCTGAAGCCTCTCAAATATC AT-3' were used as forward and reverse primers, respectively, in PCR amplification using PTC 100 thermocycler (MJ Research). Automated DNA sequencing of the PCR product was performed in ABI-377 sequencer. Both of the strands were sequenced. The sequence was submitted to GenBank (GenBank accession no. AY455754).

Crystallization of the PLA₂–*n*-tridecanoic acid complex

The crystals of the PLA₂-TDA complex were obtained by the sitting-drop vapor-diffusion method. The protein was dissolved at 20 mg/mL in 50 mM Tris-HCl buffer (pH 8.5) containing 1.4 M NaCl, 1 mM NaN₃. It was equilibrated with the same buffer containing 2.4 M NaCl. After 3 d, the crystals were found to have grown to the largest size of 0.3 × 0.2 × 0.2 mm³.

Data collection and processing

The crystals of the complex were mounted in glass capillaries. X-ray intensities were measured at 285 K using a 345-mm MAR Research imaging plate scanner mounted on a Rigaku RU-300 X-ray generator equipped with focusing mirrors. The data were processed and scaled using DENZO and SCALEPACK (Otwinowski and Minor 1997). The crystals belong to space group P4₁2₁2 with unit cell dimensions of *a* = *b* = 53.8 Å, *c* = 82.5 Å. The details of data collection statistics are summarized in Table 2.

Structure determination and refinement

The structure was solved with molecular replacement using the AMoRe program (Navaza 1994) from the CCP4 software suite (Collaborative Computational Project Number 4 1994; Dodson et al. 1997). The rotation function was calculated using the coordinates of native monomeric isoform of PLA₂ from krait venom (PDB code 1FE5; Singh et al. 2001). The final rigid body refinement gave the correlation coefficient of 63.8% and an R-factor of 37.7%. This solution was applied to model coordinates using LSQKAB (Collaborative Computational Project 4 1994), and transformed coordinates were used as the starting model for the refinement using the CNS package (Brünger 1998). The reflections were treated as two data sets, one data set consisting of 5.3% of reflections (~200 reflections), which were randomly selected for the free R calculations (Brünger 1992), and the other set consisting of the rest of the data (3200 reflections). During the refinement, the bond lengths and bond angles were restrained close to the standard values, and restraints were also placed on the planarity of groups and nonbonded contacts as defined by Engh and Huber (1991). Several cycles of refinement using rigid-body conjugate-gradient minimization, simulated-annealing, and model building with the program O (Jones et al. 1991), brought the R-factor down to 0.30 and the R-free to 0.415 for all of the data in the resolution range 20.0–2.7 Å. At this stage, the individual isotropic B-factor refinement was carried out, which reduced the R-factor to 0.260 and R-free to 0.334. Manual model building using 12Fo–Fcl and lFo–Fcl electron density maps and further refinement by simulated annealing with molecular dynamics using a slow-cooling protocol from 3000 to 300 K (Brünger et al. 1990), brought the R-factor down to 0.234 and R-free to 0.321 for the reflections in the resolution range 20.0–2.7 Å. An extra nonprotein electron density was observed at the hydrophobic substrate-binding channel of PLA₂ at about 3.0 σ cut off in the lFo–Fcl map into which a molecule of fatty acid, *n*-tridecanoic acid, was readily fitted (Fig. 3). A clear density for sodium ion was observed in the calcium-binding loop at the 4 σ cut off. Together with these, the positions of 60 water molecules were also determined in the model for which the peaks were >2.5 σ in the lFo–Fcl map, were at approx-

Table 3. Refinement statistics

Refinement	
Resolutions limits (Å)	20.0–2.7
R factor (%)	19.8
Free-R factor (%)	24.1
No. of protein atoms	912
No. of sodium ions	1
No. of ligand (<i>n</i> -tridecanoic acid) atoms	15
No. of water molecules	60
Stereochemistry	
Bond lengths (Å)	0.009
Bond angles (deg)	1.4
Average B factors (Å ²)	
B-factor from Wilson's Plot	38.8
Main chain atoms	33.3
Side chain atoms and water	35.7
For all atoms	34.6
Residues in Ramachandran Plot	
Most favored regions (%)	87.6
Additional allowed regions (%)	11.4
Generously allowed regions (%)	1.0

priate distances from the hydrogen-bond partners, and B values were $<50 \text{ \AA}^2$ after refinement. The refinement improved further and finally converged to R and R-free factors of 0.198 and 0.241, respectively. The final refinement statistics for the structure are listed in Table 3. The refined coordinates have been deposited with the PDB and assigned the entry code 1TC8.

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