Crystal Structure of a Class XIB Phospholipase A$_2$ (PLA$_2$)

RICE (ORYZA SATIVA) ISOFORM-2 PLA$_2$ AND AN OCTANOATE COMPLEX

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Phospholipase A$_2$ catalyzes the specific hydrolysis of the sn-2 acyl bond of various glycerophospholipids, producing fatty acids and lysophospholipids. Phospholipase A$_{2s}$ (PLA$_{2s}$) constitute a large superfamily of enzymes whose products are important for a multitude of signal transduction processes, lipid mediator release, lipid metabolism, development, plant stress responses, and host defense. The crystal structure of rice (Oryza sativa) isoform 2 phospholipase A$_2$ has been determined to 2.0 Å resolution using sulfur SAD phasing, and shows that the class Xib phospholipases have a unique structure compared with other secreted PLA$_{2s}$. The N-terminal half of the chain contains mainly loop structure, including the conserved Ca$^{2+}$-binding loop, but starts with a short 3$_{10}$-helix and also includes two short anti-parallel $\beta$-strands. The C-terminal half is folded into three anti-parallel $\alpha$-helices, of which the two first are also present in other secreted PLA$_{2s}$ and contain the conserved catalytic histidine and calcium liganding aspartate residues. The structure is stabilized by six disulfide bonds. The water structure around the calcium ion binding site suggests the involvement of a second water molecule in the mechanism for hydrolysis, the water-assisted calcium-coordinate oxyanion mechanism. The octanoate molecule in the complex structure is bound in a hydrophobic pocket, which extends to the likely membrane interface and is proposed to model the binding of the product fatty acid. Due to the differences in structure, the suggested surface for binding to the membrane has a different morphology in the rice PLA$_2$ compared with other phospholipases.

Phospholipase A$_2$ (PLA$_2$)$^3$ catalyzes the specific hydrolysis of the sn-2 acyl bond of various glycerophospholipids, producing fatty acids and lysophospholipids. PLA$_{2s}$ are widely distributed in nature and constitute a large superfamily of enzymes whose products are important for a multitude of signal transduction processes, lipid mediator release, lipid metabolism, and host defense (1). In plants they are implicated in plant growth, development, stress responses, and defense (2–7). Translocation, secretion, and catalytic activation by many different stimuli control the activities of PLA$_{2s}$. Based on sequence, and specific characteristics, 15 distinct groups of PLA$_{2s}$ are defined, and these are divided into 5 main clades within the superfamily: secreted sPLA$_{2s}$, cytosolic cPLA$_{2s}$, calcium-independent iPLA$_{2s}$, platelet-activating factor acetylhydrolases, and the lysosomal PLA$_{2s}$ (8). The sPLA$_{2s}$ are small secreted proteins of 14–18 kDa that usually contain 5–8 disulfide bonds and an active site His/Asp dyad and are dependent on binding of Ca$^{2+}$ ions for activity (1). The sPLA$_{2s}$ display different tissue distribution patterns and distinct physiological functions. The sPLA$_{2}$ contain PLA$_{2}$ groups I–III, V, and IX–XIV, including the snake venoms PLA$_{2s}$ (9) and the mammalian pancreatic PLA$_{2}$s (10). Members of this family were first studied nearly 100 years ago, and a wealth of information on their structures and molecular action is available; to date, searching the Protein Data Bank for phospholipase A$_2$ yields 230 structure hits, the majority of which refer to sPLA$_{2s}$.

The eukaryotic sPLA$_{2s}$ all contain highly conserved Ca$^{2+}$-binding loop (XCGXGG) and catalytic site (DXCCXHXD) motifs. The catalytic mechanism was originally proposed by Verheij et al. in 1980 (11) and subsequently modified from available crystal structures (12, 13). In the catalytic cycle, substrate hydrolysis proceeds through the activation and orientation of a water molecule by hydrogen bonding to the active site histidine. Adjacent to this histidine there is a conserved aspartate residue, which, together with main-chain carbonyl-oxygen atoms from the Ca$^{2+}$-binding loop, acts as ligands for Ca$^{2+}$. The calcium ion assists by polarizing the scissile bond and by stabilizing the negative charge developing in the transition state during phospholipid hydrolysis (14). More recently, a second water molecule, bridging the Ca$^{2+}$-coordinated catalytic water to the active site histidine, has been inferred to be involved in catalysis, the water-assisted calcium-coordinate oxyanion mechanism of PLA$_{2}$ (15–18). An interesting aspect of PLA$_{2}$ catalysis is interfacial activation; i.e. the activity on monomeric substrates is low but hugely increases on aggregated substrates (19).

The three-dimensional structures of class I, II, and X PLA$_{2s}$ are very similar and are mainly folded into three long $\alpha$-helices, a two-stranded $\beta$-sheet referred to as the $\beta$-wing, and a conserved calcium-binding loop (9–10, 12, 20–21). The same motifs are also present in the class III enzymes (13), although these proteins are more divergent. A structure of a prokaryotic...
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sPLA₂ (class XIV) showed a different, all α-helical fold (22), whereas structures of PLA₂s from the other sPLA₂ families have not yet been determined.

Only a few plant sPLA₂s have been characterized (23–28), and these have been assigned as subfamilies X1a and X1b based on sequence alignments (7–8). In rice (Oryza sativa) a minimum of three isoforms of sPLA₂ are present (24). Isoform 2 (class X1b) contains 128 amino acids preceded by a 25-amino acid signal peptide and contains the conserved active site and calcium ion binding motifs of sPLA₂s but otherwise shows low homology to other classes of sPLA₂ (24). Here we present the crystal structure of the mature isoform 2 PLA₂ from rice (rPLA₂) as well as that of a complex with octanoic acid.

EXPERIMENTAL PROCEDURES

Cloning and Expression—A synthetic cDNA coding for the predicted mature rPLA₂ protein, amino acids 1–128 without the signal peptide, was designed with optimized codons for high level expression in Escherichia coli. The cDNA was generated by a sequence of five PCR reactions using ten synthesized single-stranded oligonucleotides, each about 60 nucleotides in length. These are listed as supplemental material in Table IS, and the cDNA sequence obtained for the mature rPLA₂ is shown in supplemental Fig. S1a. The amplification started with the two most central oligonucleotides, 5’-5 and 3’-1, designed with 20 nucleotides overlap, being mixed and amplified with a 1 to 1 mixture of Taq and Pfu DNA polymerases. For each PCR cycle the growing fragment was extended by ~40 nucleotides in each direction. After each PCR reaction the obtained DNA fragment was separated on agarose gel, excised, and purified, before use as template in the next PCR amplification. In the second PCR oligonucleotides 5’-4 and 3’-2 were used, in the third PCR 5’-3 and 3’-3, in the fourth PCR 5’-2 and 3’-4, and finally in the fifth PCR oligonucleotides 5’-1 and 3’-5 were used. The final DNA fragment was then amplified using two different 5’ PCR primers together with a 3’ PCR primer (supplemental Table IS) to obtain two constructs with slightly different N-terminal amino acid sequences. The A construct has a start methionine and an alanine in front of Asn-2 of the rPLA₂ sequence, and the B construct has a start methionine and a glycine in front of Leu-1, see supplemental Fig. S1b. The two cDNA constructs were gel-purified, blunt-end-polished with T4 DNA polymerase, phosphorylated, and then blunt-end-cloned into the EcoRV-digested and dephosphorylated PETBlue-1 vector. The sequences of the inserts and the cloning borders with the plasmid were verified by sequence analysis.

The E. coli strain Tuner (DE3) LacI was used as host for the expression of the rPLA₂ constructs. A preculture of 30 ml of Luria broth media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol was inoculated from fresh single colonies and grown at 37 °C until the A600 reached ~0.5. The preculture was used to inoculate 1 liter of the same media, which was grown until A600 reached 0.5–0.8 before isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. The culture was then grown for an additional 4 h after which the cells were harvested by 15-min centrifugation at 3000 × g at 4 °C, frozen, and stored at −80 °C until further use.

Refolding and Purification—Purification, solubilization, and refolding were done according to Valentin et al. (29) with some modifications. The cell pellet was thawed and resuspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 1% deoxycholate) by stirring for 1 h at 4 °C. Cells were broken by passing the cell slurry twice through a precooled French press and an inclusion body pellet was obtained by centrifugation at 10,000 × g for 20 min. The inclusion body pellet was washed by four cycles of resuspension in lysis buffer (two times with and two times without detergents), followed by sonication for 10 intervals of 15 s on ice with 15 s of cooling in between. After each cycle the inclusion bodies were pelleted by centrifugation at 10,000 × g for 20 min. The washed inclusion body pellet was either used directly or frozen at −80 °C.

Cysteine residues of the rPLA₂ were then sulfonated. The washed inclusion body pellet was dissolved to a concentration of ~10 mg of wet pellet/ml in 50 mM Tris-HCl, pH 8.0, 6 M guanidine chloride, 0.3 M Na2SO3 by stirring at room temperature. A 0.05 volume of Thannhauser reagent (30) was added, and the solution was stirred at room temperature for a further 1 h. The extract was then dialyzed overnight against 50 volumes of 1% acetic acid at 4 °C. Precipitated sulfonated protein was collected by centrifugation at 10,000 × g for 20 min.

The protein pellet was dissolved to a protein concentration of 10 mg/ml in 50 mM Tris-HCl, pH 8.0, 6 M guanidine chloride at room temperature by end-over-end rotation for 1 h. The protein solution was added dropwise at one drop per second to refolding buffer (0.9 M guanidine chloride, 50 mM Tris-HCl, pH 8.0, 10 mM CaCl2, and 5 mM freshly added L-cysteine, 30% acetonitrile) to a final concentration of ~0.02 mg/ml with constant stirring at room temperature. The refolding solution was then stirred for a few minutes before it was allowed to stand at room temperature for 2–3 days. The refolding was monitored by measuring PLA₂ activity.

Lubrol PX 0.01% and 1 mM of methionine were added to the solution, which was then loaded into an Amicon-stirred cell with a YM-3 filter (Millipore) and concentrated ~100-fold. The concentrate was dialyzed (cut-off of 10 kDa) overnight against 10 mM Tris/HCl, pH 8.0, containing 1 mM methionine. A freshly packed 10-ml Q-Sepharose Fast Flow (Amersham Biosciences) column was run with buffer A (20 mM Tris/HCl, pH 8.0, with 1 mM methionine and acetonitrile 20%) and buffer B (as buffer A plus 1 M NaCl) and eluted with a 30-ml gradient from 0 to 25% B. During elution 1-ml fractions were collected, and the fractions with peaking PLA₂ activity were pooled.

The pooled Q-Sepharose Fast Flow eluate was injected onto a C4 reversed-phase high-performance liquid chromatography column (0.46 × 10.0 cm, Vydac, Hesperia, CA), previously equilibrated with 0.1% trifluoroacetic acid. The column was developed at 1.0 ml/min with a 30-min gradient (35–41% of acetonitrile in 0.1% trifluoroacetic acid). Absorbance was monitored at 214 and 280 nm, peak fractions were collected manually, and their pH was adjusted by adding 10 µl of Tris base per ml of eluate. The acetonitrile content was reduced by evaporation in a SpeedVac concentrator. The pooled fractions from several high-performance liquid chromatography runs were dialyzed against 10 mM Tris-HCl, pH 8.0, overnight at 4 °C,
divided into aliquots, and stored at −80 °C. Purity of the sample was confirmed by SDS-PAGE and the protein concentration was calculated from the absorbance measured at 280 nm of a small volume of sample diluted in 20 mM sodium phosphate, pH 6.5, 6 M guanidine chloride, using the extinction coefficient 5120 M⁻¹ cm⁻¹. Prior to crystallization the protein was concentrated to 7–9 mg/ml in 10 mM Tris-HCl, pH 8.0, and filtered through a 0.1-μm centrifugal filter.

PLA₂ Assay—PLA₂ assays were performed according to Ståhl et al. (23). In brief, 10 nmol of 1-palmitoyl-2-[14C]oleoylphosphocholine dissolved in assay buffer (50 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, and 0.06% (w/v) Lubrol PX) was incubated with enzyme fractions (0.5–10 μl) in a total volume of 50 μl at 30 °C for 5 min. The reaction was stopped, and the lipids were extracted according to the method of Bligh and Dyer (31). The lipid-containing chloroform phases from PLA₂ assays were divided into aliquots, and stored at −80 °C. Purity of the sample was confirmed by SDS-PAGE and the protein concentration was calculated from the absorbance measured at 280 nm of a small volume of sample diluted in 20 mM sodium phosphate, pH 6.5, 6 M guanidine chloride, using the extinction coefficient 5120 M⁻¹ cm⁻¹. Prior to crystallization the protein was concentrated to 7–9 mg/ml in 10 mM Tris-HCl, pH 8.0, and filtered through a 0.1-μm centrifugal filter.

Phasing, Model Building, and Refinement—Phases were obtained by sulfur-SAD phasing using data collected to 2.2-Å resolution from a single hexagonal crystal on beamline BM14, which is equipped with a Mar225 charge-coupled device detector. 395 degrees of data were collected at a wavelength of 1.771 Å using a 1° oscillation angle, to give a single highly redundant dataset. Higher resolution native data were later collected to a resolution of 2.0 Å on the micro focus beamline ID23-2, using a second crystal. Native data from the orthorhombic crystal form were collected on beamline ID29 to a resolution of 2.3 Å. In each case the data were processed using the HKL2000 suite (32). Where necessary, the scaled intensities were converted to CCP4 format using Scalepack2MTZ from the CCP4 suite (33), and structure factors were calculated using TRUNCATE. Data from the octanoic acid soaked crystals were collected on beamline ID14-1 at European Synchrotron Radiation Facility and processed using Mosflm (34) and SCALA from the CCP4 suite (33). Table 1 shows a summary of the statistics for each of the four datasets.

| Data set | Sulfur SAD | High resolution | Octanoic acid soak | Crystal form 2 |
|----------|------------|-----------------|-------------------|---------------|
| Space group | P6₃ | P6₃ | P6₃ | P2₁2₁2₁ |
| Unit cell (Å) | 108.5, 108.5, 41.54 | 108.7, 108.7, 41.65 | 109.2, 109.2, 41.39 | 169.1, 41.49, 53.12 |
| Molecules in asymmetric unit | 2 | 2 | 2 | 3 |
| Resolution (Å) | 50-2.2 (2.28-2.2) | 30-2.0 (2.07-2.0) | 30-2.0 (2.11-2.0) | 30-2.3 (2.42-2.3) |
| Rmerge (%) | 0.092 (0.320) | 0.079 (0.334) | 0.074 (0.237) | 0.090 (0.326) |
| Completeness (%) | 99.1 (93.5) | 99.7 (98.0) | 98.4 (90.8) | 95.0 (93.2) |
| Multiplicity | 39.1 (4.3) | 13.91 (1.96) | 12.6 (4.2) | 17.4 (5.4) |
| Anomalous completeness (%) | 22.7 (12.5) | 3.4 (2.9) | 3.8 (2.4) | 4.8 (4.9) |
| Anomalous multiplicity | 99.0 | 99.0 | 99.0 | 99.0 |

Data Collection and Processing—All x-ray diffraction data were collected at the European Synchrotron Radiation Facility, Grenoble, France. The structure of the rice PLA₂ was solved by sulfur-SAD phasing using data collected to 2.2-Å resolution from a single hexagonal crystal on beamline BM14, which is equipped with a Mar225 charge-coupled device detector. 395 degrees of data were collected at a wavelength of 1.771 Å using a 1° oscillation angle, to give a single highly redundant dataset. Higher resolution native data were later collected to a resolution of 2.0 Å on the micro focus beamline ID23-2, using a second crystal. Native data from the orthorhombic crystal form were collected on beamline ID29 to a resolution of 2.3 Å. In each case the data were processed using the HKL2000 suite (32). Where necessary, the scaled intensities were converted to CCP4 format using Scalepack2MTZ from the CCP4 suite (33), and structure factors were calculated using TRUNCATE. Data from the octanoic acid soaked crystals were collected on beamline ID14-1 at European Synchrotron Radiation Facility and processed using Mosflm (34) and SCALA from the CCP4 suite (33). Table 1 shows a summary of the statistics for each of the four datasets.
nation of the partial model and refined sulfur sites using PHASER_EP, and then phase improvement with PIRATE and, once the non-crystallographic symmetry operator became known, DM (41) with NCS. Tight NCS restraints were applied during refinement. After a few rounds, the electron density maps became of excellent quality allowing unambiguous chain tracing and sequence fitting. Final refinement with inclusion of water molecules was performed using the high resolution data set to 2.0 Å with medium main-chain and loose side-chain NCS restraints. A final check of the model building was performed by calculating a composite omit map in CNS (42).

The structure of the second crystal form was determined by molecular replacement in MOLREP (43), using the monomer of the refined P6₂ structure as the search model. The initial search placed two monomers in the asymmetric unit, but manual inspection of the resulting maps showed that a third monomer was present, albeit with weaker density. A second MOLREP search, performed after fixing the positions of both previously identified monomers, was successful in placing the final copy of the protein. The structure was refined with alternating cycles of restrained refinement in REFMAC5 (40) and manual inspection and model building in COOT. Initial refinement included the use of medium NCS restraints on all monomers, but this was later relaxed for chain C, in which crystal-packing effects result in slight differences for the other two chains. In the final stages of refinement, atomic displacement parameters were refined in REFMAC by the TLS (translation, libration, and screw) method (44) with each of the three monomers in the asymmetric unit treated as a single TLS group.

The structure of the octanoic acid complex was solved by molecular replacement, using the monomer of the refined P6₂ structure as the search model. Restrained refinement with NCS was performed in REFMAC5 and model building in COOT.

The geometry of each model was checked using the validation functions of COOT in addition to a final analysis in PROCHECK (45), and the fit between the data and model was assessed in SFCHECK (46). The results from refinement are detailed in Table 2. Structure alignments were carried out using the SSM superposition function in COOT, and LSQ in the program O (47). The figures were prepared by using PyMOL.4

Structure factors and the coordinates of the final models have been deposited in the Protein Data Bank (http://www.rcsb.org) with accession codes native P6₂: 2wg7; native P2₁2₁2: 2wg8; and octanoic acid complex: 2wg9.

RESULTS AND DISCUSSION

Cloning, Refolding, and Purification—To produce enough material for crystallization trials of a plant sPLA₂, a synthetic gene approach, with optimized codons for high levels of expression, was used to produce large quantities of recombinant mature rPLA₂ enzyme in E. coli. This strategy has been successfully used for several animal sPLA₂ enzymes (49–53). The synthetic gene (supplemental Fig. S1a) was generated by five consecutive PCR reactions using ten oligonucleotides (supplemental Table IS). To optimize the chance of obtaining correct refolding of the protein, removal of the start methionine was desired, and this is known to occur more likely with a small amino acid in the second position of the sequence (51). To achieve this, two different variants were generated with slightly different N-terminal amino acid sequences (supplemental Fig. S1b). In construct A, Leu-1 of rPLA₂ was mutated to an alanine, whereas in construct B an extra glycine was inserted between the start methionine and Leu-1.

The proteins were produced in E. coli as inclusion bodies and therefore easily purified to a high level by simple washing of the inclusion body pellet. After solubilization in guanidine hydrochloride, and reduction and sulfonation of cysteine residues, the protein was refolded and purified. The yield of refolded and purified protein was ~10 mg/liter E. coli expression culture, and both were shown to be homogeneous by SDS-PAGE. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry confirmed that the start methionine was removed from both constructs. The A and B variants of the mature rPLA₂ showed similar specific activity after refolding, at best ~33 μmol/min/mg.

Crystallization and Data Collection—Initial crystallization trials failed to produce crystals but did yield spherulites in a single condition for the B-construct. Crystals were ultimately achieved by using Hampton Crystal Screen HT as an additive screen, which resulted in the unusual crystallization condition described under “Experimental Procedures.” From the 96 conditions of the Hampton screen, I gave a single, small, cluster of crystals when mixed with the original well solution at a 1 to 3 ratio. Streak seeding gave the same crystals that were used for data collection. The hexagonal, rod-shaped crystals grew to approximate maximum dimensions of 20 × 20 × 80 μm and diffracted in space group P6₂ or P6₄ with unit cell dimensions of a = 108.67 Å, b = 108.67 Å, and c = 41.65 Å. The asymmetric unit contained two monomers, and the solvent content of the crystals has been estimated at 52%.

Crystals of the A construct of the protein were obtained from cross-seeding with the hexagonal crystals obtained with the B construct and using the same crystallization conditions. The crystals grew to approximate maximum dimensions of 80 × 40 × 5 μm and diffracted in space group P2₁2₁2 with unit cell dimensions of a = 169.09 Å, b = 41.49 Å, and c = 53.12 Å. The asymmetric unit contained three monomers, and the solvent content of the crystals has been estimated at 45%.

| Table 2 | Refinement and model building statistics |
|---------|----------------------------------------|
| Data set | Crystal form P6₂ | Crystal form P2₁2₁2 | Octanoic acid soak |
| Refinement statistics | | | |
| Reflections in working set | 18,172 | 15,596 | 18,061 |
| Reflections in test set | 986 | 846 | 963 |
| R-factor/R-free | 0.185/0.230 | 0.190/0.245 | 0.186/0.220 |
| Number of protein atoms | 1,826 | 2,477 | 1,757 |
| Number of calcium ions | 2 | 3 | 2 |
| Number of waters | 176 | 206 | 176 |
| Average B-factor | 23.68 | 26.49 | 21.67 |
| r.m.s.d. values from ideal | | | |
| Bond lengths (Å) | 0.008 | 0.009 | 0.008 |
| Bond angles (°) | 1.021 | 1.195 | 1.005 |
| Ramachandran plot | | | |
| Preferred regions | 95.98% | 96.24% | 95.35% |
| Allowed regions | 4.04% | 3.76% | 4.65% |
| Outliers | 0.0% | 0.0% | 0.0% |

4 W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
Quality of the Electron Density Map and Model—The final electron density maps from the 2.0-Å resolution data in the P6$_2$ space group, containing two subunits in the asymmetric unit, allowed unambiguous tracing of residues 13–125 in both subunits with the exception of some flexible side chains at the surface. In addition, the positions of the main chain of residues 2–7 at the N terminus could be defined. Two calcium ions, 176 bound water molecules, and 1 sodium ion on the surface of the molecule were modeled. The resulting R and R$_{free}$ values were 18.4 and 23.0, respectively. The geometry of the model is good with 96% of the residues in the most favored regions and none in the disallowed regions of the Ramachandran plot. The two subunits superimpose with an r.m.s.d. of 0.28 Å for all main-chain atoms. In the orthorhombic space group, containing three subunits in the asymmetric unit, residues 15–121 in all three subunits are defined and very similar to those in the hexagonal lattice (r.m.s.d. of ~0.5 Å for the main-chain atoms, except subunit C, which is less ordered and distorted by packing interactions in the crystal) and the N-terminal residues 1–8 could be modeled in subunit B. Three calcium ions and 206 water molecules were modeled. The subunit structures in the octanoic acid complex were virtually identical to the unliganded structure (r.m.s.d. of ~0.23 Å for the main-chain atoms), but the N-terminal residues were not defined. The refinement results are shown in (Table 2). Due to the higher resolution of the data in the hexagonal space group, we will use this in the description of the structure.

Overall Structure—A schematic picture of the structure of rPLA$_2$ is shown in Fig. 1. The chain starts with a weakly defined 3$_{10}$-helix (residues 2–7), which makes few interactions to the rest of the molecule and which is stabilized by crystal contacts. After a five-residue gap, for which no electron density is visible, the chain continues with a loop (residues 12–21) held in place by two disulfide bonds, Cys$_{17}$–Cys$_{45}$ and Cys$_{21}$–Cys$_{51}$. Following two short anti-parallel $\beta$-strands (residues 22–25 and 32–35 respectively), the chain forms the Ca$^{2+}$-binding loop (residues 36–44). After a surface loop, the remainder of the protein chain is folded into three anti-parallel $\alpha$-helices (residues 52–69, 74–88 and 99–125 respectively) with connecting loops. The last of these helices is particularly long and its C-terminal protrudes from the core of the structure. All 12 cysteines participate in disulfide bonds; Cys$_{17}$–Cys$_{45}$, Cys$_{21}$–Cys$_{51}$, Cys$_{26}$–Cys$_{98}$, which connect the N- and C-terminal part of the protein, Cys$_{38}$–Cys$_{58}$ anchors the Ca$^{2+}$-binding loop to $\alpha$-helix 1, and Cys$_{57}$–Cys$_{84}$ and Cys$_{64}$–Cys$_{77}$, which tether $\alpha$-helices 1 and 2. In the crystal, the two monomers in the asymmetric unit form a dimer burying a surface area of about 1000
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\textbf{A} Calcium binding loop

\begin{center}
\begin{tabular}{c}
\text{Asp 62} \hspace{1cm} \text{His 61} \\
\text{Ca$^{2+}$} \\
\text{W$_c$} \hspace{1cm} \text{W$_a$}
\end{tabular}
\end{center}

\textbf{B} Calcium binding loop

\begin{center}
\begin{tabular}{c}
\text{Asp 62} \hspace{1cm} \text{His 61} \\
\text{Ca$^{2+}$} \\
\text{Octanoate}
\end{tabular}
\end{center}

\textbf{C}

\begin{center}
\begin{tabular}{c}
\text{Asp 62} \hspace{1cm} \text{His 61} \\
\text{Cys 58} \hspace{1cm} \text{Ca$^{2+}$} \\
\text{Cys 38} \hspace{1cm} \text{Cys 47} \\
\text{Ile 111} \hspace{1cm} \text{Ile 107} \\
\text{Val 110} \hspace{1cm} \text{Val 106} \\
\text{Leu 32} \hspace{1cm} \text{Ala 29}
\end{tabular}
\end{center}

FIGURE 3. \textbf{A}, the catalytic residue His$^{61}$ and bound Ca$^{2+}$ with ligands. Only the main-chain atoms are shown for the Ca$^{2+}$-binding loop. Polar interactions are shown with dashed lines. The catalytic water and the assisting water are labeled W$_c$ and W$_a$, respectively. \textbf{B}, octanoate bound to Ca$^{2+}$. Polar interactions are shown with dashed lines. \textbf{C}, the hydrophobic walls of the active-site cleft with bound octanoate. The unbiased 2Fo - Fc map at 1.2$\sigma$ is shown as green mesh.

Å$^2$. This positions the openings to the two active sites at opposite ends of the dimer. Because there are no hints from gel-filtration experiments that the enzyme is dimeric, it does not seem likely that the dimer observed in the asymmetric unit is physiologically relevant. Another subunit-subunit interaction, observed in the hexagonal lattice and for one subunit in the orthorhombic crystal, is obtained by crystal symmetry and is made by the hydrophobic surface of the amphiphilic N-terminal 3$_{10}$-helix of one subunit and the hydrophobic tunnel leading to the active site of a neighboring subunit. At high concentrations of rPLA$_2$, this interaction might become of importance by blocking the catalytic site. This N-terminal 3$_{10}$-helix is obviously quite dynamic, and we believe it is involved in anchoring the rPLA$_2$ to the membrane interface as discussed below.

Comparison to Other PLA$_2$ Structures—The structure of rPLA$_2$, distinct from those of other sPLA$_2$s. A DALI search (54) against the Protein Data Bank gives the highest scores to vaccular protein sorting factor 4a, toxin B, and vaccular protein sorting-associated protein 4 due to the similar orientations and lengths of the three $\alpha$-helices between these structures. In the fourth position is a group III PLA$_2$, bee venom PLA$_2$ (PDB id 1poc) (13), which has an r.m.s.d. from DALI of 2.9 Å for 80 superposed $\text{Ca}$ atoms with 15% sequence identity. Number 13 in the list is PLA$_2$ from the venom of Ophiophagus hannah (PDB id 1m8t) (55) with an r.m.s.d. of 3.0 Å for 62 superposed $\text{Ca}$ atoms with 29% sequence identity. In both cases $\alpha$-helices 1 and 2 superpose well, and the general location of the Ca$^{2+}$-binding loop is conserved (Fig. 2). Although the Ca$^{2+}$-binding loop of rPLA$_2$ contains a one-residue insertion compared with other eukaryotic PLA$_2$s, and thus has a different conformation, the Ca$^{2+}$-ion is bound in the same position. The 30 first residues of rPLA$_2$ are not present in the class III bee venom PLA$_2$. The C-terminal third helix is present in both proteins but is much longer in rPLA$_2$, whereas the bee PLA$_2$ instead continues with a very long $\beta$-hairpin-like structure (Fig. 2B). The C-terminal $\alpha$-helix 3 of rPLA$_2$ is in the same location as the shorter N-terminal $\alpha$-helix in the class I, II, and X sPLA$_2$s but is in the reverse direction and with different orientation of the axis (Fig. 2C). This arrangement positions the N and C termini of rPLA$_2$ on opposite sides of the active site compared with these sPLA$_2$s. Furthermore, the so-called $\beta$-wing, present in other eukaryotic sPLA$_2$s, is absent from rPLA$_2$. The structure of rPLA$_2$ is thus different from the known sPLA$_2$s. Three disulfides are conserved in all three proteins, Cys$^{38}$–Cys$^{58}$ anchoring the Ca$^{2+}$-binding loop to $\alpha$-helix 1 and Cys$^{57}$–Cys$^{94}$ and Cys$^{64}$–Cys$^{77}$ between $\alpha$-helices 1 and 2. The only other conserved residues between all three proteins are the catalytic His$^{61}$, the Ca$^{2+}$-ligand Asp$^{62}$, and Gly$^{39}$ and Asp$^{55}$, which are important for maintaining the structure of the Ca$^{2+}$-binding loop.

Active Site and Substrate Channel—Besides the conserved Asp$^{62}$, the Ca$^{2+}$-ion is ligated by the main-chain carboxyl oxygen atoms of Tyr$^{37}$, Gly$^{39}$, and Tyr$^{41}$ from the Ca$^{2+}$-binding loop, and two water molecules (Fig. 3A). The Ca$^{2+}$-bound water molecule, which acts as a nucleophile in the reaction, is in turn hydrogen-bonded to three other water molecules. The positions of these waters are similar in the two monomers of the asymmetric unit, but the density indicates that their occupancies differ. Strong density is observed in both subunits for the Ca$^{2+}$-bound water and for another that is bound to it. In subunit A, clear density is observed for a third, which interacts with the Ca$^{2+}$-bound water, the side chains of His$^{61}$ and Asp$^{62}$ and the main-chain carboxyl of Cys$^{38}$, whereas weak density suggests the presence of a fourth water molecule interacting only with the other waters. In subunit B the latter has strong density,
whereas very little density is seen for the water coordinating His<sup>61</sup>, Asp<sup>62</sup>, and Cys<sup>58</sup>. The water at this position is interesting, because it corresponds to the proposed assisting water molecule, and its presence supports the water-assisted calcium-coordinate oxyanion mechanism of PLA<sub>2</sub> (15–18). However, this assisting water has been assumed to be present only in the activated form of sPLA<sub>2</sub> (56) and to be the result of the interfacial activation process (57). In rPLA<sub>2</sub>, His<sup>61</sup> is held in its proper orientation by interaction with the side-chain oxygen atom of Asn<sup>78</sup>, which substitutes the aspartate residue in the catalytic Asp/His dyad of other eukaryotic sPLA<sub>2</sub>s. Asn<sup>78</sup> is replaced by a serine residue in some of the other plant sPLA<sub>2</sub> enzymes, and substitution of this serine to alanine or aspartate in the Arabidopsis thaliana PLA<sub>2</sub>-α resulted in considerable loss of activity (58) confirming the importance of this interaction. The hydrophobic part of the active site is lined by the disulfide Cys<sup>38</sup>–Cys<sup>58</sup>, together with residues Leu<sup>12</sup>, Ile<sup>40</sup>, Leu<sup>81</sup>, Ile<sup>85</sup>, Val<sup>106</sup>, Ile<sup>107</sup>, Val<sup>110</sup>, and Ile<sup>111</sup> (Fig. 3C) at the inner surface. It is then extended by a hydrophobic channel (Fig. 4A), comprising residues Ala<sup>29</sup>, Pro<sup>30</sup>, Val<sup>65</sup>, Tyr<sup>72</sup>, Leu<sup>41</sup>, and Ala<sup>114</sup>, that leads to what we propose is the interfacial binding site. The hydrophobic character of the residues in this crevice and lining the active site is conserved in the whole class XIB, but their identities vary, which might give rise to differences in substrate preference. In other eukaryotic sPLA<sub>2</sub>s there is a “flap” residue, a tyrosine or a lysine, that is suggested to facilitate the dynamic transfer of substrates from the membrane to the active site and to bind the phospholipid phosphate head-group in the transition state (48). In a corresponding spatial position in rPLA<sub>2</sub> is Tyr<sup>72</sup>, which is conserved in the class XIB sPLA<sub>2</sub>s. In the ligand-free enzyme it is well defined but with some disorder in the side chain, stabilized by interactions with the N-terminal 3<sub>10</sub>-helix of a symmetry related subunit that binds with its hydrophobic face to the channel (Fig. 4A). Changing the rotamer conformation of Tyr<sup>72</sup> to that most commonly observed in structures of sPLA<sub>2</sub>s would put the hydroxyl group in the same position as in the transition state analogue complex PDB id 1mkv (48), interacting with the phospholipid head-group. In the octanoate complex, where this 3<sub>10</sub>-helix is disordered, the Tyr<sup>72</sup> side chain is also more or less disordered.

Octanoic Acid Complex—Unbiased 2Fo–F<sub>c</sub> electron density maps clearly defined the binding mode of octanoate in the crystals of the complex (Fig. 3C). Binding of the product molecule octanoic acid does not introduce any large structural changes in the protein. The N-terminal residues are not defined in the complex structure, although the resolution and packing are the same as for the unbound structure, the binding of octanoate might interfere with this subunit interaction. The octanoate molecule binds in the active site (Fig. 3B), with one carboxylate oxygen replacing the catalytic water as ligand to the Ca<sup>2+</sup>-ion and the second carboxylate oxygen substituting the assisting water molecule (Fig. 3B), which is in accordance with the mechanism with the Ca<sup>2+</sup>-bound water molecule as the nucleophile attacking the ester bond. The position of the carboxylate group of octanoate is very similar to the phosphate group of the transition state analog in PDB id 1mkv (48), and in PDB id 1pob (12), and to the inhibitor MJ33 in PDB id 1poc (13), but different from that in PDB id 1fxp (56).

The hydrophobic tail of octanoate makes favorable interactions to the hydrophobic walls of the active site, residues Ala<sup>29</sup>, Gly<sup>39</sup>, Ile<sup>40</sup>, Cys<sup>58</sup>, Leu<sup>81</sup>, Ile<sup>107</sup>, and Ile<sup>111</sup>, replacing several water molecules in the structure of the unbound active site crevice (Fig. 3C). It seems likely that the structure represents the true conformation of a bound fatty acid product.

The Membrane Interface Site—The hydrophobic crevice described above allows the transfer of phospholipid substrates from the membrane to the active site. The channel
leads to a predominantly hydrophobic outer surface, which is likely to face the lipid-aqueous interface of the membrane or substrate aggregates. The flexible N-terminal \(3_{10}\)-helix, part of the \(\alpha\)-helical C-terminal, Tyr\(^{72}\) and Leu\(^{83}\) in the loop between \(\alpha\)-helix 1 and 2, and Phe\(^{35}\), Ile\(^{38}\), and Leu\(^{31}\) in the loop between the two anti-parallel \(\beta\)-strands, constitute the most probable interacting surface, the i-face (Fig. 4B). These parts of the structure also display the highest flexibility as indicated by the main-chain \(B\)-factors. The surface formed by these residues is not flat; it is rather concave with Tyr\(^{72}\) in the middle of the surface. The conserved residues Tyr\(^{72}\), the “flap residue,” and Arg\(^{33}\), basic residues at the C terminus, also present in the whole class, and conserved polar residues of the dynamic N-terminal \(3_{10}\)-helix, might be the residues anchoring the enzyme to the polar head-groups at the membrane surface. Upon close contact of the i-face to the membrane, desolvation, and perhaps curvature of the membrane, allows for tight association, which permits the substrate to diffuse into the active site. Due to the different architectures of the rPLA\(_2\) and other sPLA\(_2\)s, this i-face is somewhat different from that defined by e.g. the crystal structure of the anion-assisted dimer of porcine pancreatic PLA\(_2\) (56). It has been suggested that the allosteric coupling between the i-face and the active site is mediated by hydrogen-bond networks (57) and that the resulting higher activity at the interface is due to the presence of the “activating water” (56). Similar hydrogen-bond networks are present in rPLA\(_2\), however, in rPLA\(_2\), the activating water appears to be present already in the un-activated form, and the activation process remains unknown, unless the interaction with the neighboring subunit N terminus (Fig. 4B) induces an activated state.

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