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1.72 Å Resolution Refinement of the Trigonal Form of Bovine Pancreatic Phospholipase A_2

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Abstract

The trigonal crystal structure of the recombinant bovine pancreatic phospholipase A_2 has been re-refined at a slightly higher resolution (1.72 Å). The crystals are trigonal, space group $P3_121$, unit-cell parameters $a = b =$ 46.78 and $c = 102.89$ Å and are isomorphous to the previous structure. The structure was refined to a final crystallographic R value of 19.5% ($R_{free} = 28.4\%$) using 10 531 reflections. A total of 106 solvent molecules were included in the refinement compared with the earlier refinement which contains only 85 water molecules and 8 925 reflections at 1.8 Å resolution. The root-mean-square deviation from the ideal bond lengths and bond angles is considerably better in the present refinement. The active site is extended (~14 Å) from Ala1 to the calcium. The three catalytic residues (Asp99, His48 and the catalytic water) are connected by the conserved structural water and the N-terminal Ala1 on one side, and by the calcium through an equatorial water on the other. The water molecules play a role in the activity of the enzyme PLA2. The Ala1 end of the extended active site performs the activation of the phospholipid membranes while the opposite end performs the hydrolysis of the monomeric phospholipids.

1. Introduction

Phospholipase A_2 (PLA2) is implicated in a variety of physiologically important cellular processes. It is a hydrolytic enzyme that specifically cleaves the *sn*-2 aliphatic chain of naturally occurring L-glycerophospholipid in the presence of the cofactor calcium to form a lysophospholipid and a fatty acid (Verheij *et al.*, 1980; Dijkstra, Drenth *et al.*, 1981). The released fatty acid, arachidonic acid, is a precursor of leucotrienes, thromboxanes and prostaglandins which are responsible for inflammation and blood platelet aggregation. The X-ray structures of several different extracellular PLA2's from a variety of sources are available (Dijkstra, Kalk *et al.*, 1981; Dijkstra *et al.*, 1983; Keith *et al.*, 1981; Wery *et al.*, 1991; Brunie *et al.*, 1985; White *et al.*, 1990; Scott, White *et al.*, 1990; Scott, Otwinowski *et al.*, 1990; Noel *et al.*, 1991) and they display a high degree of structural homology. In the pancreas, PLA2 occurs as a pro-enzyme zymogen, with seven N-terminal amino-acid residues, which, upon secretion into the duodenal tract, becomes activated by the removal of these seven residues (de Haas *et al.*, 1968). The enzyme PLA2 is responsible for the interfacial recognition and activation of the substrates and catalysis. The N-terminal Ala1 with the conserved structural water is believed to be responsible for the former step (Dijkstra, Drenth *et al.*, 1981; Sekar *et al.*, 1997) while the latter step involves the essential calcium, the catalytic dyad residues (Asp99–His48) and the catalytic water (Dijkstra, Drenth *et al.*, 1981; Scott, White *et al.*, 1990). The extended active site contains the catalytic dyad at the center, hydrogen bonded to the catalytic water and the equatorial calcium water at one end, while the other end is hydrogen bonded to the conserved structural water. The first crystal structure of PLA2 from bovine pancreas was determined by Dijkstra and co-workers (Dijkstra, Kalk *et al.*, 1981) on the orthorhombic form. They also determined the crystal structure of the pro-PLA2 (zymogen) in the trigonal form at 3.0 Å resolution where the seven N-terminal residues were not visible (Dijkstra *et al.*, 1982). Our recombinant bovine pancreatic PLA2 crystals (Noel *et al.*, 1991) had the same space group of $P3_121$ and unit-cell parameters $a = b = 46.52$ and $c = 102.20$ Å as the pro-PLA2. We solved our structure by the molecular-replacement technique (Noel *et al.*, 1991) using their structure, and refined with the high-resolution coordinates of the orthorhombic PLA2 (Dijkstra, Kalk *et al.*, 1981). Here we report the re-refinement of the structure using a slightly higher resolution data.

2. Materials and methods

The trigonal form of the recombinant PLA2 crystals were grown by the vapor-diffusion method using the conditions described earlier (Noel *et al.*, 1991). Large single crystals were obtained by the hanging-drop method from droplets containing 5 µl of the protein, (15 mg ml^{-1} of the protein), 5 m*M* $CaCl_2$, 50 m*M* Tris buffer, pH 7.2 and 2 µl of 2-methyl-2,4-pentane diol (MPD) (75%). The reservoir contained 50% MPD. The crystals belong to the trigonal system with space group

$P3_121$ and cell parameters $a = b = 46.78$, $c = 102.89$ Å and are isomorphous to our earlier structure (Noel *et al.*, 1991). A crystal of size $0.25 \times 0.25 \times 0.50$ mm was mounted in a thin-walled glass capillary with mother liquor and sealed at both ends for data collection. X-ray diffraction data were collected on an R-AXIS IIc imaging-plate system at room temperature (291 K) out to a resolution of 1.72 Å using Cu $K\alpha$ radiation from a Rigaku X-ray generator operating at 50 kV and 100 mA. A total of 11 337 [$F \geq 2\sigma(F)$] unique reflections were obtained from 37 578 observations with an R_{merge} of 7.9%.

The atomic coordinates of the earlier trigonal form (Noel *et al.*, 1991) (PDB entry 2BPP) were used as the starting model. 7% of the reflections (806) were used for the free R-factor calculation (Brünger, 1992*b*). After a few cycles of rigid-body refinement and 150 cycles of Powell minimization, the R factor and the R_{free} were 25.1 and 30.1% between the resolutions 8.0 and 1.72 Å. Simulated annealing was performed, starting from a temperature of 4000 K and slowly cooling in decreasing steps of 25 K to 300 K (Brünger *et al.*, 1990). The R factor was 24.1% (R_{free} = 31.1%). The protein model was checked and fitted using the difference omit electron-density maps (omitting ten residues at a time) on our Silicon Graphics workstation by employing the molecular graphics program *FRODO* (Jones, 1985). During the progress of the refinement, solvent molecules were picked, when the peak electron density in the $|F_o| - |F_c|$ difference map was greater or equal to 3σ and at a hydrogen-bonding distance of $\leq$3.6 Å to polar atoms of the protein or other water molecules. The solvent peaks were also checked using $2|F_o| - |F_c|$ electron-density maps. A total of 106 solvent molecules were located and included in the refinement which dropped the R factor to 19.5% (R_{free} = 28.4%) for all 10 531 reflections between 8.0 and 1.72 Å resolution. The refinement was performed using the program *X-PLOR* 3.1 (Brünger, 1992*a*) and the Engh and Huber parameter file (Engh & Huber, 1991). A satisfactory analysis of the overall stereochemical quality of the model was obtained with the program *PROCHECK* (Laskowski *et al.*, 1993). The crystallographic parameters are summarized in Table 1. The atomic coordinates and the structure factors of the present refinement have been deposited in the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977).†

Table 1. *Crystal and other relevant refinement parameters for the trigonal PLA2*

Unit-cell dimensions (Å)	$a = b = 46.78$ $c = 102.89$
Space group	$P3_121$
Resolution range (Å)	8.0–1.72
Observed reflections	37 578
Reflections [$F > 2\sigma(F)$]	11 337
R_{merge} (%)	7.9
Cumulative completeness (%)	79
Completeness (%) (1.8–1.72 Å)	55
R factor (%) (10 531 reflections)	19.5
R_{free} (%) (806 reflections)	28.4
Refined model	
Protein atoms	957
Water molecules	106
Bound calcium ion (Ca^{2+})	1
Parameter file	parhcsdx.pro
Topology file	tophcsdx.pro
R.m.s. deviation of the model	
Bond lengths (Å)	0.010
Bond angles (°)	1.6
Dihedral angles (°)	22.7
Improper angles (°)	1.3
Average atomic temperature factors of the refined model (Å^2)	
Main chain	23.2
Side chain	26.6
Water molecules	38.8
Calcium ion	20.1

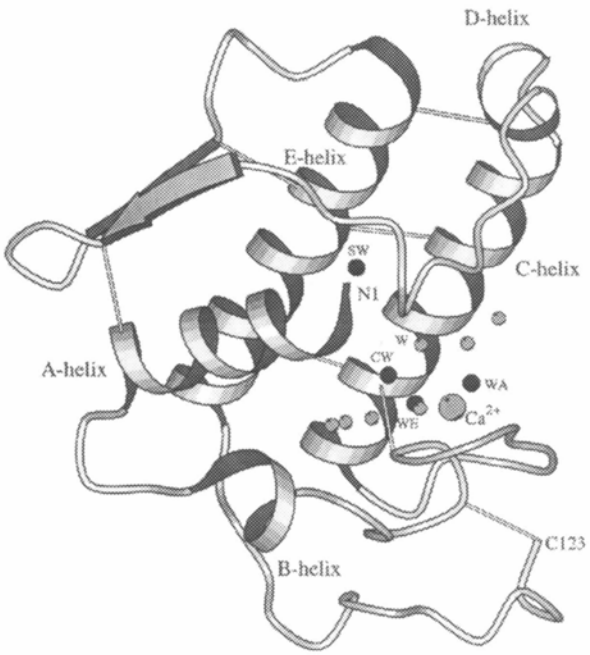

Fig. 1. The structure of recombinant bovine pancreatic PLA2 showing the essential calcium (big grey circle), the active-site water molecules (dark circle), the conserved structural water and the N-terminal Ala1 are shown. The other water molecules (small grey circle, also Fig. 3) and the disulfide bridges (open bonds) are also shown. This figure was produced using the program *MOLSCRIPT* (Kraulis, 1991).

† Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1MKT, R1MKTSF). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: GR0758). At the request of the authors the structure factors will remain privileged until 6 September 2001.

3. Results and discussion

3.1. *The overall structure*

The estimated coordinate error of the model is 0.2 Å (Luzzati, 1952). The (φ,ψ) angles are within the allowed regions of the Ramachandran map. The ribbon diagram of the structure is shown in Fig. 1. The average B values of the main chain, the side chain and the water molecules are given in Table 1. The surface loop residues (62–67) are disordered as in all the mutant structures (Sekar *et al.*, 1997). The essential Ca^{2+} ion has seven ligands that exhibit a pentagonal bipyramidal coordination as in the other PLA2 structures (Fig. 2*b*). Three ligands are from the backbone carbonyl O atoms of Tyr28, Gly30 and Gly32, two from the carboxylate O atoms of the same Asp49 and the remaining from two

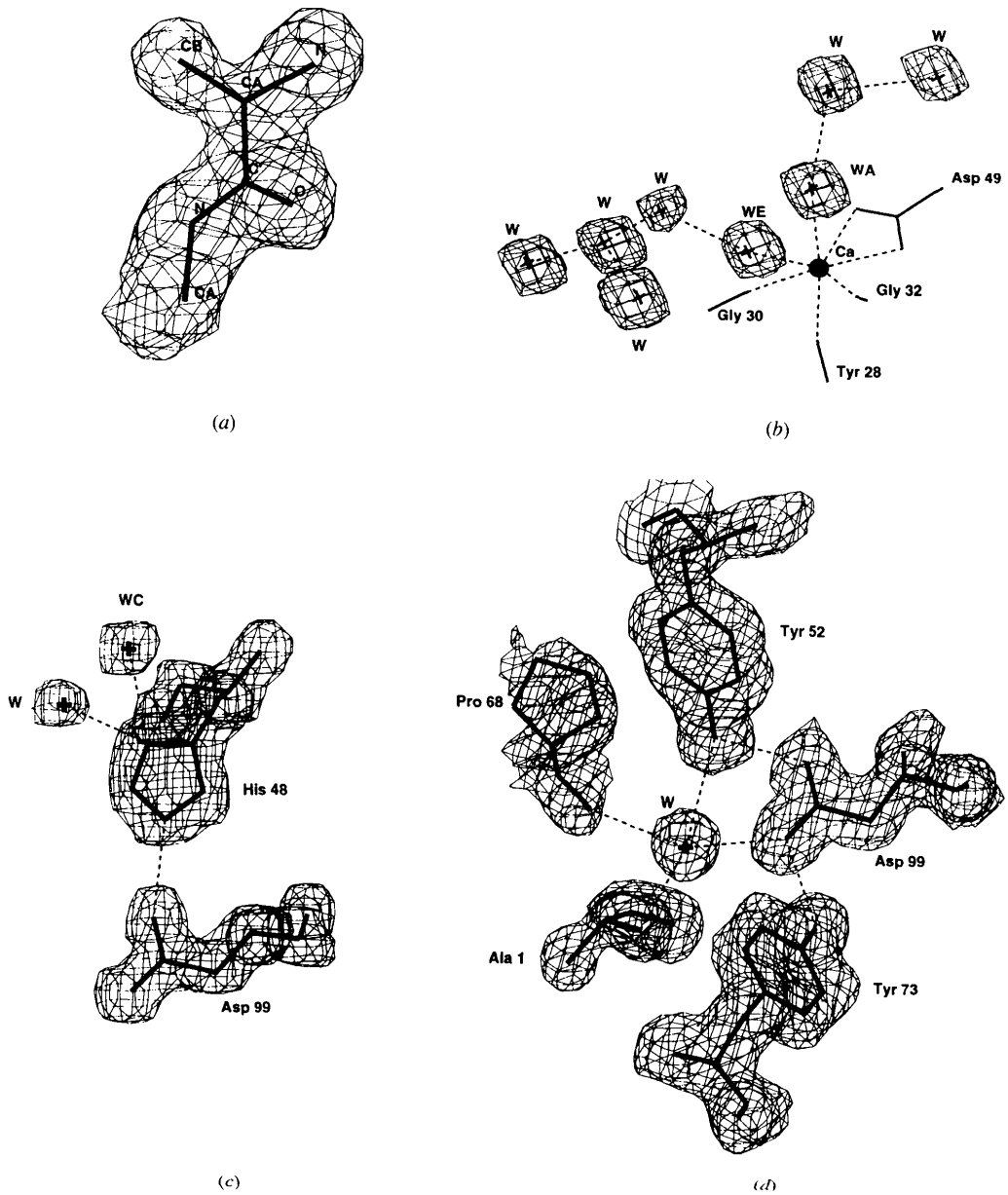

Fig. 2. (*a*) The $2|F_o| - |F_c|$ omit electron density for the N-terminal Ala1. (*b*) The omit electron-density map showing the water molecules around the pentagonal bipyramidal calcium (filled circle). The equatorial pentagonal plane is formed by the backbone carbonyl O atoms of Gly30, Gly32, both carboxylate O atoms of Asp49 and the water WE, while the axial ligands are from the carbonyl of Tyr28 and the water WA. (*c*) The $2|F_o|-|F_c|$ omit electron-density map for the catalytic dyad Asp99–His48 and the catalytic water (WC). The second water (W) hydrogen bonded to $N^{\delta 1}$ of His48 is also shown (see text). (*d*) The omit electron density, contoured at 1.0σ level, for the residues around the conserved structural water is shown. The dashed lines represent the hydrogen bonding.

water molecules. The calcium–ligand distances vary between 2.16 and 2.59 Å (average 2.38 Å), the longest being that of the first ligand (2.59 Å) of Asp49.

3.2. *Comparison with our earlier structure*

10 531 reflections were used in the present refinement between 8.0 and 1.72 Å resolution compared with our earlier refinement (Noel *et al.*, 1991) where only 8 925 reflections between 5.0 and 1.8 Å resolution were used. The root-mean-square (r.m.s.) deviations from ideal bond lengths and bond angles of the present model are 0.01 Å and 1.6° which are better than our earlier refinement (0.014 Å and 2.68°). A superposition of the two protein models gave an r.m.s. deviation of 1.05 Å. The electron density in general is more clear in the present refinement, in particular, the N-terminal Ala1 (Fig. 2*a*). Only the coordinates of the protein model from the previous refinement were deposited with the Brookhaven Protein Data Bank (PDB entry 2BPP) and not the 85 water molecules.

The present protein model is hydrated by 106 water molecules, 83 water molecules of which are directly involved in hydrogen bonding with the protein atoms, forming the first shell of hydration. Of these 83 water molecules, 42 are directly hydrogen bonded to the backbone atoms of the protein and the remaining 41 water molecules are hydrogen bonded to the side-chain atoms as observed in other protein structures (Thanki *et al.*, 1988). In addition to hydrogen bonding of the catalytic water to $N^{\delta 1}$ of His48, a second water (Fig. 2*c*) also shares $N^{\delta 1}$ of His48 in hydrogen bonding. The two histidine water molecules lie on either side of the histidine plane (Fig. 2*c*), displaced by −0.51 and 2.24 Å and subtending an angle of 68° at $N^{\delta 1}$. The enzyme PLA2 has an elongated 'active site' consisting of the 'conserved' structural water, and catalytic residues (the catalytic dyad, Asp99 and His48, and the catalytic water) and the calcium. The N-terminal Ala1 of the active site (Fig. 2*d*) is connected to the 'conserved' structural water and the catalytic residues, which in turn is connected to the equatorial coordinated calcium water through the catalytic water (Fig. 3). It is found in this refinement that the axial calcium water is hydrogen bonded to two other water molecules which are linked to each other (Fig. 3). The equatorial calcium water is at a junction, besides its coordination to calcium, it is hydrogen bonded to the catalytic water on one side and it is linked to four water molecules on the other side (Fig. 3).

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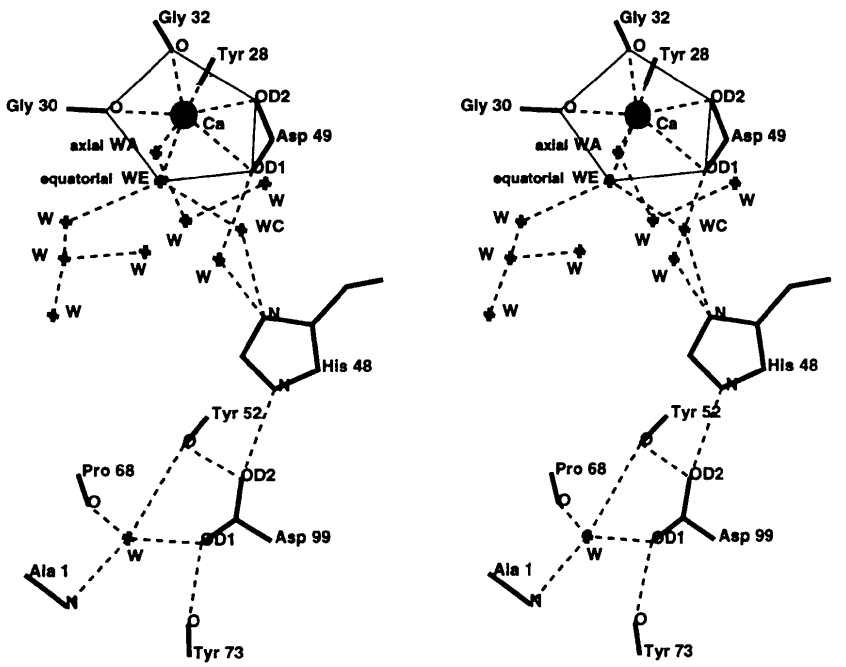

Fig. 3. A stereoview showing the hydrogen-bonding network of the elongated active site.

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