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High-resolution refinement of orthorhombic bovine pancreatic phospholipase A_2

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The X-ray structure of recombinant bovine pancreatic phospholipase A_2 (PLA2), which specifically catalyzes the cleavage of the *sn*-2 acylester bond of phospholipids, has been refined at 1.5 Å resolution. The crystal belongs to the space group $P2_12_12_1$ with unit-cell parameters a = 47.12, b = 64.59 and c = 38.14 Å similar to the native enzyme reported previously by Dijkstra *et al.* [*J. Mol. Biol.* (1981), **147**, 97–123]. The refinement converged to an R value of 18.4% ($R_{\rm free}$ = 22.8%) for 16 374 reflections between 10.0 and 1.5 Å resolution. The surface-loop residues (60–70) are ordered in the present orthorhombic recombinant enzyme, but disordered in the trigonal recombinant enzyme. The active-site residues, His48, Asp99, and the catalytic water superimpose well with the trigonal form. Besides the catalytic water which is hydrogen bonded to His48, it is often seen that there is a second water attached to the same N atom of His48 and simultaneously hydrogen bonded to the O atom of Asp49. It is thought that the second water facilitates the tautomerism of His48 for enzyme catalysis. The catalytic water is also hydrogen bonded to the equatorial water coordinated to the calcium ion. In addition to the equatorial water, there is also an axial calcium water and the additional structural water. These five common water molecules are hydrogen bonded to the additional 16 water molecules in the present orthorhombic structure which may further enhance the structural integrity of the active site. Besides the protein and one calcium ion, a total of 134 water molecules were located in the present high-resolution refinement.

1. Introduction

Bovine pancreatic phospholipase A_2 (PLA2) is a 14 kDa enzyme, containing 123 amino-acid residues. The enzyme is cross-linked by seven disulfide bonds, conserved in about 40 species of secretory PLA2s, which endows it with exceptional stability (Davidson & Dennis, 1990). The enzyme PLA2 displays a high degree of structural homology for the binding of glycerophospholipids and specifically hydrolyzes its *sn*-2 acylester bond. Apparently the wild-type bovine pancreatic PLA2 readily crystallizes in the orthorhombic form (Dijkstra *et al.*, 1981), while the recombinant PLA2 crystallizes in both the orthorhombic (present work) and trigonal forms (Noel *et al.*, 1991; Sekar *et al.*, 1998). The first crystal structure of PLA2 from bovine pancreas was determined in the orthorhombic form at 1.7 Å resolution (Dijkstra *et al.*, 1981). Since 1989, we have been studying the trigonal crystal form of the recombinant enzyme (Noel *et al.*, 1991). During our work on the trigonal form, we stumbled across the orthorhombic form, which diffracted to a very high resolution. We collected the

Table 1
Observed reflections in various resolution ranges and completeness (%).

Resolution range (Å)	No. of observed reflections	Shell completeness	Cumulative completeness
3.20–10.00	1957	96.1	96.1
2.55–3.20	1915	97.8	96.9
2.23–2.55	1874	97.2	97.0
2.03–2.23	1848	96.9	97.0
1.89–2.03	1810	95.0	96.6
1.78–1.89	1801	94.6	96.3
1.69–1.78	1787	94.4	96.0
1.62–1.69	1788	94.8	95.9
1.55–1.62	1616	86.0	94.8
1.50–1.55	1176	62.6	91.6

1.5 Å resolution data and report here the refinement and the location of more water molecules compared with the earlier refinement by Dijkstra *et al.* The possible role of the water molecules is also reported.

2. Materials and methods

Recombinant bovine pancreatic PLA2 was kindly supplied by Dr M.-D. Tsai of the Chemistry Department. Crystals were grown within a week at room temperature (291 K) by the hanging-drop method from droplets containing 5 µl of the protein (15 mg ml^{-1}), 5 m*M* $CaCl_2$, 50 m*M* Tris buffer, pH 7.2, and 3 µl of MPD (50%). The reservoir MPD concentration was 50%. For data collection, a crystal of size 0.3 × 0.3 × 0.5 mm was mounted in a quartz capillary with mother liquor

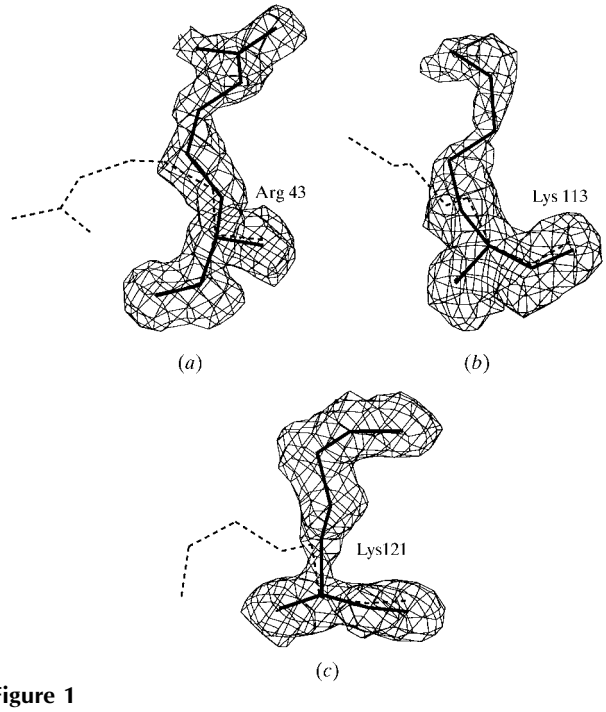

Figure 1
The omit electron-density map in the orthorhombic form showing the three side chains (*a*) Arg43, (*b*) Lys113 and (*c*) Lys121 (thick lines) compared with those observed (dashed lines) by Dijkstra *et al.* (1981). Contours are at the 1.0σ level.

Table 2
Crystal, relevant geometrical and refinement parameters.

$R_{merge} = \sum_{hkl} |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity from observations of symmetry-related reflections.

Unit-cell dimensions (Å)	a = 47.12, b = 64.59, c = 38.14
Space group	$P2_12_12_1$ (orthorhombic)
Resolution range (Å)	10.0–1.5
No. of observations	68591
Unique reflections	17572
R_{merge} (%)	4.6
Cumulative completeness at 1.5 Å resolution (%)	92
R_{work} (16 374 reflections) (%)	18.4
R_{free} (1198 reflections) (%)	22.8
Protein model	
Protein atoms	957
Water molecules	134
Bound calcium ion (Ca^{2+})	1
Parameter file	parhcsdx.pro
Topology file	tophcsdx.pro
R.m.s. deviations from ideal geometry (Å, °)	
Bond lengths	0.0009
Bond angles	1.78
Dihedral	21.6
Improper	1.31
Average temperature factors of the refined model (Å^2)	
Main chain	14.6
Side chain	18.4
Water molecules	30.7
Calcium ion	12.2

at both ends and sealed. The crystal unit-cell parameters of the recombinant PLA2, a = 47.12, b = 64.59 and c = 38.14 Å, space group $P2_12_12_1$, V_m = 2.1 Å^3 Da^{-1} are similar to those of the wild-type enzyme (a = 47.08, b = 64.44 and c = 38.15 Å) (Dijkstra *et al.*, 1981). The X-ray intensity data were collected at room temperature (291 K) on our R-axis IIc image plate with a Rigaku rotating Cu anode operating at 50 kV and 100 mA (λ = 1.5418 Å). The crystal-to-detector distance was 55 mm. Each frame was exposed for 25 min and oscillated by 3°. Altogether, 68 591 observations were made which yielded 17 572 [$F \geq 1\sigma(F)$] unique reflections in the resolution range 10.0–1.5 Å with an R_{merge} of 4.6%. The data completeness between 10.0 and 1.5 Å was 92% (Table 1).

The refinement was started with the atomic coordinates of the orthorhombic wild-type structure (Dijkstra *et al.*, 1981) using *X-PLOR* version 3.1 (Brünger, 1992*a*) on a Silicon Graphics workstation. 7% (1198 out of 17 572) of the reflections were used for the calculation of R_{free} (Brünger, 1992*b*). The remaining reflections in the resolution range 10.0–1.5 Å were used throughout the refinement. 50 cycles of rigid-body refinement and 50 cycles of Powell energy minimization on atomic positions lowered the R factor to 33.0% (R_{free} = 35.4%). Next, simulated annealing was performed, slow cooling from 4000 to 300 K in steps of 25 K, followed by refinement of positional and individual isotropic temperature factors, giving an R factor of 26.2% (R_{free} = 29.6%). The protein model was fitted into the difference electron-density maps by the molecular-modeling program *FRODO* (Jones, 1985). The water molecules were picked from $|F_o| - |F_c|$ difference electron-density maps. These water peaks were also

checked using the $2|F_o| - |F_c|$ difference electron-density maps and were included in the refinement in stages. In this manner, 134 water molecules were located. Omit electron-density maps showed no residual electron densities which could be interpreted as buffer or MPD molecules used in the crystallization. At the end of the final refinement, the R factor converged to 18.4% ($R_{\rm free}$ = 22.8%) for 16 374 reflections in the resolution range 10.0–1.5 Å. The final protein model contains 957 non-H atoms (123 residues), one Ca^{2+} ion and 134 water O atoms.

The quality of the protein model was very good as assessed by the program *PROCHECK* (Laskowski *et al.*, 1993). A Ramachandran plot showed that all the φ, ψ angles are in the most favored regions. A Luzzati plot (Luzzati, 1952) indicated an estimated error of 0.19 Å in the atomic coordinates. A summary of the refined model and the relevant geometrical parameters are given in Table 2. The atomic coordinates and structure factors have been deposited with the Protein Data Bank.

3. Results and discussion

3.1. Comparison with the earlier structures

The overall fold of the refined recombinant PLA2 is similar to the orthorhombic wild type (Dijkstra *et al.*, 1981). The backbone atoms superimpose with a root-mean-square (r.m.s.) deviation of 0.15 Å while all the atoms, including the side-chain atoms, superimpose with an r.m.s. deviation of 0.85 Å. The largest deviations are in the three side chains of Arg43, Lys113 and Lys121 (Fig. 1) and the overall r.m.s. deviation reduces to 0.59 Å if these residues are omitted. A total of 134 water molecules were located in the present refinement compared with 106 in the earlier report (Dijkstra *et al.*, 1981); of these, 73 are within 1.0 Å of each other. Most of the 134 water molecules are in the first hydration sphere of the protein while only 12 are in the second hydration sphere. About half the first hydration-sphere water molecules are hydrogen bonded to the peptide backbone atoms while the remaining are hydrogen bonded to the side-chain atoms (Thanki *et al.*, 1988).

The backbone atoms superimpose on the trigonal form (Sekar *et al.*, 1998) with an r.m.s. deviation of 0.73 Å; including the side-chain atoms, the r.m.s. deviation is 1.40 Å. The disorder of the surface loop in the trigonal structure is responsible for the large deviation. In fact, the r.m.s. deviation reduces to 0.43 Å for the backbone atoms, if the surface-loop residues are omitted. There are 40 water molecules within 1.0 Å in the present orthorhombic and trigonal forms.

3.2. The surface loop

The electron density of the 85 atoms of the surface-loop residues (60–70) is clear and ordered (Fig. 2*a*). Indeed, the average B value for the surface loop is 18.6 Å^2 in the orthorhombic form, while it is 58.3 Å^2 in the trigonal form. The volume per Dalton of the orthorhombic form (2.1 Å^3) is less than that of the trigonal form (2.24 Å^3) by 8%. This indicates that the orthorhombic form is more tightly packed than the trigonal form. There are 12 symmetry-related molecules involved in intermolecular contacts ($\leq$ 3.6 Å) in the orthorhombic form while there are only eight in the trigonal form (Fig. 3). Four of the 12 are involved in close contacts with the surface-loop residues whereas only two are involved in the

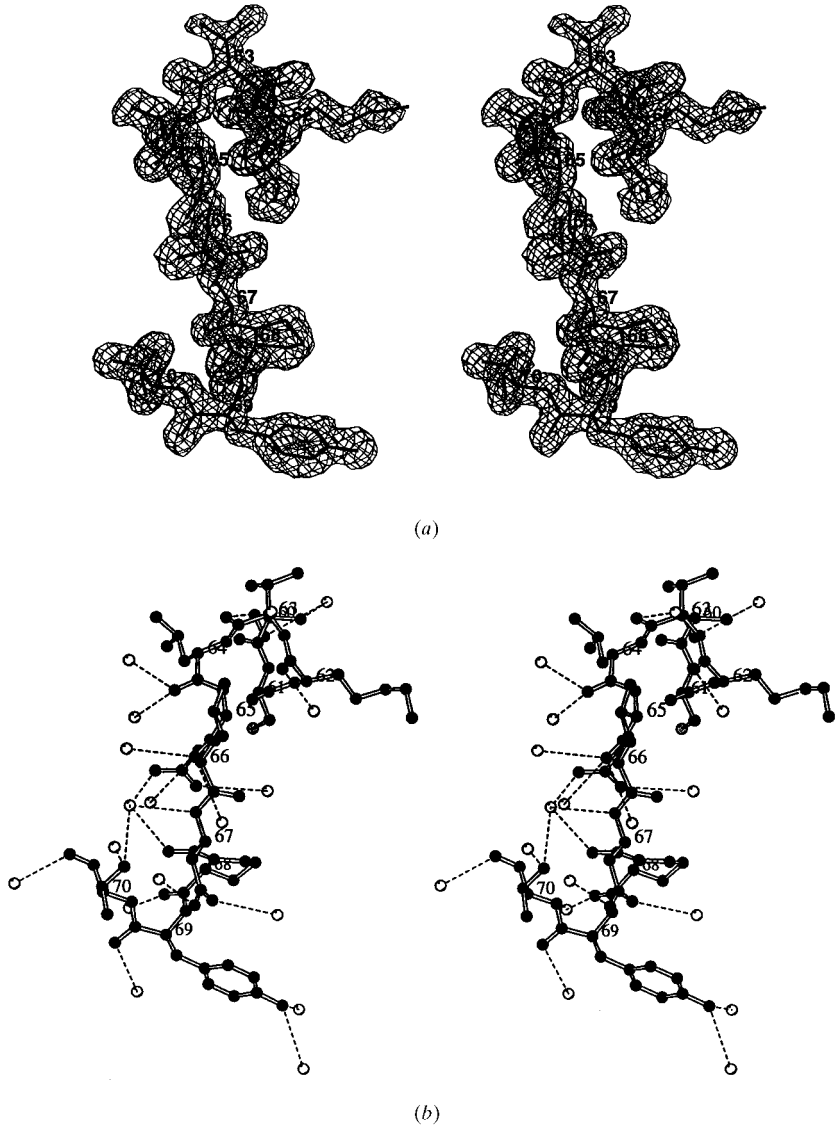

Figure 2
A stereoview of (*a*) the omit electron-density map showing the ordered surface-loop residues 60–70 in the present orthorhombic form, contoured at the 1.0σ level, and (*b*) the water molecules (open circles) involved in hydrogen bonding with the polar atoms of the surface-loop residues produced using the program *MOLSCRIPT* (Kraulis, 1991).

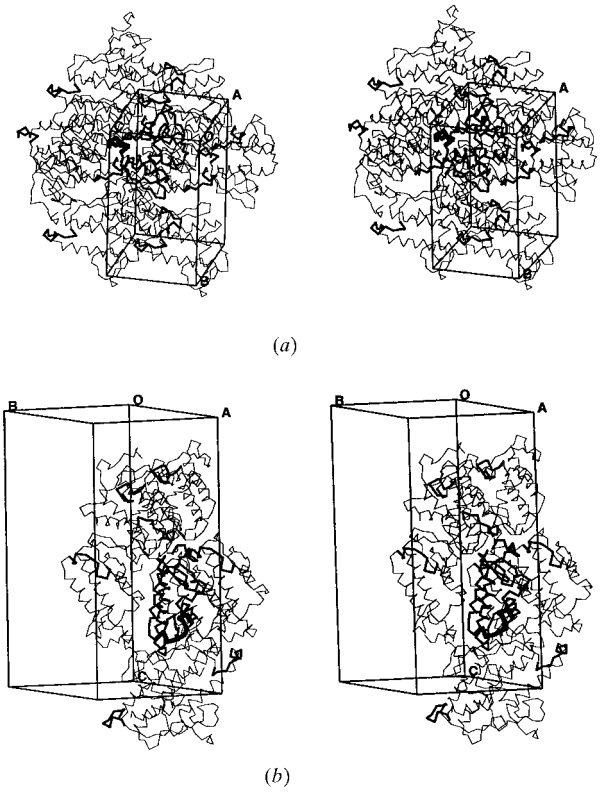

Figure 3
A stereoview of the packing diagram showing the reference molecule (thick lines) and the symmetry-related molecules (thin lines) in (*a*) the orthorhombic and (*b*) the trigonal forms with the surface loop highlighted.

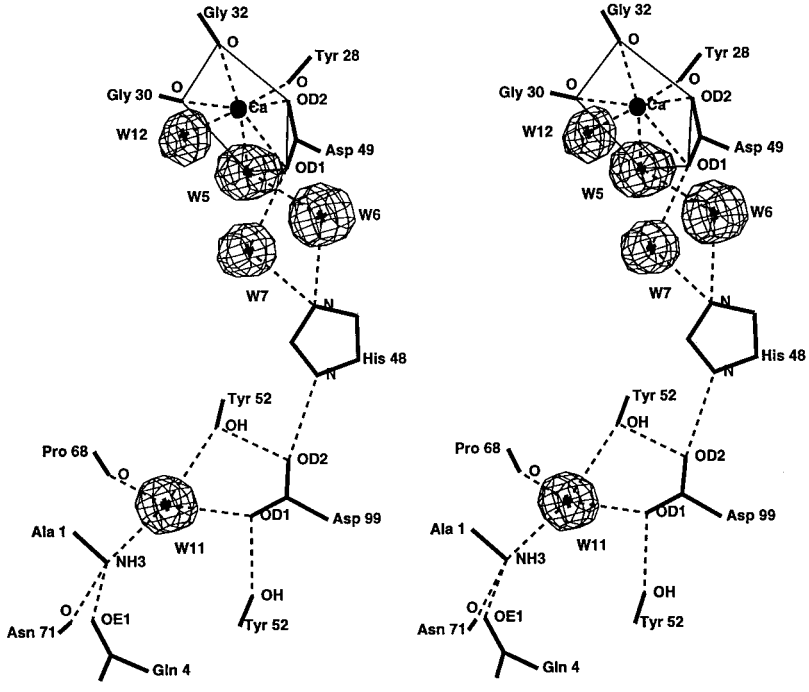

Figure 4
A stereoview of the hydrogen-bonding (dashed lines) network connecting the active/catalytic site and the calcium coordination sphere. The omit electron-density map shows the five water molecules commonly found in the active site: the structural water (*W*11), the two calcium-coordinated waters (*W*5 and *W*12), the catalytic water (*W*6) and the second water (*W*7). Contours are shown at the 1.0σ level.

trigonal form. Thus, the larger number of intermolecular contacts in the orthorhombic form may be responsible for the ordering of the surface loop. However, in the orthorhombic mutants, Q4E (Liu *et al.*, 1995), Y52 and 73F/D99N (Sekar *et al.*, 1997), solved so far, the surface loop is disordered. This could mean that the packing may not entirely explain the ordering of the surface loop.

The orthorhombic high-resolution structure has 18 water molecules surrounding the ordered surface loop, compared with only seven in the trigonal structure. The 18 water molecules make 22 hydrogen bonds with the backbone and the side chains (Fig. 2*b*), while only seven hydrogen bonds are found in the trigonal structure. To what extent the water molecules contribute to the ordering of the surface loop in the orthorhombic structure is not clear.

3.3. Active-site water molecules

The active-site cleft consists of the commonly found five water molecules: the catalytic water *W*6, a second water *W*7 hydrogen bonded to the same imidazole $N^{\delta1}$ atom of histidine, the two calcium-coordinated water molecules (equatorial *W*5 and axial *W*12), and the conserved structural water *W*11 (Fig. 4). The His48 water molecules subtend an angle of 69° at $N^{\delta1}$ and we conjecture that the water molecule *W*7 could be assisting the catalytic water *W*6 in the tautomerization of His48 for enzyme catalysis. Like the catalytic water hydrogen bonded to the equatorial calcium water, the second water *W*7 is hydrogen bonded to the carboxylate $O^{\delta1}$ atom of Asp49, which is coordinated to the calcium. In the serine proteases (Fujinaga & James, 1987; Teplyakov *et al.*, 1990), besides serine which replaces the catalytic water, another water molecule is hydrogen bonded to the catalytic histidine, corresponding to the second water molecule in PLA2, which may have a similar role.

The equatorial water *W*5 bridging the catalytic water and the calcium ion is in the interior of the protein and has a low temperature factor (10.2 Å^2). On the other hand, the axially coordinated calcium water *W*12 is exposed to the solvent channel and has a higher temperature factor (19.6 Å^2). The equatorial calcium water is hydrogen bonded to eight other water molecules of the active site forming a branch. The axial water is connected to the second histidine water *W*7 through a water molecule which is hydrogen bonded to the phenolic hydroxyl of Tyr69 (Fig. 5). This water is hydrogen bonded to a string of six water molecules. The conserved structural water, *W*11, is hydrogen bonded to the active-site residues and to two other water molecules that are linked together on the surface of the enzyme (Fig. 5). Therefore, on one end of the catalytic dyad (Asp99–His48) is the catalytic water and the equatorial calcium water, and on the other end is the conserved structural water making an elon-

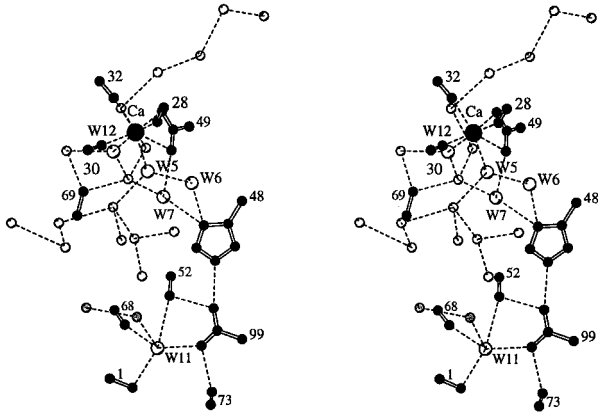

Figure 5
A stereoview showing the hydrogen-bonding network of the 16 active-site water molecules (small open circles) and the commonly observed five water molecules, (large open circles) using the program *MOLSCRIPT* (Kraulis, 1991). The two surface water molecules linked to the structural water, *W*11, are represented by shaded circles.

gated enzyme active site including calcium (Figs. 4, 5).

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