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Structures of the catalytic site mutants D99A and H48Q and the calcium-loop mutant D49E of phospholipase A_2

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PDB References: phospholipase A_2 mutant D99A, 1kvx; phospholipase A_2 mutant H48Q, 1kvy; phospholipase A_2 mutant D49E, 1kvw.

Crystal structures of the active-site mutants D99A and H48Q and the calcium-loop mutant D49E of bovine phospholipase A_2 have been determined at around 1.9 Å resolution. The D99A mutant is isomorphous to the orthorhombic recombinant enzyme, space group $P2_12_12_1$. The H48Q and the calcium-loop mutant D49E are isomorphous to the trigonal recombinant enzyme, space group $P3_121$. The two active-site mutants show no major structural perturbations. The structural water is absent in D99A and, therefore, the hydrogen-bonding scheme is changed. In H48Q, the catalytic water is present and hydrogen bonded to Gln48 N, but the second water found in native His48 is absent. In the calcium-loop mutant D49E, the two water molecules forming the pentagonal bipyramid around calcium are absent and only one O atom of the Glu49 carboxylate group is coordinated to calcium, resulting in only four ligands.

1. Introduction

Phospholipase A_2 (PLA2) releases arachidonic acid and lysophospholipids on cleavage of the S_N2 ester bond of L-phosphoglycerides. These products are important in transmembrane signalling and causing inflammation. The active site of PLA2 contains the catalytic dyad, Asp99 and His48, and a catalytic water W6 which acts as a nucleophile during the

Table 1
Crystal data for three mutants of PLA2.

	D99A	H48Q	D49E
Cell dimensions (Å)	a = 46.56, b = 64.57, c = 37.99	$a = b$ = 47.12, c = 102.88	$a = b$ = 46.87, c = 103.04
Resolution range (Å)	10.0–1.90	10.0–1.95	10.0–1.90
Space group	$P2_12_12_1$	$P3_121$	$P3_121$
Observed reflections	20534	21567	43149
Unique reflections	8110	7424	10009
R_{merge}† (%)	6.7	8.2	6.1
Completeness (%)	86	74	92
R_{work} (%)	20.0	20.9	19.8
R_{free} (%)	31.3	31.4	27.7
Protein model			
Protein atoms	954	956	958
Water molecules	98	68	70
Bound calcium ion (Ca^{2+})	1	1	
R.m.s. deviations			
Bond lengths (Å)	0.011	0.013	0.013
Bond angles (°)	1.67	1.91	1.73
Torsion angles (°)	22.2	23.0	23.1
Improper angles (°)	1.26	1.71	1.2
Parameter file	parhcsdx.pro	parhcsdx.pro	parhcsdx.pro
Topology file	tophcsdx.pro	tophcsdx.pro	tophcsdx.pro
Average temperature factors (Å^2)			
Main chain	21.2	23.2	28.6
Side chain	24.9	26.0	32.7
Water molecules	33.6	35.6	40.5
Calcium ion	18.9	28.0	47.6

enzymatic reaction (Fig. 1*a*) (Dijkstra *et al.*, 1981; Scott *et al.*, 1990), similar to the catalytic triad (Asp, His, Ser) in serine proteases (Fujinaga & James, 1987; Teplyakov *et al.*, 1990). The catalytic water is hydrogen bonded to His48 $N^{\delta1}$ on one side and $N^{\varepsilon2}$ is hydrogen bonded to Asp99 on the other. Both the carboxylate O atoms of Asp99 are involved in a two-way hydrogen bonding with His48 and the surrounding residues (Fig. 1*a*). It has been shown that Asp is involved in stabilizing the appropriate tautomer of histidine in phospholipases (Li & Tsai, 1993) and in serine proteases (*e.g.* Sprang *et al.*, 1987).

The calcium cofactor is essential for both phospholipid binding and catalysis. It is also in the active-site cleft and is involved in a pentagonal bipyramidal coordination with both carboxylate O atoms of Asp49, the carbonyl O atoms of Tyr28, Gly30 and Gly32 and two water molecules (Fig. 3*b*). During catalysis, the two anionic O atoms of the phosphate of the substrate replace the two water molecules coordinated to calcium (Scott *et al.*, 1990; Sekar, Eswaramoorthy *et al.*, 1997). The only ligand that is conserved in all PLA2s is the bidentate Asp49 (Davidson & Dennis, 1990). The mutants D49A, D49N, D49Q and D49K have no calcium affinity, but the mutant D49E has an affinity weakened by a factor of 12 (Li *et al.*, 1994). The activity of D49E is reduced by a factor of 10^6 relative to that of wild-type PLA2.

It was of interest to study the crystal structures of the active-site mutants (D99A and H48Q) and the calcium-loop mutant D49E to gain a better understanding of the nature of the structural perturbations caused by these mutants and to study the effect of the extra methylene group of Glu49 on the calcium coordination.

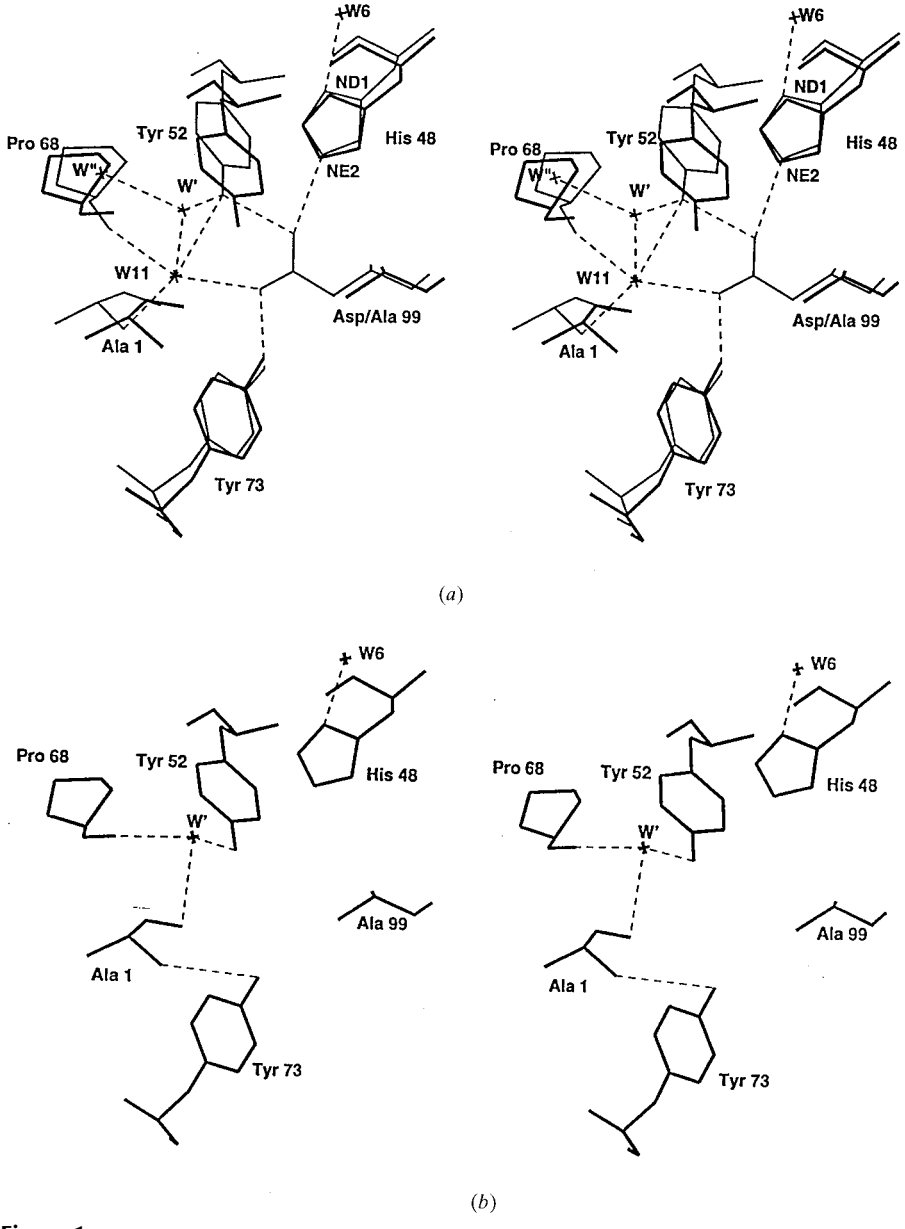

Figure 1
(*a*) A stereoview of the superposition of a portion of the active-site residues of the orthorhombic form of the recombinant PLA2 (thin lines) and the mutant D99A (thick lines). Note the large movements of the residues Pro68, Tyr52 and Ala1 in the mutant D99A. (*b*) A stereoview of the hydrogen-bonding scheme observed in the mutant D99A. Notice that in the absence of the structural water W′ is involved in hydrogen bonding.

2. Materials and methods

The mutants were supplied by Professor M. D. Tsai of the Chemistry Department, Ohio State University. Crystals of the active-site mutant D99A were obtained using the hanging-drop vapor-diffusion method at room temperature (291 K). The droplet contained 5 µl of the protein (15 mg ml^{-1}) in 50 m*M* Tris buffer, 5.0 m*M* $CaCl_2$ and 2.0 µl of 50% MPD equilibrated against 75% MPD in the reservoir. The mutants H48Q (Li & Tsai, 1993) and D49E (Li *et al.*, 1994) were crystallized under similar conditions. The intensity data for all three mutants were collected using an R-AXIS IIc imaging-plate system powered by a Rigaku generator at 50 kV and 100 mA. Crystal data and pertinent parameters are summarized in Table 1.

2.1. Refinement of D99A

The atomic coordinates of the recently refined orthorhombic recombinant PLA2 (PDB code 1UNE; Sekar & Sundaralingam, 1999) were used as the starting model for the refinement of D99A. 813 reflections were used to calculate the R_{free} value (Brünger,

1992*a*) to monitor the progress of the refinement. Initially, 25 cycles of rigid-body refinement were carried out, followed by 100 cycles of positional refinement by Powell energy minimization. Without the mutated residue Ala99, the *R* value dropped to 31.5% (R_{free} = 35.8%) for 7297 reflections in the resolution range 10.0–1.9 Å. Ala99 was then inserted and fitted into the difference electron-density map, and the model was subjected to simulated annealing by heating the system to 4000 K and slowly cooling to 300 K in steps of 25 K. 100 cycles of positional refinement lowered the *R* factor to 27.6% (R_{free} = 32.8%). The calcium ion was included and water molecules were added from the difference electron-density maps at various stages of the refinement. In all, 98 water molecules were included in the refinement and the final *R* value was 20.0% (R_{free} = 31.3%). The refined model consists of 954 non-H protein atoms, one calcium ion and 98 water molecules. The average error for the atomic coordinates is 0.24 Å (Luzzati, 1952). The Ramachandran (φ,ψ) plot (Ramakrishnan & Ramachandran, 1965) calculated using *PROCHECK* (Laskowski *et al.*, 1993) showed that the main-chain dihedral angles are in the allowed regions, with the exception of residue Val65. The electron density for Tyr52 is not clear and the surface-loop residues 60–70 are disordered. The overall tertiary fold is similar to the recombinant enzyme with an r.m.s. deviation of 0.47 Å.

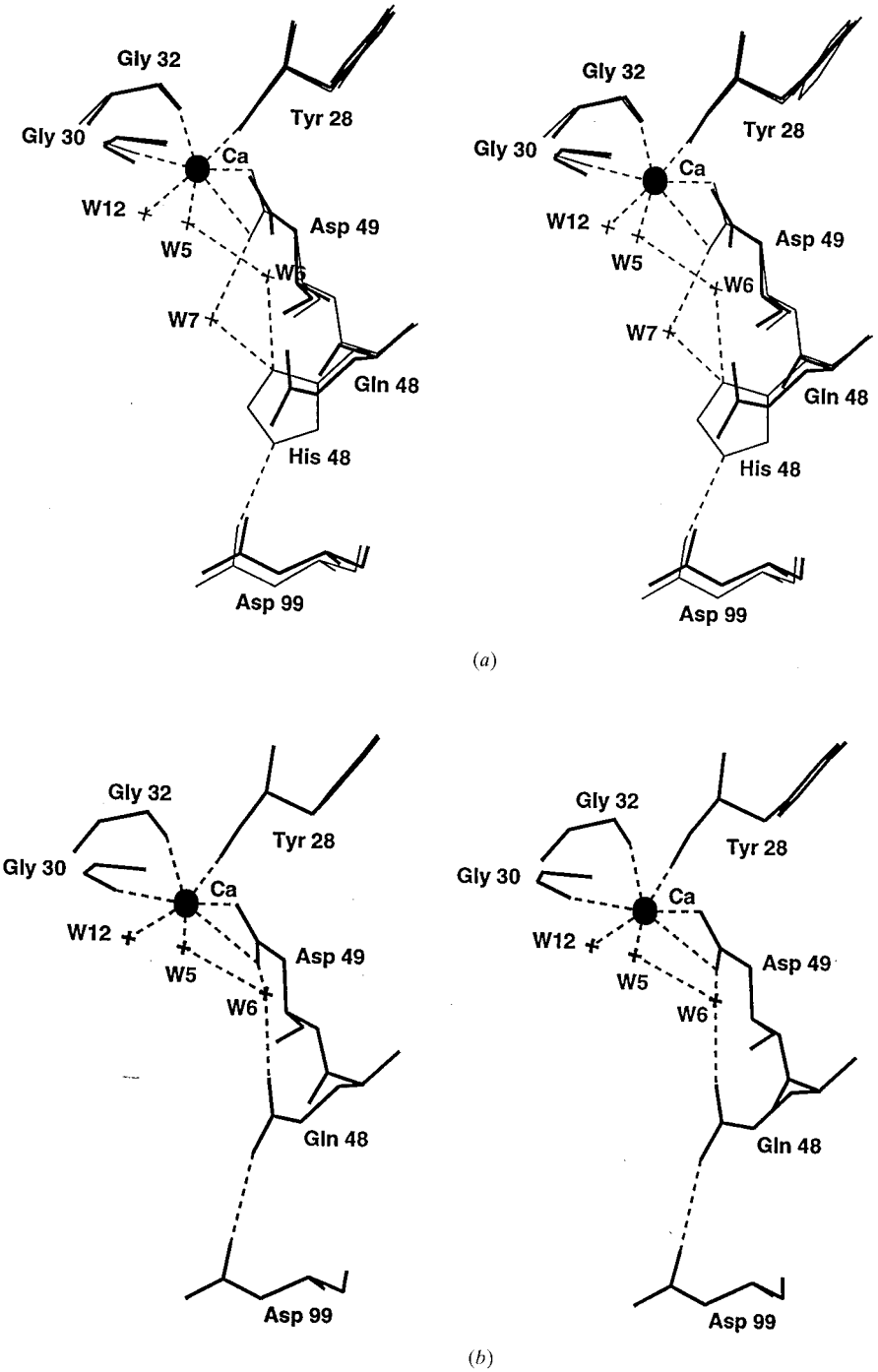

Figure 2
(*a*) A stereoview of the superposition of the active-site calcium coordination and the catalytic dyad residues of the trigonal form of the recombinant PLA2 (thin lines) with the single mutant H48Q (thick lines). (*b*) A stereoview of the same in the mutant H48Q. Note the second histidine water W7 (Fig. 2*a*) is missing and the catalytic water W6 is moved to form a hydrogen bond to the carboxylate O atom of Asp49. The calcium ion is shown as a solid circle.

2.2. Refinement of H48Q

The H48Q refinement was carried out starting from the atomic coordinates of the recombinant PLA2 (PDB code 1MKT; Sekar *et al.*, 1998). A randomly selected 5% of the reflections (347) were used to calculate R_{free} (Brünger, 1992*a*). Refinement protocols as above gave a final *R* value of 20.9% (R_{free} = 31.4%), including 68 water molecules with 956 non-H atoms of the protein and one calcium ion. The estimated error in the atomic coordinates is 0.2 Å. All the non-glycine and non-proline residues are in the allowed regions of the Ramachandran plot except residues Val63, Asn67 and Asn79, which are not clear in the electron-density maps. The electron density for the mutated residue is clear but the N-terminal Ala1 is not unlike that in the recombinant trigonal form (Sekar *et al.*, 1998). The overall tertiary fold of the H48Q mutant is very similar to trigonal recombinant PLA2 (r.m.s. deviation = 0.55 Å).

2.3. Refinement of D49E

The D49E mutant was refined starting with the atomic coordinates of the trigonal recombinant enzyme (PDB code 1MKT; Sekar *et al.*, 1998). 7% of the

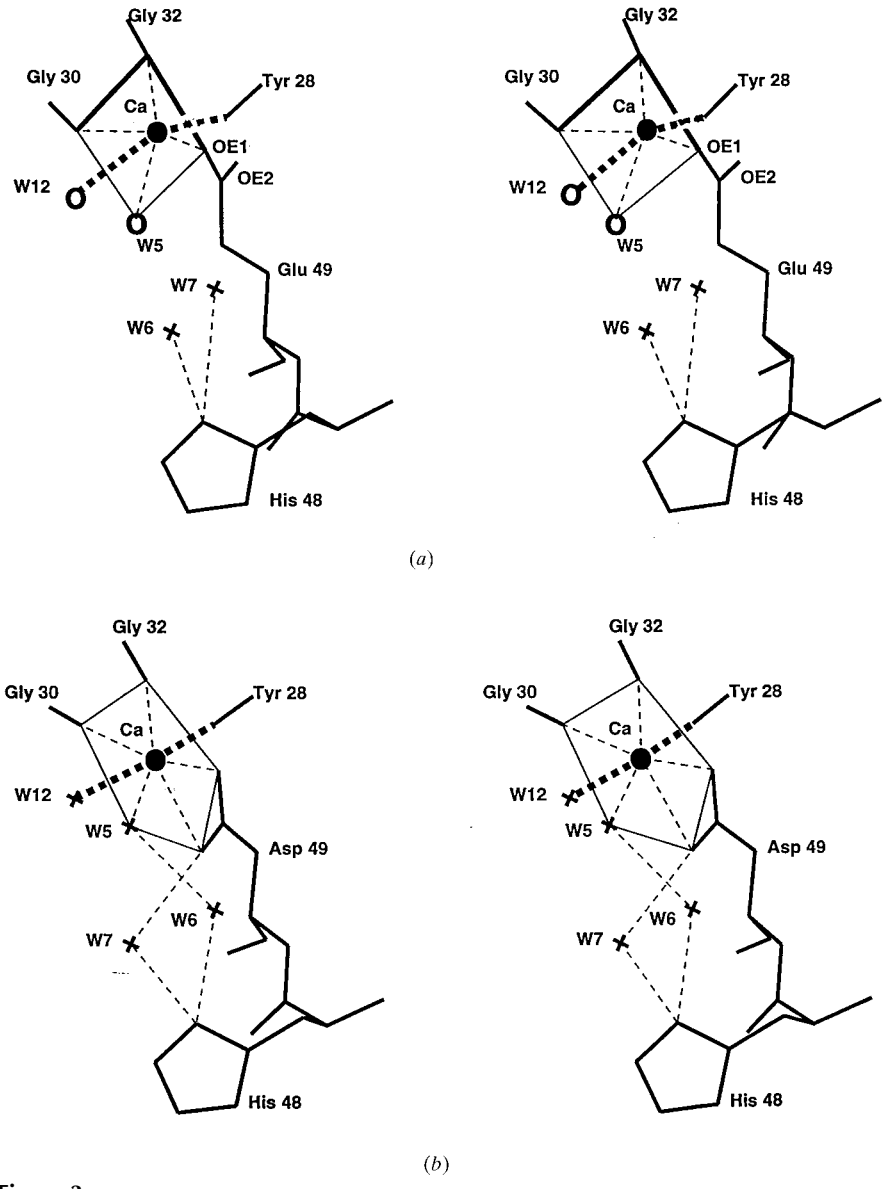

Figure 3
The same stereoview of the calcium coordination in (*a*) the mutant D49E and (*b*) in the trigonal recombinant PLA2 (Sekar *et al.*, 1998). The calcium ion is shown as a solid circle. The equatorial calcium water W5 and the axial calcium water W12 are missing in the mutant and are shown as open circles. In the mutant, only one of the carboxylate O atoms is liganded to calcium, compared with the recombinant enzyme where Asp49 forms a bidentate ligation. Thus, calcium has only four ligands in the mutant while it has seven ligands in the recombinant enzyme.

reflections (688) were used to calculate $R_{\rm free}$ (Brünger, 1992*a*) to monitor the refinement. The refinement converged to an R value of 19.8% ($R_{\rm free}$ = 27.7%) for 958 protein atoms, 70 water molecules and a calcium ion. The estimated error in the atomic positions is 0.2 Å (Luzzati, 1952). All the main-chain torsion angles are in the allowed regions of conformational space. Even though the calcium coordination is affected, the fold of the mutant D49E is similar to the recombinant enzyme (Sekar *et al.*, 1998), with an r.m.s. deviation of 0.42 Å for all non-H atoms.

In all cases, the program *X-PLOR* 3.1 (Brünger, 1992*b*) was used for the refinement and the program *FRODO* (Jones, 1985) was used for molecular modelling. The atomic coordinates and the structure factors for D99A, H48Q and D49E have been deposited with the Protein Data Bank (Bernstein *et al.*, 1977).

3. Results and discussion

3.1. Orthorhombic form of D99A mutant

In the orthorhombic D99A mutant the calcium coordination is unaffected compared with the recombinant enzyme, and the five protein ligands to calcium superimpose with an r.m.s. deviation of 0.17 Å. The calcium–ligand distances vary between 2.10 and 2.53 Å with an average of 2.33 Å. Pro68 and Tyr52 are not very clear in the electron-density map. The active-site residues Ala1, His48, Ala99 and Tyr73 are slightly perturbed and the structural water is missing.[1] This causes a change in the hydrogen-bonding network; the backbone carbonyl groups of Pro68 and Ala1 and the phenolic hydroxyl group of Tyr52 are hydrogen bonded to the water molecule W′ (Fig. 1*b*) which is directly linked to the structural water found in the recombinant enzyme (Sekar & Sundaralingam, 1999). It should be mentioned that in the orthorhombic recombinant PLA2, the conserved structural water W11 (Fig. 1*a*) is linked to two additional water molecules (W′ and W″) (Fig. 1*a*). In summary, Asp99 is crucial to retain the structural water W11 and form the catalytic triad.

3.2. Trigonal form of H48Q mutant

The crystal structure of the trigonal H48Q mutant shows that Gln48 can hydrogen bond on both sides of the carboxamide. The oxygen ($O^{\varepsilon1}$) of Gln hydrogen bonds to the catalytic water, while the N atom ($N^{\varepsilon2}$) hydrogen bonds according to the scheme observed in His48 of the recombinant enzyme (Fig. 2*a*). Unlike His, Gln does not appear to accept the proton from the catalytic water, which is not deprotonated, therefore. This leads to the dramatic loss of biological activity of the Gln mutant (Li & Tsai, 1993). In addition, the second water W7 found to be linked to His48 in the recombinant enzyme is absent (Fig. 2*b*). This water may play a role in assisting the catalytic water W6 in the tauto-

[1] The structural water is also found to be missing in the single mutant D99N (Kumar *et al.*, 1994) and the triple (D99N/Y52,73F) mutants (Sekar, Yu *et al.*, 1997) where the asparagine amide group pushes the structural water out.

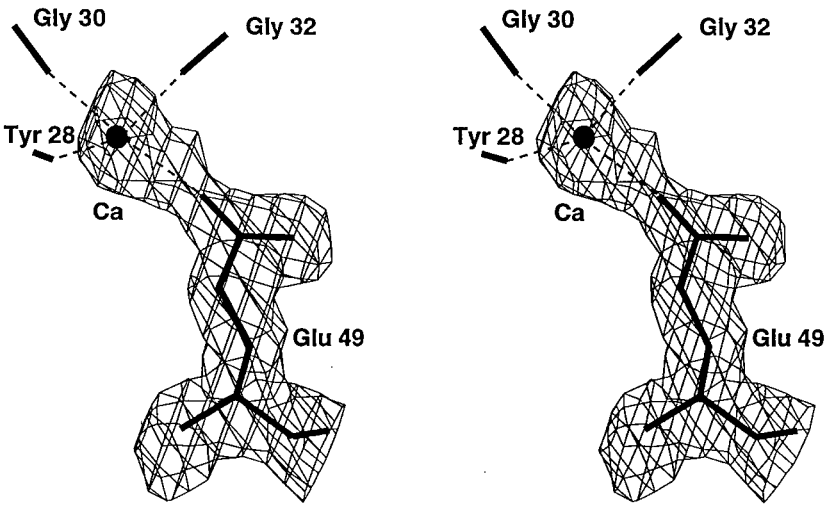

Figure 4
The omit electron density of the mutated residue Glu49 and the calcium ion. Contours are shown at the 1.0σ level. Note that there are only four ligands around the calcium ion.

merization of His48 (Sekar & Sundaralingam, 1999). Furthermore, the second water is absent owing to the large movements of the side chain of Gln48.

3.3. Trigonal form of D49E mutant

In the recombinant PLA2, the calcium ion is seven-coordinated and forms a pentagonal bipyramid (Fig. 3*b*). One of the waters W5 is in the equatorial plane with the ligands Gly30, Gly32 and Asp49 (both carboxylate O atoms), while the other water W12 is in the axial position opposite Tyr28 (Fig. 3*b*). The two disulfide bonds, Cys27–Cys123 and Cys29–Cys45 apparently confer additional stability to the calcium loop. During catalysis, the two water molecules involved in the calcium coordination are substituted by the phosphate O atoms of the substrate/inhibitor (Dijkstra *et al.*, 1981; Scott *et al.*, 1990; Sekar, Eswaramoorthy *et al.*, 1997). Asp49 is invariant and provides both of its carboxylate O atoms to the calcium. It is the only side-chain residue available for mutation, but mutating D49 to D49A, D49N, D49Q or D49K gave no enzymatic activity (Li *et al.*, 1994).

The crystal structure shows that the mutated Gln49 residue provides only one ligand ($O^{\varepsilon 1}$) (Fig. 4) to the calcium, compared with Asp49 in the recombinant enzyme which provides a bidentate ligand ($O^{\delta 1}$ and $O^{\delta 2}$). Thus, in the mutant, calcium is more labile which is reflected in the increased thermal vibration of 47.6 Å^2 compared with 20.1 Å^2 in the recombinant enzyme. All the four ligands to calcium also have higher temperature factors than in the recombinant enzyme. The two water molecules liganded to calcium are missing (Fig. 3*a*). It is possible that they might exhibit large thermal vibrations or disorder. The unidentate ligand of the mutated residue Glu49 is displaced by 0.52 Å from Asp49, while the other carboxylate O atom is too distant, 4.36 Å, to be coordinated to the Ca^{2+} ion. The four atoms of the mutant (Gly30, Gly32, calcium ion and the unidentate ligand of Glu49) are in a plane, while the backbone carbonyl group of Tyr28 is displaced by 2.5 Å (Fig. 3*a*). This work indicates how Asp49 in the calcium coordination is essential for the mechanism of substrate hydrolysis and cannot be replaced by Glu49.

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