

were within a spherical volume of radius $<25\text{ }\mu\text{m}$. The pipette was always positioned such that it was 20 to 25 μm from the dendritic knob and that the solution passed over the cilia before reaching somatic membrane. Since the distance from the cilia to the dendrite and soma was no more than 15 to 30 μm , the force of ejection was sufficient to cause the solution to arrive within fractions of a millisecond everywhere in the region of the cell. We included the dye fast green in the pipette and affirmed that the pulsed solution rapidly engulfed the entire cell. If the solution only partially surrounded the cell, our estimates for concentration would be low.

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Enhanced Activity and Altered Specificity of Phospholipase A₂ by Deletion of a Surface Loop

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Protein engineering and x-ray crystallography have been used to study the role of a surface loop that is present in pancreatic phospholipases but is absent in snake venom phospholipases. Removal of residues 62 to 66 from porcine pancreatic phospholipase A₂ does not change the binding constant for micelles significantly, but it improves catalytic activity up to 16 times on micellar (zwitterionic) lecithin substrates. In contrast, the decrease in activity on negatively charged substrates is greater than fourfold. A crystallographic study of the mutant enzyme shows that the region of the deletion has a well-defined structure that differs from the structure of the wild-type enzyme. No structural changes in the active site of the enzyme were detected.

THE LIPOLYTIC ENZYME PHOSPHOLIPASE A₂ (PLA₂) specifically cleaves the 2-acyl linkage of phosphoglycerides in a calcium-dependent reaction (1). Phospholipases occur both extracellularly and intracellularly. The extracellular PLA₂'s from mammalian pancreas and from snake venom (1, 2), and also the mammalian intracellular PLA₂'s (3–5), exhibit a high degree of sequence homology. A difference between snake venom PLA₂'s and the pancreatic enzymes is that the former ones in general have higher turnover numbers and a greater affinity for phospholipid molecules aggregated in micelles than the pancreatic ones (6, 7). In the pancreas but not in snake venom the enzyme occurs as a precursor that is activated in the duodenal tract by the

tryptic removal of a small activation peptide from the NH₂-terminus.

The x-ray analyses of several PLA₂'s from pig, ox, and *Crotalus atrox* venom (8, 9) show that these enzymes are structurally similar

(10). A comparison of the structures of active bovine PLA₂ and that of inactive bovine precursor (11) shows that in the active enzyme the NH₂-terminal helix and loop 62 to 72 are well defined, whereas in the precursor this latter loop and the first three residues of the NH₂-terminal α helix are mobile. Because pro-PLA₂, contrary to active phospholipase, does not bind to aggregates of zwitterionic phospholipids, it has been suggested that a low mobility of the NH₂-terminal helix and the surface loop are required for efficient binding (12). A similar immobilization of the substrate binding domain has also been observed for trypsin after activation of trypsinogen (13).

Lipolytic enzymes hydrolyze aggregated substrates such as micelles, vesicles, and liposomes at much higher velocities than monomeric substrate molecules. Hydrolysis of these aggregated phospholipids requires the binding of the enzyme to the lipid-water interface. After binding of the enzyme to the lipid aggregates, one single substrate molecule is thought to diffuse into the active site, where it is hydrolyzed. Many of the residues involved in the binding to aggregated phospholipids have been identified by chemical modification studies of phospholipases from many sources. On the basis of these studies, it has been concluded that the porcine enzyme residues Leu², Trp³, Arg⁶, Leu¹⁹, Met²⁰, Leu³¹, and Tyr⁶⁹ are involved in the interaction of the enzyme with lipid-water interfaces (2). The three-dimensional (3-D) structures of the pancreatic phospholipases show that these residues are all located at one face of the molecule around the active site (14). From these observations it has been inferred that the binding site of the enzyme for aggregated substrates is an extended region around the entrance of the

Table 1. Kinetic properties of two native and one mutant PLA₂ enzymes acting on various substrates (20). Assays on monomeric diC6dithioPC were carried out in the presence of 100 mM CaCl₂, 100 mM NaCl, and 200 mM tris-HCl at pH 8.0 in a spectrophotometric assay at substrate concentrations not exceeding 0.8 mM (23). Assays on micelles were carried out in the presence of 1 mM sodium borate, 25 mM CaCl₂, and 100 mM NaCl at pH 8.0. The syntheses of these substrates and details of the kinetic analyses are in (24). The K_m values are based on the concentration of micelles after correction for the concentration of free monomers. The K_m values are apparent because more than one equilibrium is involved (1). Enzymes: Wild-type, porcine pancreatic PLA₂; $\Delta 62-66$, $\Delta 62-66$ PLA₂; *N. melanoleuca*, fraction DE-III from the venom of *N. melanoleuca* (6). Standard errors of all values were less than 10% of the reported value. ND, not determined.

Enzyme	Monomeric diC6dithioPC			Micelles							
	diC6PC			diC7PC		diC8PC		diC7GS			
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ M ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)
Wild type	0.62	0.7	890	5	14	25	3.7	410	3.2	45	ND*
$\Delta 62-66$	0.90	0.5	1790	80	8	240	1.9	980	1.9	10	ND*
<i>N. melanoleuca</i>	ND†	ND†	ND†	830	ND*	980	ND*	3490	ND*	1	ND*

*Because of the formation of lipid-protein aggregates below the critical micelle concentration, K_m values could not be obtained. †Monomers of zwitterionic substrates induced the formation of lipid-protein aggregates for this venom enzyme, so that no true data for monomers could be obtained.

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active site. The 3-D structures also show that several other residues, which were not detected by chemical modification, are part of this face of the enzyme. In particular, the residues 65, 67, 70, and 72, which are part

of the 62 to 72 surface loop mentioned above, are of interest, because these residues presumably also participate in the binding of phospholipid aggregates (14).

Snake venom PLA₂'s (1) and also the

mammalian intracellular rat platelet PLA₂ (4) lack part of the 62 to 72 surface loop. The deletion of five amino acid residues is the most conspicuous difference between pancreatic and elapid venom phospholipases. In an attempt to improve the catalytic properties of pancreatic phospholipases, we have studied the effect of deleting residues 62 to 66 from porcine pancreatic PLA₂. In redesigning the primary sequence, we made other adjustments to maintain maximal sequence homology with elapid venom PLA₂'s in the region of the loop (Fig. 1). Thus the mutations D59S, S60G, and N67Y were introduced simultaneously (15). The desired mutant (Δ 62–66 PLA₂) was expressed and isolated as described for the wild-type enzyme (16).

To analyze the 3-D structure of the mutant at the atomic level, we determined the crystal structure of the mutant PLA₂. The crystals of the mutant enzyme Δ 62–66, which are of much higher quality than those of the wild-type porcine phospholipase, diffract to resolution below 2.1 Å. A comparison of the crystal structures of wild-type and mutant porcine PLA₂ at 2.5 Å resolution is given in Fig. 2. The folding of both molecules is similar, with the largest differences occurring at the site of the deletion. The root-mean-square (rms) difference is 0.45 Å for all C α backbone atoms, excluding residues 58 to 70. The structure of this loop appears to be intermediate between those in porcine and *C. atrox* phospholipases (Fig. 3). The *C. atrox* PLA₂ lacks three more residues in this region than the mutant and *Naja melanoleuca* PLA₂ (Fig. 1). Compared with the wild-type PLA₂, the disulfide bond between residues 61 and 91 in the mutant PLA₂ adopts a different conformation to accommodate the shortened loop. Moreover, the Tyr⁶⁹ side chain has moved outward toward the surface of the molecule. In contrast to the residues in or near the deletion loop, the region around the α -NH₃⁺ group of residue Ala¹ and the NH₂-terminal α helix are virtually the same. The residues in the active site are also, within error, in the same position as in the wild-type structure. The rms difference for all atoms of the active site residues and for those forming the hydrophobic wall of the active site (8, 14) is 0.40 Å.

Kinetic parameters (catalytic rates k_{cat} and Michaelis constants K_m) of the mutant enzyme were determined both on monomeric and on micellar substrates (Table 1). To allow comparison, the values of the wild-type pancreatic PLA₂ and of a venom PLA₂ are also included. The data obtained with monomeric substrate show that Δ 62–66 PLA₂ has a fully functional active site and has a specificity constant twice that of the

	50	55	60	65	70
a	H D N C Y R D A K N L D S C K F L V D N P Y T E S Y				
b	* * * * * K Q * * K * * * * * V * * * * * N N *				
c	* * * * * G E * E K I S G * - - - - W * * I K T *				
d	* * C * * G K * T - - * - * - - - - * * K * V * *				
e	* * * * * * * * * * S G * - - - - Y * * * * *				

Fig. 1. Comparison of part of the sequences of a mutated PLA₂ and of four native PLA₂s. (a) Porcine pancreas, (b) bovine pancreas, (c) *N. melanoleuca*: fraction DE-III, (d) *C. atrox*, and (e) mutant Δ 62–66 PLA₂. *Naja melanoleuca* is a member of the Elapidae, whereas *C. atrox* belongs to the Crotalidae. The full sequences of the native PLA₂'s have been compared (1). The sequence numbering is according to Renetseder *et al.* (10). An asterisk denotes homology with the porcine sequence. Absent amino acid residues are indicated by a dash. After subcloning the pro-PLA₂-cDNA into the M13-vector mp8, mutagenesis was performed according to the gapped-duplex method (25) with a 44-nucleotide mutagenic primer: 5'-GCTTTCGGTGTAGGGATA* Δ ACAGCC*T*G*A*CAGGTTCTTGGCATCTC 3'. In this oligonucleotide an asterisk denotes a nucleotide change compared with the cDNA sequence of the wild-type PLA₂, and the Δ indicates a gap of 15 nucleotides. *Escherichia coli* K12 strains PC2494 (Phabagen Collection, Utrecht) and HB2154 (26) and M13 mp18 Replicative Form (RF)-DNA were used for mutagenesis and preparation of single-stranded and RF-DNA's. Screening for mutants was performed by Pvu II restriction endonuclease digests, and positive clones were sequenced after plaque purification. From the sequenced DNA a Bam HI–Hind III fragment, containing the entire phospholipase cDNA (16), was cloned into the expression vector. Mutant pro- Δ 62–66 PLA₂ was isolated as a fusion protein; active Δ 62–66 PLA₂ was obtained after tryptic cleavage of reoxidized fusion protein followed by purification by O-carboxymethylcellulose and DEAE-cellulose chromatography as described for the wild-type enzyme (16).

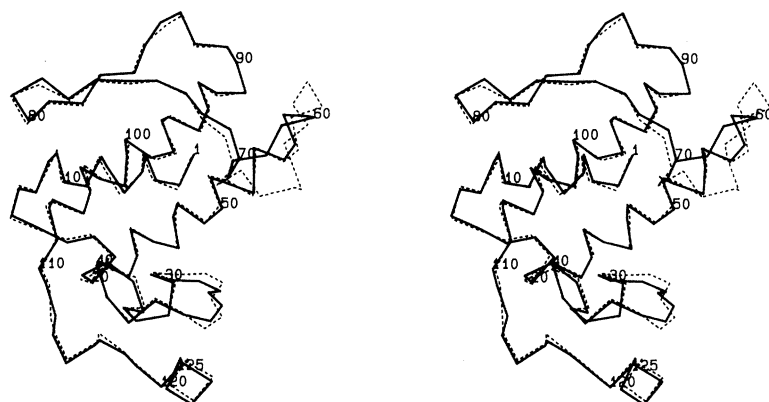


Fig. 2. Comparison of a C α backbone tracing of wild-type (dotted lines) and mutant porcine pancreatic PLA₂ (heavy lines). The structure of wild-type porcine PLA₂ was from Dijkstra *et al.* (8). The mutant PLA₂ was crystallized in a hanging drop setup from 50% methanol in 100 mM Bistris buffer, pH 7.0, with 5 mM CaCl₂. The space group was *P*₂₁ with unit cell dimensions *a* = 45.7 Å, *b* = 73.5 Å, *c* = 37.1 Å, and β = 107.1°. There are two PLA₂ molecules per asymmetric unit. X-ray diffraction data to 2.5 Å resolution were collected from one single crystal (0.4 mm by 0.3 mm by 0.15 mm) on a fast area-sensitive television detector system (Enraf-Nonius, Delft, The Netherlands). The data set was 88.5% complete up to 2.5 Å resolution. The 3-D structure of the mutant PLA₂ was solved by molecular replacement methods [rotation (27) and translation functions (28)] with the wild-type phospholipase as the starting model. The position of the second molecule with respect to the first molecule in the asymmetric unit was found with a correlation function search (29). The position and orientation of both molecules were refined with the rigid body refinement option of the TNT refinement program package (30). This same program package was used in the subsequent crystallographic refinement. At intervals, the fit of the model to its electron density was analyzed and improved with the molecular graphics program FRODO (31), running on an Evans & Sutherland PS390 picture system. The final crystallographic *R* factor is 0.237 for all data to 2.5 Å resolution, with tight restraints on the molecular geometry (for example, the rms deviation from ideal bond lengths is 0.009 Å). Coordinates have been deposited with the Brookhaven Data Bank.

wild-type enzyme. The dissociation constant for Ca^{2+} ions at pH 6.0 decreased from 1.8 mM for the wild-type to 0.8 mM for the mutant enzyme. These changes might indicate an improved positioning of the amino acid side chains in the active site. Such conformational changes could remain undetected by x-ray crystallography at 2.5 Å resolution.

The wild type, the mutant $\Delta 62-66$, and the *N. melanoleuca* PLA₂'s were tested on a series of synthetic short-chain lecithins at concentrations above the critical micelle concentration of these zwitterionic substrates. The apparent K_m 's of $\Delta 62-66$ and of wild-type PLA₂ measured on zwitterionic substrates differ only slightly. However, $\Delta 62-66$ has much greater activity than wild-type PLA₂, although not quite as great as for *N. melanoleuca* PLA₂. Furthermore, the difference between $\Delta 62-66$ and wild-type PLA₂ increases with decreasing acyl chain length in the substrate, the ratio of these activities being as great as 16 for dihexanoyllecithin. Despite the removal of one net negative charge in the mutant, $\Delta 62-66$ PLA₂ is much less active on the negatively charged substrate diheptanoylglycosulfate than the wild-type enzyme. A mutant PLA₂ containing only the mutations D59S and S60G (17) displayed 35% activity on diC8PC micelles and 80% activity on diC7GS micelles relative to the wild-type enzyme. Thus deletion of the loop, rather than the removal of one negative charge, induced the considerable change in the preference of the enzyme for zwitterionic and negatively charged phospholipids and conferred properties to the porcine enzyme similar to those in *N. melanoleuca* (6) and *Crotalus adamanteus* (18, 19) phospholipases.

Whereas the mutant is only twofold more active than the wild-type enzyme on monomeric substrates, a further eightfold increase in relative activity is observed with aggregated dihexanoyllecithin as the substrate. The question arises how the increased activity on these micelles can be explained. Answering this question is hampered by the lack of structural knowledge of the enzyme-micelle complex. What is clear, however, is that the mutations and deletions in the loop 62 to 72 affected part of the putative binding site for phospholipid aggregates: Asn⁶⁷ was replaced by Tyr, Val⁶⁵ was deleted, and the x-ray analysis shows that Tyr⁶⁹ in the mutant enzyme has a position and orientation that differ from those of the wild-type enzyme. All of these changes could cause a different orientation of the active site of the mutant with respect to the lipid aggregate, allowing a more efficient interaction with the individual phospholipid molecules. Another effect of the mutations is that the residues in the

putative binding site for aggregated substrates in mutant and wild-type enzyme more or less lie in one plane (Fig. 4). Only the residues in the loop region of the wild-type enzyme protrude from this smooth surface. This protrusion might destabilize the complex with loosely packed micelles, which would explain the rather low catalytic efficiency of the wild-type enzyme on diC6PC micelles (20). However, the hydrophobicity of this loop could contribute to the binding of the wild-type enzyme to relatively tightly packed micelles, composed of phospholipids with longer acyl chains.

With regard to the lowered activity on negatively charged aggregated substrates, several factors could contribute to this phenomenon. First, the deletion removed Lys⁶² from a position where all pancreatic phospholipases have conserved a positively charged residue. This Lys residue is proba-

bly important for the interaction of pancreatic phospholipases with negatively charged mixed micelles of bile salts and phospholipids, the natural substrate for pancreatic phospholipases, but not for venom phospholipases. Second, negatively charged and zwitterionic substrates might bind differently in the active site of the enzyme, which might result in a different effect of the mutation of the hydrolysis rate toward these substrates. Third, the packing of diC7GS in micelles might be quite different from that of the equivalent diC7PC micelles.

A rational alteration of enzyme specificity and activity requires a thorough knowledge of how the amino acid sequence relates to folding, conformation, and mechanism of catalysis. Despite limited insights in these processes, the introduction of point mutations has proved to be a powerful tool to improve the catalytic efficiency, change the

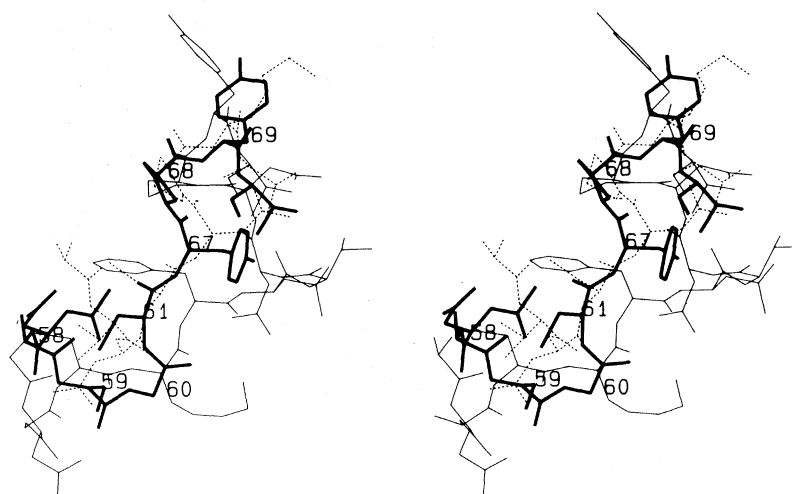


Fig. 3. Stereoview of a comparison of the conformation of residues 57 to 70 in wild-type porcine PLA₂ (thin lines), *C. atrox* phospholipase A₂ (dotted lines), and mutant porcine PLA₂ (heavy lines). Sequence numbering is according to Renetseder *et al.* (10).

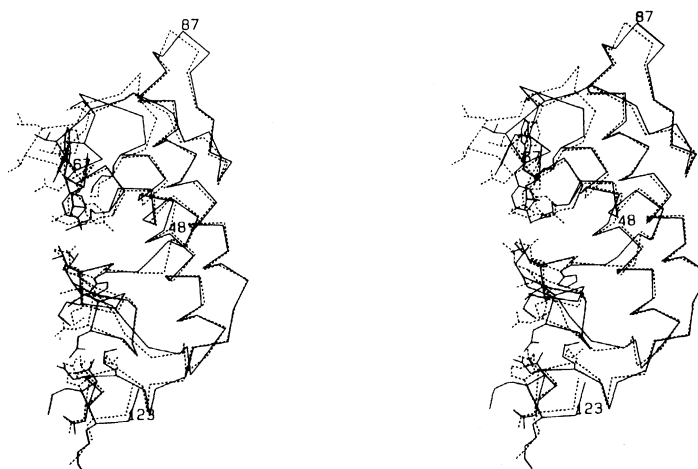


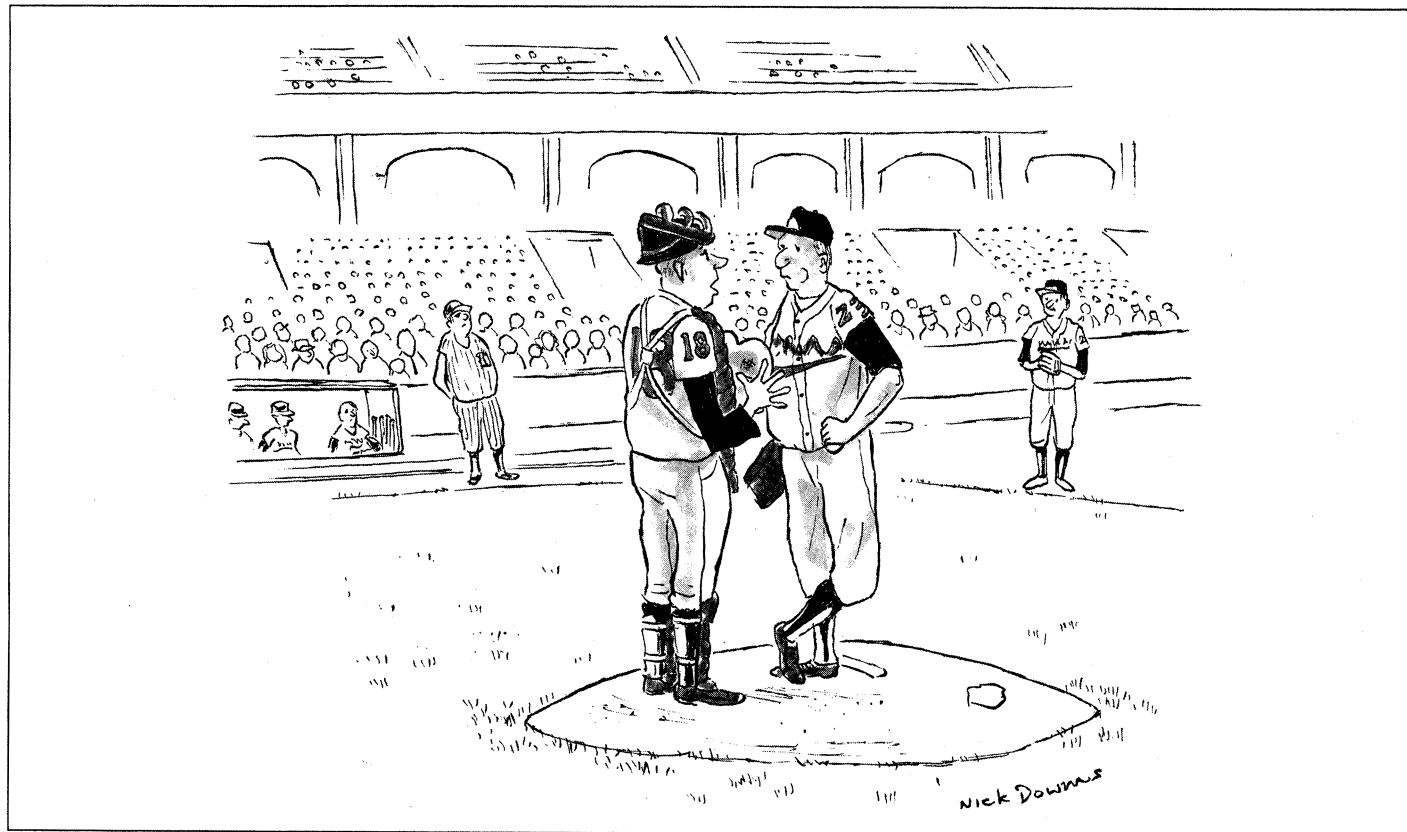
Fig. 4. The putative binding site for aggregated phospholipids in wild-type (dotted lines) and mutant PLA₂ (solid lines). The side chains are shown only for those residues presumed to be involved in the binding of phospholipid aggregates.

specificity, or alter the stability of enzymes (21). Our results show that natural variations in homologous proteins can provide a guide in creating enzymes with increased activity or altered specificity, which are major goals of protein engineering. This study demonstrates that the deletion of a surface loop can improve crystallization properties of a protein significantly. Intracellular phospholipases, which are thought to play an important role in inflammation processes, lack part of surface loop 62 to 72. In the absence of 3-D structures of the intracellular phospholipases, the structural data of the extracellular ones are being used for the design of specific inhibitors for the intracellular PLA₂'s (22). A refined 3-D structure of the deletion mutant could be used for this purpose.

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"Throw the ball in such a way that it rotates in a forward direction, allowing the air to pass underneath the ball easier than the air above it, thereby creating pressure which will press the ball downward in a sharper arc, or curve, than would normally occur from gravitational pull alone."