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PHOSPHOLIPASE A_2—A STRUCTURAL REVIEW

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R. K. Arni and R. J. Ward. Phospholipase A_2—A structural review. *Toxicon* **34**, 827–841, 1996.—Phospholipases A_2 (PLA_2) are widely distributed in nature and are well characterized proteins with respect to their catalytic and pharmacological activities. A wealth of structural information has recently become available both from X-ray diffraction and NMR studies, and although a detailed model of the catalytic mechanism of PLA_2 has been proposed, the structural bases of other aspects of PLA_2 function, such as interfacial activation and venom PLA_2 pharmacological activities, are still under debate. An appreciation of the PLA_2 protein structure will yield new insights with regard to these activities. The salient structural features of the class I, II and III PLA_2 are discussed with respect to their functional rôles. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Phospholipases A_2 (PLA_2 EC 3.1.1.4, phosphatide *sn*-2 acylhydrolases) specifically hydrolyze the *sn*-2 ester bond of phospholipids (van Deenen and de Haas, 1963), displaying enhanced activity towards lipids in lamellar and micellar aggregates both in membranes and at other lipid–water interfaces (Ramirez and Jain, 1991; Jain *et al.*, 1995). These enzymes are widely distributed in nature and have been traditionally classified as 'intracellular' or 'extracellular'. Intracellular PLA_2s are often membrane associated and are involved in phospholipid metabolism, signal transduction and other varied essential cellular functions [see Mukherjee *et al.* (1994) for recent review]. Extracellular PLA_2s are abundant in mammalian pancreatic juices and in the venoms of snakes and insects and display diverse rôles, including blood platelet aggregation [reviewed by Waite (1988)]. Their catalytic activity results in the release of arachidonic acid, a precursor of eicosanoids, which is implicated in triggering inflammatory reactions (Kudo *et al.*, 1993). Extracellular PLA_2s have been divided into classes I, II and III, based on their amino-acid sequence and disulphide bonding pattern (Renetseder *et al.*, 1985). With the discovery of an 85000 mol. wt intracellular PLA_2, sensitive to micromolar Ca^{2+} concentrations both in rat kidney and human platelets (Clark *et al.*, 1991; Sharp *et al.*, 1991), an additional group of PLA_2, class IV, has been proposed (Dennis, 1994). As the number of protein sequences available grows rapidly, the PLA_2 classification system can be expected to expand further.

Class I, II and III PLA_2s are small proteins of 119 to 143 amino acids, with molecular weights ranging between 1200 and 1500 mol. wt. The Class I enzymes have been isolated from *Elapidae* and *Hydrophidae* snake venoms and mammalian pancreas, whereas class II PLA_2s are present in snake venoms of *Crotalidae* and *Viperidae* species. More recently, they have been shown to be present in a variety of mammalian non-pancreatic tissues (Johansen *et al.*, 1992; Kudo *et al.*, 1993). The class III enzymes have principally been isolated from lizard and bee (*Apis mellifera*) venoms.

Apart from their primary catalytic function, snake venom PLA_2s often display additional pharmacological activities. Hemorragic (Gutiérrez *et al.*, 1980), myotoxic (Mebs, 1986; Gutiérrez and Lomonte, 1995), hemolytic (Condrea *et al.*, 1981), odema formation (Lloret and Moreno, 1993), hypotensive (Huang, 1984), pre-synaptic (Chang *et al.*, 1977) and post-synaptic neurotoxicity (Bon *et al.*, 1979), cardiotoxic (Fletcher *et al.*, 1981), platelet aggregation (Yuan *et al.*, 1993; Gerrard *et al.*, 1993) and convulsant (Fletcher *et al.*, 1980) activities have been reported, implicating the action of PLA_2s in many of the pharmacological effects seen on snake evenomation. These effects may be independent of catalytic activity (Lomonte and Gutierrez, 1989; Homsi-Brandenburgo *et al.*, 1988; Chwetzoff *et al.*, 1989). Although the primary sequences of a large number of PLA_2s are currently available, prediction of the structural determinants of pharmacological activities based on sequence comparisons (Kini and Iwanaga, 1986; Kini and Evans, 1987), distribution of charged residues or hydrophobic characteristics (Tsai *et al.*, 1987) have not proven to be generally applicable. Knowledge of the three-dimensional structures of PLA_2s will prove invaluable in improving our understanding of the molecular bases of these pharmacological activities, and we provide here an overview of the secondary, tertiary and quaternary aspects of PLA_2 structures.

PRIMARY STRUCTURE

More than 150 PLA_2 amino acid sequences are currently available in the protein sequence databases (June 1995 releases of SWISSPROT and Brookhaven protein sequence databases). Although the rapid expansion of molecular biological techniques offers various strategies for the isolation and subsequent sequencing of PLA_2 genes, direct protein sequencing continues to contribute to the expansion of the sequence databases (Heinrickson, 1991). Amino-acid sequence comparisons of class I and II PLA_2s are based on a homology numbering scheme derived from the bovine pancreatic PLA_2 sequence (Dufton and Hider, 1983; Renetseder *et al.*, 1985), and have been comprehensively reviewed (e.g. Heinrickson, 1991; Harris, 1991). The reader is referred to these reviews for more detailed information, and we include here only a brief summary of the results of sequence comparisons which are relevant to the context of this review.

The division between Class I and II PLA_2s is based on two structural criteria which are readily identified in the amino acid sequences:

1. The positions of the seven disulphide bonds differ, class II PLA_2s lack the Cys11–Cys77 disulphide, which is present in class I, but possess an alternative disulphide bridge between Cys51–Cys133. It should be noted, however, that three sequences from the related viperidae species *Bitis gabonica* (Botes and Viljoen, 1976), *Bitis nasicornis* (Joubert *et al.*, 1983) and *Cerastes cerastes* (Siddiqi *et al.*, 1991) possess only six disulphide bridges, lacking the 61–91 link.

2. In the region 52–65, class I proteins display a two to three amino acid inse 'elapid' loop), which is extended by a further five amino acids in the case of m pancreatic PLA_2s (the 'pancreatic' loop). This loop is truncated in the class which in addition display a 5–7 amino acid C-terminal extension. This C extension is linked to the body of the protein by the Cys51–Cys133 disulphi mentioned above.

Class III enzymes display a low degree of homology both as a group, a compared to the class I and II PLA_2s. Nevertheless, two common motifs are ev active site regions 32–39 in the class III and 44–51 in classes I and II (-CCxxHD the calcium binding loop 10–14 in class III and 28–32 in classes I and II (-W/Y Despite the overall differences in primary sequence, crucial secondary anc structural elements involved in catalysis and calcium ion binding are conservec classes I, II and III PLA_2s (Scott *et al.*, 1990b).

An analysis of amino-acid homology in a subset of 72 class I and II PLA_2 s is presented as a histogram in Fig. 1, in which identical or highly conserved am at a given position in the sequence results in high frequency (for details of the

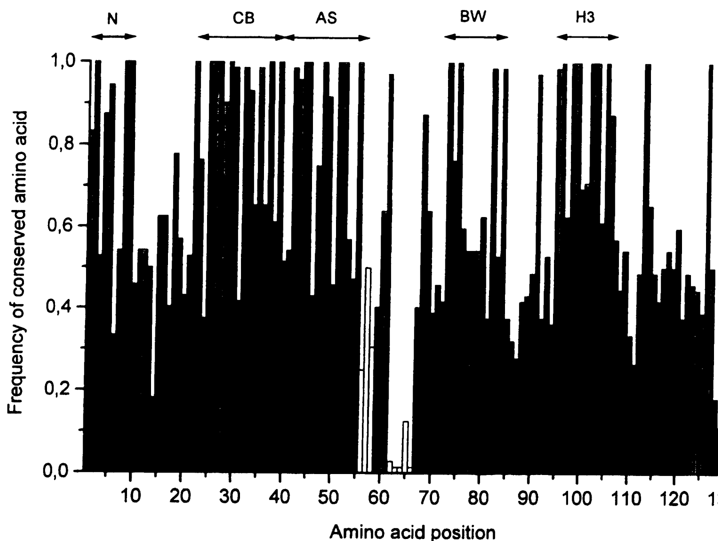

Fig. 1. Frequency of occurrence of the conserved amino acids plotted against amino acid posi in a sequence alignment. All available PLA_2 sequences were extracted from the June 1995 rele of SWISSPROT and Brookhaven protein sequence databases, and an initial alignment made u PileUp in the GCG suite of programs (GCG, 1994). Using the dendogram output from Pile highly similar sequences representing isoforms along with partial sequences were elimin together with partial sequences. A final manual alignment of 72 sequences was included using homologous regions as defined by Renetseder *et al.* (1985). The amino acids at each positio the alignment were allocated to one of six groups, hydrophobic (G, A, L, I, V, M, P), arom (F, Y, W), polar (H, T, S, N, Q), negatively (D, E) and positively (R, K) charged and cyst (C). The frequency of occurrence was calculated and the highest frequency at each position plot Regions of high homology are the N-terminal helix (N), calcium binding loop (CB), active site (β-wing (BW) and helix 3 (H3). The elapid and pancreatic loops are shown as white, and cyste as black bars.

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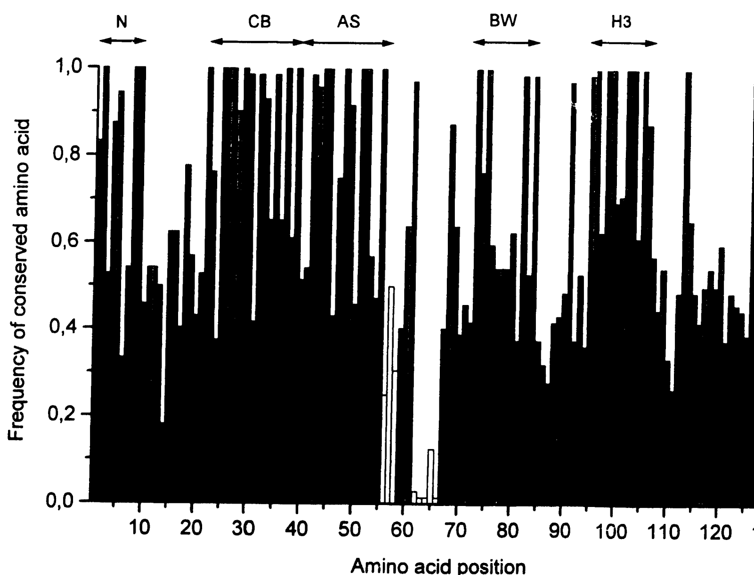

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see legend of Fig. 1). Despite their diversity of sources, three regions in class I and II PLA_2s retain a significantly high degree of amino-acid sequence homology. These regions contribute to the formation of the highly conserved secondary and tertiary structural elements including the N-terminal helix, calcium binding and active site regions together with the amino-acids forming the 'hydrophobic channel' which binds the fatty-acyl chains of the phospholipid, as discussed in more detail later. The regions displaying a lower degree of amino acid homology correspond to structurally less conserved elements, and are likely determinants of the diverse pharmacological effects exhibited by venom PLA_2s.

SECONDARY AND TERTIARY STRUCTURE

Due to their abundance in a number of natural sources, relative ease of purification and high stability, PLA_2s are amongst the best studied enzymes. A variety of techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), molecular dynamics (Demaret and Brunie, 1990) and small angle X-ray scattering (SAXS) have been combined along with spectroscopic and biochemical data to probe their structure–function relationship. Structures of PLA_2s have been determined in the presence (Thunnissen *et al.*, 1990) and absence of both specific inhibitors (Dijkistra *et al.*, 1981; Brunie *et al.*, 1985) and transition state analogues (White *et al.*, 1990; Scott *et al.*, 1990b). More recently, advances in NMR instrumentation and techniques have opened an alternative and complementary method for the three-dimensional structure determination of PLA_2 in solution (e.g. Peters *et al.*, 1992).

Representative structures of all three classes of PLA_2 have been determined and the atomic coordinates are available from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). The overall three-dimensional fold of class I (*Naja naja atra*), II (*Bothrops asper* Myotoxin II) and III (bee venom) PLA_2s are presented as ribbon representations in Fig. 2, where the major structural elements are labelled in the case of class II PLA_2. SAXS studies of (class II) PLA_2s in solution (Beltran-Abrego, 1993) indicate that they resemble a flattened ellipsoid with approximate dimensions of 45 Å × 30 Å × 20 Å (1 Å = 10^{-10} m). In the crystalline state, both class I and II possess *ca.* 50% α-helical and 10% β-pleated sheet structure.

A structurally conserved PLA_2 catalytic motif

The major structural feature of the Class I/II enzymes is a platform defined by two long antiparallel disulphide linked α-helices (helices 2 and 3, residues 37–54 and 90–109), with a distance between the helical axes of about 10 Å (see Fig. 2). Although these two α-helices do not display a clear amphipathic character, the hydrophilic amino acid side chains are generally exposed to the solvent and the hydrophobic residues point into the protein core. Crucial exceptions include the amino acids forming the catalytic network (His48, Asp49, Asp99 and Tyr52) which are located on these two helices (see Fig. 2). Figure 1 indicates that the amino acid sequences in these regions are highly conserved. Superpositioning of these helices from class I/II PLA_2s results in a root-mean-square (r.m.s.) difference in the atomic positions of the Cα atoms of *ca.* 0.4 Å. For comparison, superpositioning of the main-chain Cα atoms from class I/II PLA_2s as a whole results in an r.m.s. difference ranging between 2 and 6 Å.

Calcium is an essential cofactor for catalysis, and its substitution by other divalent ions such as barium or cadmium results in a significant reduction of activity (Yu *et al.*, 1993).

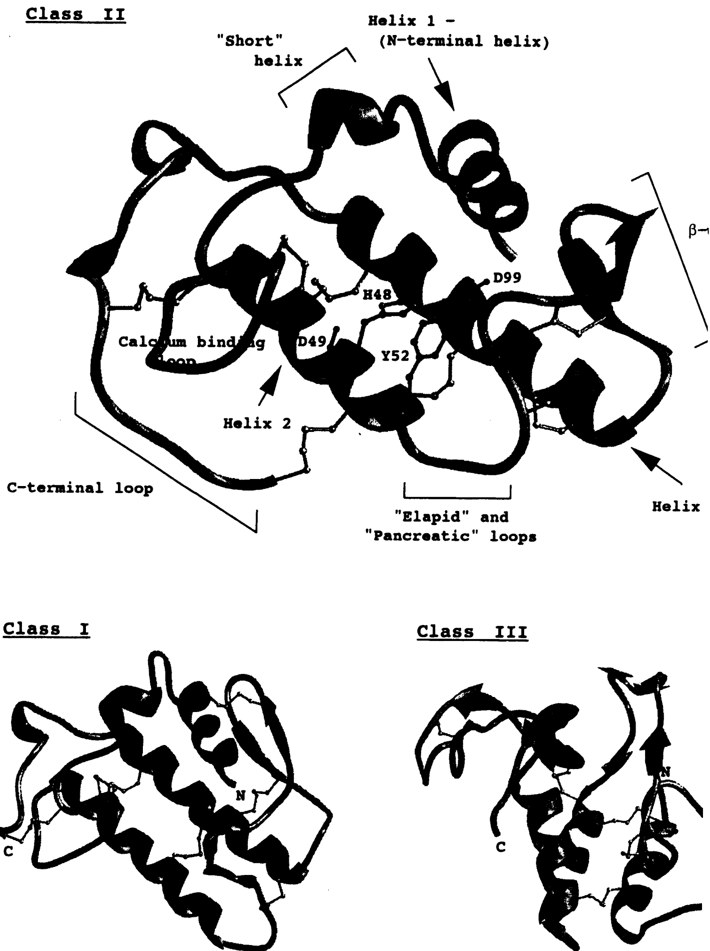


Fig. 2. Ribbon representations of Class II (*C. atrox*), Class I (porcine pancreatic) and (*Apis melifera* venom) PLA_2s. In each case, the orientation approximates 'the lipids eye all three classes the disulphide bridges are shown with light shaded bonds. In the Class I the salient structural features are labelled and the amino acids involved in catalysis a binding are shown with dark bonds.

Figure 3(a) illustrates the primary calcium binding site, in which the ca coordinated by the two carboxylate oxygen atoms of Asp49 and three main-c atoms from the appropriately named calcium binding loop (region 2 structurally conserved solvent water molecules complete the coordination s Ca^{2+} ion forming a pentagonal bipyramid. A disulphide bridge (Cys27→Cy the correct relative orientation of the calcium binding loop in relation to the which form the catalytic network. Glycine residues lend this region tl conformational flexibility, permitting the three main-chain carbonyl oxyg contribute to the stabilization of the bound calcium ion.

Asp49 is essential for Ca^{2+} binding, and the conservative substitution Asp49→Glu49 results in a 12-fold decrease in calcium affinity ($K_{dCa} = 23$ mM) with a concomitant loss of catalytic activity (Li *et al.*, 1994). Furthermore, Ala, Asn, Gln, Lys mutants at this position fail to bind Ca^{2+}. Naturally occurring PLA_2-homologues in which Asp49 is changed to Lys (Maraganore *et al.*, 1984; Francis *et al.*, 1991; Homsi-Brandenburgo *et al.*, 1988; Ward *et al.*, 1995), Ser (Krizaj *et al.*, 1991) or Ala (Liu *et al.*, 1991) are therefore catalytically inactive. However, they retain cytolytic activity (Lomonte *et al.*, 1994b) and destroy the integrity of synthetic liposome membranes by a poorly understood calcium-independent process (Rufini *et al.*, 1992; Pedersen *et al.*, 1994). Crystal structures of Lys49 PLA_2-homologues reveal that the Nζ atom of Lys49 occupies the position of the calcium ion in the catalytically active Asp49 PLA_2s [see Fig. 3(b)] (Holland *et al.*, 1990; Scott *et al.*, 1992; Arni *et al.*, 1995).

The structural motif formed by the two antiparallel disulphide linked helices in conjunction with the Ca^{2+} binding loop is conserved in the bee venom class III PLA_2 structure, as illustrated in Fig. 4 (helices 26–39 and 62–76, loop 8–14) (Scott *et al.*, 1990b). The two regions of significant sequence homology between classes I, II and III (refer to Section 1) are located in this region. The inset to Fig. 4 illustrates that the catalytic and calcium binding site residues in class I/II PLA_2s have almost perfectly positioned counterparts in the class III enzyme. This is the only structural motif conserved between the class I/II and the class III enzymes.

The proposed catalytic mechanism of PLA_2s is based on structural studies of class I, II and III PLA_2s in the native states and complexed with transition-state analogues (Verheij *et al.*, 1980; Scott *et al.*, 1990a). In the structures of the transition-state analogue complexes, bonds between the two structurally conserved solvent water molecules which complete the calcium ion coordination are substituted by bonds to the non-bridging oxygen of the phosphate and the *sn*2 oxygen. This *sn*2 oxygen atom is additionally hydrogen bonded to the main chain nitrogen atom of Gly30, thereby ensuring the proper orientation of the substrate. The hydrocarbon chains are oriented approximately parallel

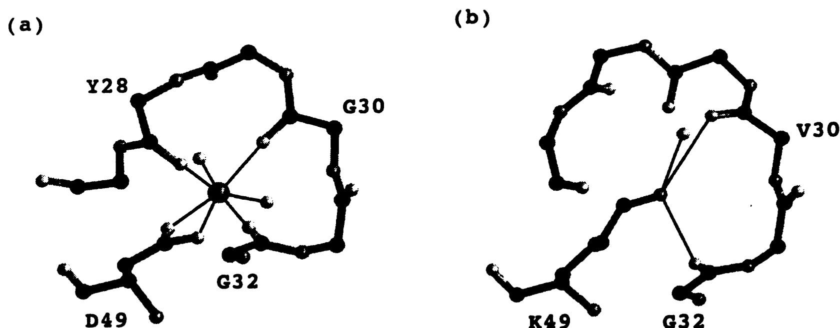


Fig. 3. Ball and stick representation of (a) the calcium binding loop from the catalytically active *N. naja naja* PLA_2. The centrally located hepta coordinated calcium ion is presented as a larger sphere forming bonds with the carbonyl oxygen atoms of Asp49, three backbone oxygen atoms and two solvent water molecules. (b) The analogous region in the catalytically inactive Lys49 PLA_2 homologue in Myotoxin II from *B. asper*. The Lys49 Nζ atom occupies the position of the calcium ion forming two bonds with the backbone carbonyl oxgen atoms and a single solvent water molecule.

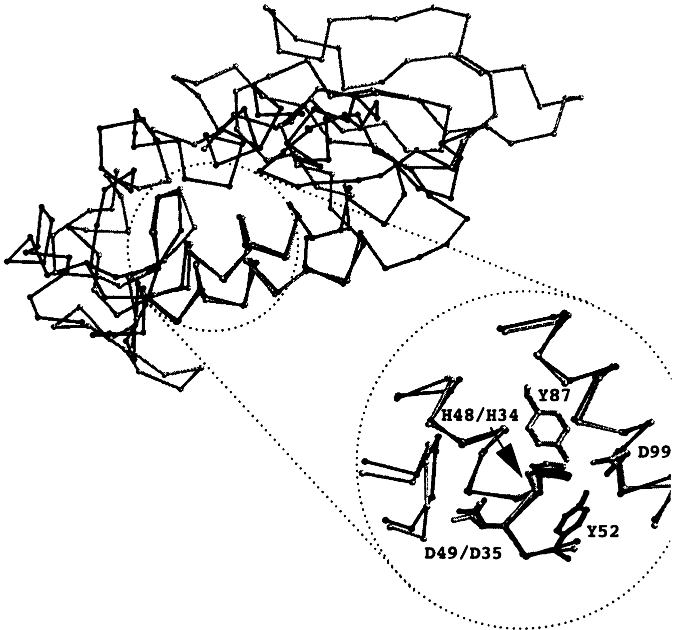


Fig. 4. Ball and stick representation of the results of the superpositioning of the porcine (dark bonds) and bee venom (light bonds) PLA_2s Cα atoms using the homologous regio Class I/II and Class III PLA_2s. The two anti-parallel helices and the calcium binding highly conserved three-dimensional structures. The inset illustrates that the residues H48 and D99 from porcine pancreatic PLA_2 and H34, D35, Y87 and D64 from the bee ve which are involved in catalysis are structurally conserved.

to each other in the hydrophobic channel that extends from the surface o to the active site (see below).

The His48 Nδ1 atom is stabilized by a bond to the carboxyl oxgen atoms 66 in bee venom PLA_2) which in turn is hydrogen bonded to the hydroxyl of the highly conserved Tyr52 (note that Tyr 87 in bee venom PLA_2 appro opposite side but serves the same function, see Fig. 4). His48 Nδ1 then behav base by polarizing and abstracting a proton from a positionally conserved w which then participates in the formation of a tetrahederal intermediate. Up the intermediate, and release of hydrolysis products, three water molecules active site (Scott *et al.*, 1990a).

The homologous hydrophobic channel

The residues of the short N-terminal amphiphilic helix (Helix 1 in Fig. 2 the highly conserved protein core and are stabilized by either a disu (Cys11→Cys77 in class I) or by hydrogen bonds (class II). In addition, th extensive contacts with the β-wing (see below). The hydrophobic residues surface of the N-terminal helix are highly conserved (see Fig. 1) and form the hydrophobic channel which provides access to the catalytic sit contributions to the hydrophobic channel include amino acid 19 which is

single helical turn (the 'short helix' in Fig. 2) following the N-terminal helix, amino acid 31 located within the calcium binding loop and amino acid 69 located in the first strand of the β-wing. Site-directed mutagenesis studies indicated that amino acid 31 is involved in binding and orientation of the lipid substrate and in shielding the active site from the solvent (Kuipers *et al.*, 1990a,b), and it has been suggested that this region becomes more ordered upon binding micelles (Kilby *et al.*, 1995). Mutagenesis of Tyr69 suggests a rôle in the control of stereospecificity in porcine pancreatic phospholipase A_2 (Kuipers *et al.*, 1989b). Additional amino acids contributing to the structurally conserved core include the highly conserved triplet Phe5, Ala104 and Phe106. Mutagenesis of Phe106 results in a reduction in the catalytic activity, emphasizing the importance of an intact hydrophobic channel for lipid binding, and the restriction of free access of solvent to the active site during catalysis (Dupureur *et al.*, 1992). In the Lys49 PLA_2-homologues, although this triplet changes to Leu5, Val104 and Leu106, the close packing of the side chains in the hydrophobic channel is maintained (Holland *et al.*, 1990; Scott *et al.*, 1992; Arni *et al.*, 1995).

In addition to their contribution to the hydrophobic core, amino acids in the N-terminal helix play a further rôle in catalysis, since the modification of residues at the $-NH_2$ terminus reduces enzymatic activity (Slotboom and de Haas, 1975). Lipid binding studies using NMR indicate a random coil $\rightarrow$ α-helix transformation of the N-terminal region of the helix (Maliwal *et al.*, 1994). However, this region is always observed to be helical in structural studies using X-ray crystallography. Furthermore, crystal structures of several Lys49 PLA_2s have indicated that Phe 3 is present in two conformations, i.e. open and closed, depending on whether a substrate is bound in the active site (unpublished results of this laboratory). This side-chain motion may complement that of Lys56, which has been demonstrated to undergo a significant conformational change on substrate binding (Noel *et al.*, 1991), forming a 'lid' which encloses the bound lipid during the catalytic cycle.

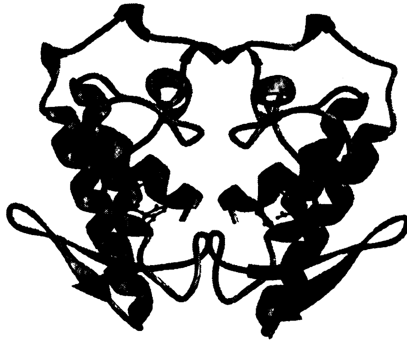
Variable loops regions

The relative orientation of the short antiparallel β-sheet, generally referred to as the β-wing (see Fig. 2), is maintained by a disulphide bridge in the class I enzymes. This disulphide bridge is absent in class II enzymes, and in this group the β-wing is found to adopt a variety of orientations in relation to the body of the protein. Both Tyr73 and an aromatic residue at position 75 are fully conserved and are located at the interface between the β-wing and body of the protein, and mutagenesis studies suggest that Tyr73 is involved in the stabilization of this region (Kuipers *et al.*, 1990b). Structural studies of Lys49 PLA_2-homologues from the venom of *Bothrops asper* (Arni *et al.*, 1995) indicate that this region forms a part of the homodimer interface [Fig. 5(b)].

The extended structures following the first long helix (helix 2 in Fig. 2) in class I PLA_2s are referred to as the 'Elapid' or 'Pancreatic' loops. Figure 2 indicates that the loop is shortened in the elapidae class I enzymes and is further truncated in the class II enzymes. Deletions in this region (amino acids 62–66) in porcine pancreatic PLA_2 resulted in enhanced catalytic activity on zwitterionic substrates (Kuipers *et al.*, 1989a). The crystal structure of this mutant indicated that the shortened loop has a well defined structure, adopting an intermediate conformation between the wild type porcine and *C. atrox* PLA_2s.

In the class II enzymes, the C-terminal loop region is extended by a further five to seven amino acids which is abundant in charged residues. It has been suggested that heparin, an inhibitor of cytolytic function in myotoxic Lys49–PLA_2s, binds to this region (Lomonte

C. atrox



***B. asper* myotoxin II**

N. naja naja

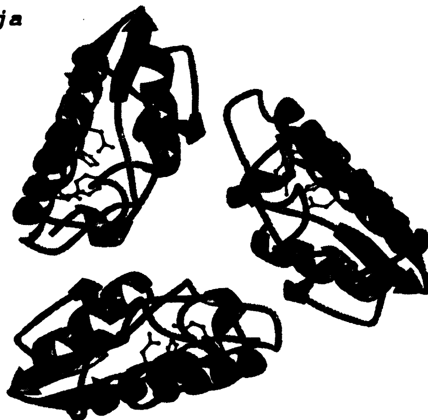


Fig. 5. Ribbon representations of the dimers from *C. atrox*, *B. asper* myotoxin II ar
from *N. naja naja*. The catalytic site residues are included as ball and stick n

et al., 1994a). Additionally, a synthetic peptide of this C-terminal fragment was demonstrated to possess specific cytolytic activity towards cultured cells, further implicating the C-terminus in myotoxic PLA_2s as a structural determinant of pharmacological activity (Gutiérrez and Lomonte, 1995).

QUATERNARY STRUCTURE

The kinetics of lipid hydrolysis by porcine pancreatic and other PLA_2s of phospholipid aggregates are preceded by an initial lag phase, and a number of models have been proposed to explain this phenomena. This latency has been shown to be accompanied by dimerization due to the autocatalytic transfer of a substrate derived acyl group to Lys56 (Tomasselli *et al.*, 1989). Transglutaminase mediated post-translational modification of PLA_2 also results in the formation of dimers, significantly increasing catalytic activity (Cordella-Miele *et al.*, 1990).

Crystal structures of monomeric (Holland *et al.*, 1990; Scott *et al.*, 1992), dimeric (Brunie *et al.*, 1985; Arni *et al.*, 1995) and trimeric (Fremont *et al.*, 1993) PLA_2s have been determined. The crystal structure of the calcium free form of PLA_2 from the venom of *C. atrox* (Brunie *et al.*, 1985) indicates the existence of the enzyme as a dimer [Fig. 5(a)]. This structure has been used as a basis for the modelling of the dimeric structure of the basic PLA_2 from *Naja nigricollis* (nigexine) venom (Demaret *et al.*, 1990). However, in the above models, the catalytic sites of the two molecules face an internal cavity and are shielded from the solvent. It has been proposed that on binding calcium, substantial alterations of the intersubunit contacts in the dimeric enzyme result in a rearrangement of the relative orientations of the molecules, thus providing access to the catalytic site (Brunie *et al.*, 1985).

The structure of myotoxin II, a dimeric Lys49 PLA_2 homologue isolated from the venom of *Bothrops asper*, demonstrated the presence of a novel dimer [Arni *et al.*, 1995—Fig. 5(b)]. This protein exists as a stable dimer in solution even when heated to 85°C for 5 min or in 2 M urea (Francis *et al.*, 1991). In the crystalline state, the dimer is formed by interactions between equivalent residues located in the N-terminal and β-wing regions in each of the two molecules. This dimeric form has since been observed in the crystal structures of two other Lys49 PLA_2-homologues which crystallize in different space groups indicating that this is not an artefact of crystal packing contacts (Arni and Ward, unpublished results). In this model, the catalytic sites are exposed and easily accessable to phospholipids in micelles or aggregated substrates.

The PLA_2 from the Indian cobra (*Naja naja naja*) in the crystalline state is present as a trimer (Fremont *et al.*, 1993). In dilute solutions, at physiological concentrations, the enzyme has been shown to exist as a monomer, forming dimeric or higher order aggregates as the concentration increases (Hazlett and Dennis, 1985). However, the functional significance of this trimeric form is currently unclear.

In addition to these homodimeric structures, the existence of heteropolymeric PLA_2s has long been established (e.g. Bon *et al.*, 1979; Pearson *et al.*, 1993). Indeed, the first venom protein to be crystallized was the heterodimeric protein crotoxin from the venom of *Crotalus durissus terrificus* (Slotta and Fraenkel-Conrat, 1938). More recently, the heterodimeric protein Vipoxin, a complex of a toxic PLA_2 with its natural inhibitor has been crystallized (Betzel *et al.*, 1993). We await the results of these studies since this structural information will contribute in improving our understanding of oligomerization and regulation of PLA_2 activities.

CONCLUSIONS AND PERSPECTIVES

X-ray crystallographic and spectroscopic studies of PLA_2s complexed and transition state analogues have contributed greatly in improving ou of the structural basis of activity. As the search for specific inhibito thearapeutic agents broadens [reviewed by Glaser *et al.* (1993); Gelb *et al* (1995)], these studies will continue to provide essential structural in application of NMR, in particular to enzyme/micelle complexes (e.g. Kil Plesniak *et al.*, 1995), will provide further information on the stuctural bas activation' (Peters *et al.*, 1992; Maliwal *et al.*, 1994).

Recent crystallographic and spectroscopic studies in this laboratory hav that the novel dimeric structure of Lys49–PLA_2s from *Bothrops* speci significant conformational change on association with natural lipids preparation, Ward and Arni). In the absence of a bound substrate the d 'open' conformation, and upon substrate binding the relative confor sub-units undergoes a significant displacement with shifts of up to 15 Å 'closed' conformation. In this model, the dimer interface behaves as a hinge relative movement of essentially rigid monomers.

Class I/II PLA_2s display a wide variety of pharmacological activies, and th considerable interest in determining the structural bases of these diverse eff that high-affinity receptors in specific target tissues mediate these path responses (e.g. Lambeau *et al.*, 1991; Lambeau *et al.*, 1995; Simpson *et al.* 1990), and recently myotoxin receptors from rabbit (Lambeau *et al.*, 19 (Ishizaki *et al.*, 1994) skeletal muscle have been cloned and sequenced. Since bind not only myotoxins but also endogenous secreted class I/II enzymes binding studies between venom myotoxins and site-directed mutants of por PLA_2 identified amino-acids in the Ca^{2+} binding loop as key residues in re (Lambeau *et al.*, 1995). Similar competitive binding studies of venom ne mutant bovine pancreatic PLA_2 have identified Tyr22 in the short helix determinant of neurotoxic activity (Tzeng *et al.*, 1995). These studies using highlight the impact molecular cloning techniques, in particular site-directe is having on expanding our understanding of PLA_2 structure–function relati the increasingly widespread application of these techniques, and the c diversity of venom PLA_2 pharmacology, there is clearly much sco investigation.

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