At the Interface: Crystal Structures of Phospholipases A2

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Phospholipases A2 (PLA2-EC 3.1.1.4) are ubiquitous enzymes which catalyse the hydrolysis of the sn-2 acyl bonds of sn-3 phospholipids (van Deenen and de Haas, 1963). Snake venoms are an abundant source of PLA2s and due to their ease of purification have been extensively studied. In addition to their catalytic activity, snake venom PLA2s exhibit a wide range of pharmacological activities which have received much attention in an effort to understand the molecular basis of the clinical effects of envenomation. These pharmacological effects include hemorrhagic (Gutiérrez et al., 1980), myotoxic (Mebs, 1986), hemolytic (Condrea et al., 1981a,b), edema 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 (Gerrard et al., 1993; Yuan et al., 1993) and convulsant (Fletcher et al., 1980).

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The proposal that toxicity results from the hydrolysis of target cell membrane lipids is supported in some cases by evidence for a correlation between toxicity and catalytic activity (e.g. Takasaki et al., 1990; Diaz et al., 1991). However, a wealth of experimental data, mainly from chemical modification of PLA2s (e.g. Condrea et al., 1981a,b; Rosenberg et al., 1983; Mollier et al., 1989; Babu and Gowda, 1994; Chang, 1996) but also from antibody binding studies (Lomonte et al., 1992) have functionally separated catalytic and pharmacological activities for several myotoxic (Babu and Gowda, 1994), anticoagulant (Diaz-Oreiro and Gutierrez, 1997) and neurotoxic (Chang, 1996) PLA2s. These results have led to the concept of specific sites on the protein which are determinants of a given pharmacological activity, and which are structurally distinct from the catalytic site.

The catalytic site of PLA2s contains a highly conserved aspartic acid at position 49 (Asp49), which is intimately involved in the binding of the essential co-factor Ca2+. After the initial report of a PLA2 with lysine at position 49 (Lys49) isolated from the venom of Agkistrodon piscivorus piscivorus (Maraganore et al., 1984), Lys49–PLA2s have been purified from the venoms of a growing number of Agkistrodon (Selistre de Araujo et al., 1996a), Bothrops (reviewed by Gutierrez and Lomonte, 1995) and Trimeresurus (Liu et al., 1991; Ogawa et al., 1992) species. These Lys49–PLA2s display little or no detectable catalytic activity against synthetic lipid substrates (Homsi-Brandenburgo et al., 1988; Francis et al., 1991), however even in the presence of EGTA they disrupt both synthetic (Diaz et al., 1991; Rufini et al., 1992) and biological membranes (Diaz et al., 1991) by a Ca2+-independent mechanism, which does not involve the hydrolysis of membrane lipids (Pedersen et al., 1994). In addition to this poorly understood Ca2+-independent membrane damaging activity, Lys49–PLA2s demonstrate myotoxic and oedema forming pharmacological activities (Liu et al., 1991; Johnson and Ownby, 1993; Gutierrez and Lomonte, 1995).

We have undertaken a systematic X-ray crystallographic study in conjunction with the application of recently developed techniques in amino acid sequence analysis in an effort to correlate structural and functional features of Lys49–PLA2s. The expanding knowledge of this sub-family of PLA2-homologues allows us to use them as a model system to study not only their pharmacological and catalytic activities, but also to investigate general features of the interaction of PLA2s with membranes.

CORRELATING THE STRUCTURE WITH THE FUNCTION OF LYS49-PLA2S

Recent advances in recombinant DNA and protein micro-sequencing technology have resulted in a rapid expansion in the numbers of DNA and amino acid sequences of PLA2s. Although the PLA2 superfamily may be divided into 4 or possibly 5 different classes (Dennis, 1994), all venom PLA2s characterized to date are of class I (snakes of families elapidae, hydrophidae), II (viperidae, crotalidae) or III (insects, lizards). The differences in the pattern of disulphide bridges and loop insertions which differentiate between the amino acid sequences of the highly conserved class I and II PLA2s isolated from snake venoms has been extensively reviewed (e.g. Harris, 1991; Heinrickson, 1991; Arni and Ward, 1996) and the reader is referred to these texts for further details. The high amino acid sequence identity is reflected in the close homology observed between the structures of class I/II PLA2s as determined by X-ray crystallography. The canonical protein fold of the class I/II PLA2 families is illustrated in Fig. 1 for the case of the Asp49–PLA2 from Crotalus atrox venom (Brunie et al., 1985). Two anti-parallel disulphide linked a-helices (helices 2 and 3) define a rigid scaffold to which the Ca2+-binding loop, the C-terminal...
loop and a single double-stranded β-sheet (the so-called β-wing) are covalently linked by disulphide bridges. In class II PLA₂s the position of the N-terminal helix (labelled as helix 1 in Fig. 1) is stabilized by extensive side-chain contacts with both the β-wing and the body of the protein. Less apparent from amino acid sequence comparisons, but evident from the structure of the type III PLA₂ from the venom of the honeybee (*Apis mellifera*) (Scott et al., 1990a), is the conservation of a PLA₂ active site motif consisting of the two disulphide linked anti-parallel α-helices in conjunction with the Ca²⁺-binding loop (Scott et al., 1990a). The highly conserved active site residues are clustered on these two helices and interact via the Asp49 (Asp 37 in the bee venom PLA₂) with the Ca²⁺-binding loop. A detailed model of catalytic activity has been proposed which involves the catalytic site

**Table 1. Lys49–PLA₂s for which crystal structures have been solved by X-ray crystallography**

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Source</th>
<th>Monomer or dimer</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>App-K49</td>
<td><em>Agkistrodon p. piscivorus</em></td>
<td>M</td>
<td>Holland et al. (1990) and Scott et al. (1992)</td>
</tr>
<tr>
<td>Myotoxin-II</td>
<td><em>Bothrops (cerrophidion)</em> godmani</td>
<td>M</td>
<td>de Azevedo et al. (1998)</td>
</tr>
<tr>
<td>Bothropstoxin-I</td>
<td><em>Bothrops jararacussu</em></td>
<td>D*</td>
<td>da Silva Giotto et al. (1997)</td>
</tr>
<tr>
<td>Myotoxin II</td>
<td><em>Bothrops asper</em></td>
<td>D</td>
<td>Arni et al. (1995)</td>
</tr>
<tr>
<td>Myotoxin II</td>
<td><em>Bothrops moojeni</em></td>
<td>D</td>
<td>de Azevedo et al. (1997a)</td>
</tr>
<tr>
<td>Piratoxin I</td>
<td><em>Bothrops pirajai</em></td>
<td>D</td>
<td>de Azevedo et al. (1997b)</td>
</tr>
</tbody>
</table>

*The dimer has been observed in two conformations in two different crystal forms of the protein.*
residues His48(36), Tyr52(84) and Asp99(66) in the formation of a tetrahedral intermediate with the lipid which is stabilized by the Ca\(^{2+}\) ion (Scott et al., 1990b). The crystal structures of 6 Lys49–PLA\(_2\)s have been solved to date (see Table 1) and as illustrated by the example of myotoxin II from Bothrops asper presented in Fig. 2(b), these structures demonstrate that the N\(_\zeta\) atom of the Lys49 is located exactly at the position occupied by the Ca\(^{2+}\) ion in the catalytically active Asp49–PLA\(_2\)s [shown in Fig. 2(a)].

![Figure 2](image)

Fig. 2. Ball-and-stick representation of residues 27–33 and residue 49 (using the homology numbering scheme of Renetseder et al., 1985), which comprises the Ca\(^{2+}\)-binding region of class I/II PLA\(_2\)s. (a) This region in the Asp49–PLA\(_2\) from Naja naja atra (Scott et al., 1990b), illustrating the pentagonal bipyramid coordination of the Ca\(^{2+}\) ion is completed by interactions with both the main-chain carbonyl oxygen atoms from the Ca\(^{2+}\) binding loop, the side chain carboxyl oxygen atoms from Asp49 and two water molecules. This is in contrast to the same region (b) of the Lys49–PLA\(_2\) from Bothrops jararajai (de Azevedo et al., 1997b), in which the N\(_\zeta\) atom of the Lys49 side chain fills the position occupied by the Ca\(^{2+}\) ion, forming hydrogen bonds (shown as dashed lines with the distances given in Å).
The positions of the other residues involved in catalytic activity are fully conserved in all Lys49–PLA2 crystal structures solved to date, and the current consensus for the lack of catalytic activity of Lys49–PLA2s suggests that the N<sub>C</sub> atom sterically hinders the binding of the essential co-factor Ca<sup>2+</sup>. This interpretation is supported by site-directed mutagenesis studies with porcine pancreatic PLA2 in which the Asp49 has been substituted by Lys (Li et al., 1994). This Asp49 → Lys mutant shows a drastic reduction in its ability to bind Ca<sup>2+</sup> together with a concomitant loss of catalytic activity (van Den Bergh et al., 1989). Recently, however, catalytic activity has been observed in various Lys49–PLA2s in vitro in the presence of polycations (Mancin et al., 1997), which clearly demonstrates that the processes of lipid binding and hydrolysis may still occur despite the substitution by Lys in the active site. This observation further indicates that the loss of Ca<sup>2+</sup> binding ability in Lys49–PLA2s is not directly responsible for their lack of catalytic activity.

Several studies have identified other amino acid substitutions which are unique to the Lys49–PLA2 subfamily (Francis et al., 1991; Selistre de Araujo et al., 1996a,b). A recent analysis using a novel multidimensional ‘sequence space’ analysis (Ward et al., 1998), has compared 72 PLA2 amino acid sequences and identified 11 residue substitutions that are highly unique to the Lys49–PLA2 subfamily. Rather than occupying positions scattered throughout the protein, these unique substitutions are concentrated in amino acid clusters (Ward et al., 1998). It is particularly significant that two of these clusters occur in the active site and lipid substrate binding regions and a list of these residues is presented in Table 2. Chemical modification of Phe5 in the lipid binding pocket of pancreatic PLA2 has demonstrated that conservation of the hydrophobic nature of this region is essential for maintained catalytic activity (van Scharrenberg et al., 1982). A site-directed mutagenesis study targeting this region in which Phe106 was substituted by Ile (Dupureur et al., 1992) suggests that not only is the hydrophobic nature of this residue important, but also that a maintained volume of the amino acid side chain at this position is crucial for effective protein function. The combined amino acid substitutions in the lipid binding region of the Lys49–PLA2s do not result in a change in the volume of this region (Holland et al., 1990; Scott et al., 1992; Ward et al., 1997), suggesting that these proteins retain the ability to bind lipids. This proposition is supported by activation of Lys49–PLA2s by polycations (Mancin et al., 1997) and by the observation that myotoxin-II from Bothrops asper is autoacylated which is suggested to be due to an ‘interrupted’ catalytic cycle (Pedersen et al., 1995).

<table>
<thead>
<tr>
<th>Residue in Lys49–PLA2</th>
<th>Residue in Asp49–PLA2</th>
<th>Cluster location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu5</td>
<td>Phe5</td>
<td>LBP</td>
</tr>
<tr>
<td>Gln11</td>
<td>Cys11*</td>
<td>DI</td>
</tr>
<tr>
<td>Glu12</td>
<td>variable</td>
<td>DI</td>
</tr>
<tr>
<td>Asn28</td>
<td>Tyr28</td>
<td>AS</td>
</tr>
<tr>
<td>Lys49</td>
<td>Asp49</td>
<td>AS</td>
</tr>
<tr>
<td>Lys53</td>
<td>variable</td>
<td>–</td>
</tr>
<tr>
<td>Trp77</td>
<td>Cys77*</td>
<td>DI</td>
</tr>
<tr>
<td>Lys80</td>
<td>Gly80</td>
<td>DI</td>
</tr>
<tr>
<td>Glu86</td>
<td>variable</td>
<td>LBP</td>
</tr>
<tr>
<td>Val102</td>
<td>Ala102</td>
<td>LBP</td>
</tr>
<tr>
<td>Leu106</td>
<td>Phe106</td>
<td>LBP</td>
</tr>
</tbody>
</table>

*Disulphide bridge formed between these two residues in class I PLA2s.
LBP: lipid binding pocket; DI: dimer interface; AS: active site.
In addition to unique clusters in the lipid binding and active site regions, another cluster of residues comprised of residues Glu12, Trp77 and Lys80 forms a motif unique to the Lys49–PLA2s (Ward et al., 1998). These residues are located at the tip of the β-wing and the N-terminal α-helix, the regions of the protein which participate in the formation of a homodimer observed in the crystal structures of several Lys49–PLA2s solved in our laboratory (see Table 2). The intermolecular contacts formed between these residues and their significance for quaternary structure stabilization are addressed in more detail in Section 3.

The highly unique residue substitutions in the active site, lipid binding pocket and dimer interface regions of the Lys49–PLA2s may reflect the structural determinants of the Ca2+-independent mechanism of action, a function which is specific to this family of proteins. In contrast, residue substitutions in more peripheral positions at the protein surface are less unique to the Lys49–PLA2s and are likely to be involved in the structural determinants of functions in common with other PLA2s. Residues less unique to the Lys49–PLA2 family include several Lys residues which are located on the surface of the protein which defines the ‘interface recognition site’ (IRS). The IRS is a region surrounding the entrance to the active site which is suggested to make contact with lipid head groups on association of the protein with the membrane (Pieterson et al., 1974; Ramirez and Jain, 1991). The high proportion of the cationic residue Lys in IRS positions observed in the Lys49–PLA2s may account for their increased membrane damaging activity against negatively charged membranes (Rufini et al., 1992). Several other residues which are less specific to the Lys49–PLA2s are also observed in clusters at the surface of the protein, in particular the two charged residue clusters Glu86/Lys93 and Arg107/Asp108. Although the combination of these residues at the given positions is totally conserved in the Lys49–PLA2s, the Glu86/Lys93 motif is also found in presynaptic neurotoxins and the Arg107/Asp108 motif is shared by venom PLA2s with broader cytolytic activity.

QUATERNARY STRUCTURAL CHANGES OF THE LYS49–PLA2 HOMODIMER

Initial reports of a highly stable dimeric solution form of myotoxin II, a Lys49–PLA2 from Bothrops asper (Arni et al., 1995) have been substantiated by subsequent observations with a range of Lys49–PLA2s from Bothrops sp (da Silva Giotto et al., 1998, see Table 1). X-ray crystallographic studies have revealed that the catalytically active Asp49–PLA2 from the venom of Crotalus atrox forms a dimer in which the catalytic sites of the two molecules face one another and are shielded from the solvent by the bulk of the protein as illustrated in Fig. 3(a) (Brunie et al., 1985). However, crystallographic studies with Lys49–PLA2s from a number of Bothrops species have identified an alternative dimer configuration presented in Fig. 3(b). In this configuration, the lipid binding channels and active sites are exposed to solvent in both molecules. As described previously (Section 2, see also Table 2), the amino acid triad comprised of Glu12, Trp77 and Lys80 is uniquely conserved in all Lys49–PLA2s. These amino acids are located at the N-terminal end of helix 1 and at the tips of the β-wings and are involved in the formation of highly conserved hydrogen-bonds and salt-bridges which stabilize the dimeric form (Arni et al., 1995; de Azevedo et al., 1997a,b,c; da Silva Giotto et al., 1997). It is worthy of note that the Lys49–PLA2s from Agkistrodon piscivorus piscivorus (Holland et al., 1990; Scott et al., 1992) and myotoxin II from Bothrops (Cerrophidion) godmani (GodMT-II; de Azevedo et al., 1997c) are observed as monomers in the crystalline state. In the case of GodMT-II, a dimeric form of the protein was identified in solution by SDS-PAGE, indicating that the
monomeric forms observed by X-ray crystallography may be an artefact resulting from the crystallization process (de Azevedo et al., 1998).

The conformations of the tips of the $\beta$-wings in all PLA$_2$s show a large degree of conformational variability. The dimer interface observed for Lys49–PLA$_2$ appears to have retained this flexible quality and functions as a molecular hinge permitting the relative motion of essentially rigid monomers. In the case of bothropstoxin-I, the Lys49–PLA$_2$ isolated from Bothrops jararacussu, a relative motion of 23° between the monomers in two different crystal forms has been observed resulting in ‘open’ and ‘closed’ forms of the dimers (da Silva Giotto et al., 1998). As illustrated in Fig. 4(a) and (b), a small

Fig. 3. Ribbon representations of the dimeric configurations observed in the crystalline states for (a) the Asp49–PLA$_2$ from Crotalus atrox (Brunie et al., 1985) and (b) the myotoxin II, a Lys49–PLA$_2$ from Bothrops asper (Arni et al., 1995). In the Lys49–PLA$_2$ dimer, the active site residues (detailed as ball-and-sticks) remain exposed to solvent.
conformation change at the interfacial ‘hinge’ between the two dimers, when amplified over the length of the monomer results in a significant difference in the relative positions of the C-terminal loop regions. Crystal forms of the dimers of Lys49–PLA2s from other Bothrops species demonstrate variations in the angle between the two monomers (de Azevedo et al., 1997a,b), suggesting that sub-states of the ‘open’ and ‘closed’ dimer configuration may exist. This is supported by spectroscopic studies which indicate an equilibrium between ‘open’ and ‘closed’ states exists in solution (da Silva Giotto et al., 1998).

A PROPOSED MODEL FOR THE CA$^{2+}$-INDEPENDENT MECHANISM OF ACTION

The quaternary structural transitions of the Lys49–PLA2 homodimer observed both in solution and in the crystalline state suggest a possible mechanism of action for the Ca$^{2+}$-independent membrane damaging activity. We have recently proposed (da Silva Giotto et
transition would result in the reorganization of 70–80 lipid molecules per Lys49–PLA2 hypothesis. Experiments may be based and work is currently in progress in our laboratories to test this model is speculative, however it does provide a framework around which future experiments may be based and work is currently in progress in our laboratories to test this hypothesis.

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