

Review

A structure-based proposal for a comprehensive myotoxic mechanism of phospholipase A₂-like proteins from viperid snake venoms



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ABSTRACT

Envenomation via snakebites is an important public health problem in many tropical and subtropical countries that, in addition to mortality, can result in permanent sequelae as a consequence of local tissue damage, which represents a major challenge to antivenom therapy. Venom phospholipases A₂ (PLA₂s) and PLA₂-like proteins play a leading role in the complex pathogenesis of skeletal muscle necrosis, nevertheless their precise mechanism of action is only partially understood. Recently, detailed structural information has been obtained for more than twenty different members of the PLA₂-like myotoxin subfamily. In this review, we integrate the available structural, biochemical and functional data on these toxins and present a comprehensive hypothesis for their myotoxic mechanism. This process involves an allosteric transition and the participation of two independent interaction sites for docking and disruption of the target membrane, respectively, leading to a five-step mechanism of action. Furthermore, recent functional and structural studies of these toxins complexed with ligands reveal diverse neutralization mechanisms that can be classified into at least three different groups. Therefore, the data summarized here for the PLA₂-like myotoxins could provide a useful molecular basis for the search for novel neutralizing strategies to improve the treatment of envenomation by viperid snakes.

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1. Introduction

Snakebite envenomings are currently recognized by the World Health Organization as an important neglected tropical disease [1]. Recent estimates indicate that at least 421,000 envenomings and 20,000 deaths by ophidian accidents occur each year in the world; however, these figures may be much higher, considering the under-reporting that occurs in many regions of Asia, Africa and Latin America [2]. The mortality caused by snakebites exceeds that attributed to other neglected tropical diseases such as dengue haemorrhagic fever, leishmaniasis, cholera, schistosomiasis, or Chagas disease; nevertheless, snakebites receive far less attention from governments, health authorities, and development agencies worldwide [3,4].

In addition to mortality, snakebites may result in disability and other permanent sequelae as a consequence of the local tissue damage that can develop in severe envenoming cases [5]. Necrosis and hemorrhage are among the most feared consequences of envenomation, depending on the inflicting snake species. The main toxins involved in the complex pathogenesis of the tissue-damaging activities have been identified as members of the phospholipase A₂ (PLA₂) and metalloproteinase families. Both toxin types are frequently the most abundant components in

snake venoms from the Viperidae family [6–8], and their rapid activities often limit the ability of antivenom to prevent tissue damage [9]. Myotoxic PLA₂s target the sarcolemma and induce an acute degeneration of skeletal muscle fibers, whereas hemorrhagic metalloproteinases degrade extracellular matrix components and lead to the loss of microvessel integrity [9].

The targeting of skeletal muscle fibers by specialized PLA₂s, whose genes were recruited, neofunctionalized, and expressed in the venom glands of snakes of the Elapidae and Viperidae families, is unsurprising, considering that muscle represents the largest proportion of the body mass of prey and that its rapid damage contributes to immobilization, capture, and the initiation of digestion of prey [10]. Two related but structurally distinct PLA₂ scaffolds were recruited in the evolution of advanced snakes: group I in the family Elapidae and group II in Viperidae. The independent acquisition of myotoxic activity by PLA₂s from both lineages represents an example of convergent evolution [11].

The ability of snake venom PLA₂s to induce skeletal muscle necrosis depends on their catalytic activity [11], i.e., the hydrolysis of glycerophospholipids at the *sn*-2 position of the glycerol backbone, releasing fatty acids and lysophospholipids [12]. The enzymatic inactivation of myotoxic PLA₂s studied to date hampers their toxicity. However, although phospholipid hydrolysis is a necessary step in their mechanism of toxicity, additional structural and functional aspects of these enzymes must be involved because not all snake venom PLA₂s are myotoxic and a poor correlation between the catalysis and toxicity

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of these enzymes is well established [13]. Thus, the mechanisms of myotoxicity exerted by venom PLA₂s are still only partially understood [14].

Within the group II PLA₂s of viperids, an intriguing subtype was discovered in 1984 [15] that conserves the basic structural fold of this family of enzymes but lacks catalytic activity. These 'PLA₂ homologues' or 'PLA₂-like' proteins were subsequently found in a large number of viperid species and have the ability to induce a rapid local myonecrosis similarly as their catalytically active PLA₂ counterparts [16–19]. The study of these PLA₂-like myotoxins has attracted growing interest, and several hypotheses have attempted to explain their catalytic-independent mechanisms of myotoxicity. This review presents a summary of the current knowledge about the structure/function relationships of the PLA₂-like myotoxins, focusing on four main topics: (a) the structural basis for their catalytic inactivity; (b) the relevance of the quaternary structure assembly for their myotoxicity; (c) their mechanism of myotoxicity; and (d) the diverse ligand interactions that block their myotoxic activity. It is hoped that insights into these topics, gathered through an increasing number of structural studies, will expand our current view of how these toxins lead to the dramatic myonecrosis that develops in snakebites and pave the way for the identification of novel agents that will improve the medical treatment of this pathology.

2. The 'PLA₂-like myotoxins' or 'PLA₂-homologues': brief history and general characteristics

A seminal paper in 1984 described a new class of PLA₂ found in the venom of the North American Eastern cottonmouth (*Agkistrodon piscivorus piscivorus*), which had a lysine instead of aspartate at position 49, an amino acid residue considered to be an obligate component of the catalytic machinery of these enzymes [15,20]. This protein turned out to be the first of a large subfamily of toxins widely distributed in the venoms of viperids, commonly referred to as PLA₂-like, PLA₂ homologues, or Lys49 myotoxins. By 2012, a review listed 65 proteins of this type [21], and this number continues to grow. In addition to the most frequent Asp49/Lys49 substitution, a few reported variants have Ser, Arg, Asn, or Gln at this position. Originally, the Lys49-PLA₂ from *A. piscivorus piscivorus* (App-K49) was reported to be enzymatically active despite its inability to bind Ca²⁺ [15,20]. Subsequently, other Lys49-PLA₂s were also reported to have low levels of PLA₂ activity in vitro [22–27]. This concept was controversial and conflicted with reports of their inability to bind Ca²⁺ and with site-directed mutagenesis experiments with porcine [28] and bovine [29] pancreatic PLA₂s showing that their corresponding D49K mutants lost catalytic activity. Moreover, the extensive purification of the App-K49 resulted in negligible enzymatic activity that was 4000 times less than that of the Asp49 enzyme isolated from the same source [28]. Due to these conflicting data, most authors studying Lys49-PLA₂s proteins in the following years cautiously referred to them as PLA₂s having "either no, or low, catalytic activity" (for a review, see [16]).

This long-standing uncertainty in the literature was finally settled after recombinant snake venom Lys49 [30] and Ser49 [31] PLA₂-like proteins were produced and demonstrated to be enzymatically inactive. The structural basis for the enzymatic inactivity of the PLA₂-like proteins is discussed in Section 3. A likely explanation for the low levels of catalytic activity initially observed for Lys49-PLA₂s is that they might have contained traces of contaminating catalytic PLA₂s. In further support of the lack of enzymatic activity for Lys49 proteins, even when acting upon their biological targets in vivo, recent studies based on sensitive mass spectrometry techniques have shown that these proteins do not hydrolyze membrane phospholipids of myogenic cells in culture or of mature muscle, unlike their catalytic PLA₂ counterparts [32].

All PLA₂-like toxins from viperid venoms tested to date display myotoxic activity in rodent assays, as evidenced by the histological evaluation of the injected skeletal muscle tissue and by the increase of

creatine kinase activity in plasma caused by the release of the CK-MM isozyme from the cytosol of damaged muscle fibers into the bloodstream [33]. The myotoxic effect of these proteins occurs only locally around the site of injection and develops very rapidly. Using intravital microscopy techniques, skeletal muscle fibers were visualized with real-time dynamics to undergo necrosis in as little as 3–4 min after exposure [34]. The localized nature of this effect, as opposed to the systemic myotoxic effect displayed by some PLA₂s or PLA₂ complexes [35,36], suggests that the PLA₂-like myotoxins not only bind to skeletal muscle fibers but also may be sequestered by binding to other cell types or to extracellular matrix, thus precluding their systemic spread and the targeting of distant muscle tissue [37,38]. This view is supported by the fact that PLA₂-like myotoxins are able to lyse a variety of cell types in culture [39,40] and to bind to mouse [41] and human erythrocytes [42], albeit without causing their lysis. Moreover, these myotoxins could not be detected in plasma after their intramuscular or intraperitoneal injection using a sensitive (10 ng/mL) enzyme-immunoassay [41], and conversely, radiolabeled variants were not distributed to skeletal muscle tissue after their intravenous injection [43].

The pathological events induced by the PLA₂-like myotoxins in skeletal muscle tissue in vivo and in myogenic cells in vitro have been studied by a variety of approaches (see recent reviews in [11,37,44]). The available evidence indicates that the sarcolemma is the primary site of action of these myotoxins, and this targeting causes a rapid loss of permeability that leads to a prominent influx of calcium ions [45–47] and an efflux of potassium ions and ATP, among several other intracellular markers [48]. Thus, sarcolemmal damage is currently considered the critical event that triggers the downstream degenerative processes that elicit muscle cell necrosis [32].

Because the PLA₂-like myotoxins are unable to catalyze phospholipid hydrolysis, their toxicity must be explained by a distinct mechanism from that of their catalytically active PLA₂ counterparts. A growing number of amino acid sequences and three-dimensional structures of the PLA₂-like proteins, obtained soon after their discovery but more intensively in recent years, have provided an essential platform for identifying the functional site responsible for their myotoxic activity. The first clue toward mapping the bioactive region of a PLA₂-like protein was obtained in studies on *Bothrops asper* myotoxin II, which identified a segment of thirteen amino acid residues near the C-terminus (¹¹⁵KKYRYLKLCKK¹²⁹; numbering system of Renetseder et al. [49]) in the form of a synthetic peptide capable of causing direct cytolysis [50]. In the same study, this protein site was demonstrated to interact with heparin, explaining the neutralizing action of this glycosaminoglycan toward the effects of myotoxin II [50]. Moreover, a peptide synthesized with the equivalent sequence from the Lys49 PLA₂-like protein from *A. piscivorus piscivorus*, ¹¹⁵KKYKAYFLKCKK¹²⁹, induced myonecrosis upon intramuscular injection in mice, unequivocally supporting that this region contains the key structural determinants needed for myotoxicity [51]. Systematic site-directed mutagenesis studies on another PLA₂-like myotoxin, bothropstoxin-I from *Bothrops jararacussu*, concurred in the functional relevance of the cationic/hydrophobic cluster near the C-terminus for its toxic activities and further narrowed the identification of key structural determinants within the sequence 117–122 of this protein [30,52].

A wealth of structural information has accumulated on a considerable number of PLA₂-like myotoxins, of which twenty Lys49-PLA₂s crystallographic structures are currently deposited in the Protein Data Bank (www.rcsb.org/pdb/), including apo forms as well as ligand-complexed forms (Table 1), one crystal structure of Ser49-PLA₂, one of an Arg49-PLA₂, and three structures of a special type of basic myotoxic Asp49-PLA₂s that could represent a new PLA₂-like class (see Section 5). In combination with functional data on these proteins and aided by bioinformatic analyses, this structural information can be integrated into a comprehensive mechanism that would explain their myotoxicity, as proposed in the following sections.

Table 1
Crystallographic models of myotoxic PLA₂-like proteins.

Class/protein	Species	PDB code	Complex	Space group	Reference
Lys49-PLA ₂					
PrTX-I	<i>Bothrops pirajai</i>	2Q2J	Native	P3 ₁ 21	[70]
		2OK9	BPB	P2 ₁	[105]
		3CYL	α-Tocopherol	P2 ₁	[70]
		3QNL	Rosmarinic acid	P2 ₁ 2 ₁ 2 ₁	[100]
PrTX-II BthTX-I		1QLL	N-tridecanoic acid	P2 ₁	[56]
	<i>Bothrops jararacussu</i>	3I3H, 3HZD	Native	P3 ₁ 21	[58]
		2H8I	PEG400	P3 ₁ 21	[68]
		3IQ3	PEG4000	P2 ₁	[58]
		3HZW	BPB	P2 ₁ 2 ₁ 2 ₁	[58]
		3CXI	α-Tocopherol	P2 ₁	[70]
MyoII	<i>Bothrops asper</i>	1CLP	Native	P2 ₁ 2 ₁ 2 ₁	[54]
		1Y4L	Suramin	P2 ₁ 2 ₁ 2 ₁	[69]
BnSP-7	<i>Bothrops neuwiedi pauloensis</i>	1PA0	Native	P3 ₁ 21	[63]
BnSP-6		1PC9	Native	P3 ₁ 21	[63]
BnIV		3MLM	Myristic acid	P2 ₁	[59]
BbTX-II	<i>Bothrops brazili</i>	4K09	Native	P3 ₁ 21	[72]
MTX-II		4K06	PEG	P2 ₁	[72]
		4DCF	PEG	C2	[83]
MjTX-II	<i>Bothrops moojeni</i>	4KF3	PEG	P2 ₁ 2 ₁ 2 ₁	[60]
		1XXS	Stearic acid	P2 ₁ 2 ₁ 2 ₁	[57]
GodMT-II	<i>Cerrophidion godmani</i>	1GOD	Native	P4 ₃ 2 ₁ 2	[57]
MyoII	<i>Atropoides nummifer</i>	2A0Z	Native	P4 ₃ 2 ₁ 2	[75]
ACL myotoxin	<i>Agkistrodon contortrix laticinctus</i>	1S8I, 1S8H	Native	P4 ₁ 2 ₁ 2	[74]
		1S8G	Lauric acid	P4 ₁ 2 ₁ 2	[74]
AppK49	<i>Agkistrodon piscivorus piscivorus</i>	1PPA	Native	P4 ₁ 2 ₁ 2	[53]
Acutohaemolysin	<i>Deinagkistrodon acutus</i>	1MC2	Native	C2	[76]
Basic Asp49-PLA ₂					
PrTX-III	<i>Bothrops pirajai</i>	1GMZ	Native	C2	[86]
BthTX-II	<i>Bothrops jararacussu</i>	2OQD	Native	C2	[85]
		3JR8	Calcium ions	C2	[87]
Arg49-PLA ₂					
Zhaoermiatoxin	<i>Zhaoermia mangshanensis</i>	2PH4	PEG	P64	[99]
Ser49-PLA ₂					
Ecarpholin	<i>Echis carinatus</i>	2QHD	Lauric acid	P3 ₁ 21	[62]
		3BJW	Suramin	P2 ₁	[62]

3. Why are PLA₂-like proteins catalytically inactive?

The inability of Lys49-PLA₂ to bind Ca²⁺ is essential to its catalytic inactivity, and this was attributed to the D49K substitution because the majority of the catalytic network residues are conserved [15]. Crystallographic studies supported this hypothesis by demonstrating that the ε-amino group of Lys49 occupies the position of the Ca²⁺ ion observed in catalytic PLA₂ structures [53–55]. In contrast with this observation, site-directed mutagenesis experiments with a Lys49-PLA₂ showed that the K49D reversion mutant remained catalytically inactive [30], demonstrating that the single D49K replacement is not the only explanation for the catalytic inactivity of these toxins. Since then, two different proposals based on distinct crystallographic structures have attempted to find additional causes for the absence of catalytic activity for Lys49-PLA₂s.

3.1. The role of Lys122

A structural study on piratoxin II (PrTX-II) structure from *Bothrops pirajai* venom [56] found a continuous electron density in its hydrophobic channel and identified it as a putative fatty acid. Based on this observation, the authors proposed a role for the Lys122 residue that is conserved in all Lys49-PLA₂s but uncommon for catalytic PLA₂s. This residue interacts with the carbonyl of Cys29, which hyperpolarizes the peptide bond between Cys29 and Gly30, leading to an increased affinity for the fatty acid head group. According to these authors, the presence of this ligand would impair access to the hydrophobic channel and lead to the catalytic inactivity of Lys49-PLA₂s. However, PrTX-II was not crystallized in the presence of a fatty acid; the electron density found at the

hydrophobic channel was only interpreted as a fatty acid. In contrast, MjTX-II from *Bothrops moojeni* venom was co-crystallized with a fatty acid (stearic acid), and its structure presented significant differences [57,58]. In the MjTX-II structure, the carboxyl oxygens of the stearic acid form hydrogen bonds with His48 and Gly30, whereas for the PrTX-II structure, a water molecule makes these interactions. Consequently, the presumed fatty acid in PrTX-II is shifted out one carbon position in relation to the stearic acid of MjTX-II. More recently, in the structure of Bn-IV from *Bothrops neuwiedi* venom co-crystallized with myristic acid [59], the binding of myristic acid was similar to that of stearic acid in MjTX-II.

Several high-resolution structures of Lys49-PLA₂s present unambiguous electron densities for PEG molecules in their hydrophobic channel [58]. Fernandes et al. [58] have suggested that PrTX-II has a PEG molecule instead of a fatty acid molecule in its hydrophobic channel because it was crystallized in the presence of PEG3350 and the electron density map is compatible with this ligand. These authors observed that Lys122 interacts with Cys29 for only 12 of the 30 different monomers for apo and complexed Lys49-PLA₂s structures deposited in the Protein Data Bank. Moreover, B-factor values of Lys122 residues for most Lys49-PLA₂s are very high compared to the average for the proteins, and for some structures, there are no electron density maps for this residue above 1.2σ. Finally, the structure of Bn-IV from *B. neuwiedi* venom co-crystallized with myristic acid [59] showed the presence of this fatty acid in the hydrophobic channel for both monomers, but Lys122 interacts with Cys29 in only one of them. Subsequently, the structure of MjTX-II complexed with PEG molecules [60] demonstrated that Lys122 interacts with Cys29 in both monomers, similarly to the MjTX-II/stearic acid structure. Then, they suggested [60] that this phenomenon may

occur with either fatty acids or PEG molecules in the hydrophobic channel of Lys49-PLA₂s. All these data show that Lys122 is a very flexible residue that can adopt diverse conformations, and consequently, the hyperpolarization of Cys29 is not an obligatory function of Lys122 even in complexed structures. However, Lys122 appears to be an important residue for Lys49-PLA₂s because it is conserved in all Lys49-PLA₂s but is rare in PLA₂s. Accordingly, site-directed mutagenesis showed that the K122A mutant displays a significant reduction in myotoxic activity [30,61]. Therefore, considering the high flexibility of Lys122, it is reasonable to assume that this residue can combine multiple functions related to myotoxic activity rather than to catalytic inactivity. These functions would include assisting fatty acid anchorage in the hydrophobic channel with an eventual hyperpolarization of Cys29, contributing to the activation of the toxin and acting as an auxiliary residue to the membrane docking site (see Section 6).

3.2. The role of Tyr28 and the calcium-binding loop

Another proposal to explain the catalytic inactivity for Lys49 myotoxins suggested that the Y28N substitution plays a key role because it affects the stability of the Ca²⁺-binding loop [58]. All PLA₂s (catalytic PLA₂s) present a tyrosine residue at position 28, whereas an asparagine occupies this position for all Lys49-PLA₂s. A structural analysis of Tyr28 in PLA₂s reveals an important stability feature for the Ca²⁺-binding loop region with which it is associated. All snake venom PLA₂ structures display an interaction between the O γ atom of Tyr28 and the amino group of Gly35 in the range between 3.1 and 3.5 Å (with the exception of a special class of myotoxic basic Asp49-PLA₂s—see Section 5). This interaction also occurs for other PLA₂s, such as pancreatic bovine PLA₂, and provides more structural stability for Ca²⁺-binding loops. This fact can be verified by the distance between the C α atoms of Tyr28 and Gly33, which reflects the aperture of this loop. This distance is approximately 4.3 Å in PLA₂s but higher than 6.3 Å for Lys49-PLA₂s, due to their Y28N substitution. This open configuration of the Ca²⁺-binding loop in Lys49-PLA₂s may be another factor responsible for the inability of Lys49-PLA₂s to bind Ca²⁺ ions in addition to the lysine at position 49. Therefore, the catalytic inactivity of Lys49-PLA₂s can be explained by two replacements, Y28N and D49K [58]. This conclusion is also supported by structural studies of Ecarpholin S, a Ser49-PLA₂ from *Echis carinatus sochureki* venom, which demonstrated that the lack of a Tyr28–Gly35 interaction was also responsible for its Ca²⁺-binding loop disarrangement [62]. Additionally, site-directed mutagenesis experiments on Ammodytin L, another Ser49-PLA₂ myotoxin, showed that H28Y/S49D mutants have restored enzymatic activity [31]. Finally, the catalytic inactivity of Arg49-PLA₂s can also be explained by Y28N and D49R replacements because these proteins also have Asn at position 28.

4. What is the oligomeric conformation of Lys49 myotoxins?

Many snake venom PLA₂s have a strong tendency to form oligomers. The majority if not all PLA₂-like myotoxins occur as dimers when analyzed by electrophoresis, and accordingly, most structures have been solved as homodimers [17,54,63]. Taking into account that the dissociation of PLA₂-like proteins into monomers has been shown to impair their bioactivities [64,65], their oligomeric assembly appears to be relevant for toxicity.

4.1. The choice between two possible dimeric conformations for bothropic Lys49-PLA₂s

Bothropic Lys49-PLA₂s are the most studied proteins of this group, and the majority of them are dimeric, as shown by electrophoresis, spectroscopic [66,67], small angle X-ray scattering [68], dynamic light scattering [58], and X-ray crystallography experiments (Table 1). The first structure of a Lys49-PLA₂ from the *Bothrops* genus was solved in

1995 [54] and was followed by several others from the same genus [56,57,63,66]. All of these structures adopted a common dimeric conformation. Da Silva-Giotto and colleagues [66] solved the structure of the BthTX-I in this assembly and presented two structures with different aperture angles between the monomers, which they referred to as “open” and “closed” conformations. Subsequently, Magro et al. [63] analyzed eight Lys49-PLA₂s structures and observed that these proteins could adopt many conformations due to the flexibility between the monomers.

In 2005, the crystal structure of BaspTX-II from *Bothrops asper* complexed to suramin showed that this ligand binds simultaneously to both monomers of the protein [69]; these protein/ligand interactions are only possible if a novel Lys49-PLA₂ quaternary assembly is considered (for a review, see [19]). In contrast to the original model of dimerization, referred to as the “conventional dimer”, this “alternative dimer” is stabilized by contacts between the putative calcium-binding loop and C-terminal regions, and its dimeric interface is formed by the hydrophobic surfaces surrounding the entrance of the “active sites”. If the “conventional dimer” had been used to solve this structure, it would not be energetically favorable because a large hydrophobic portion of this ligand would be exposed to the solvent [69]. Dos Santos and colleagues [70] solved the crystal structure of a Lys49-PLA₂ complexed to alpha-tocopherol and also observed results similar to those obtained for the BaspTX-II/suramin complex. More recently, the crystal structure of MjTX-II complexed to polyethylene glycol 4000 also revealed a binding mode that is only possible for the “alternative dimer” [60]. To address whether this conformation was only induced by binding to these particular ligands or if it can be adopted by all bothropic Lys49-PLA₂s, the structures of these proteins available in the PDB (Table 1) were inspected by crystallographic symmetry application of the “alternative dimer” conformation for all the proteins analyzed [19]. Bioinformatic analyses [70] using the PISA program [71] showed that all Lys49-PLA₂s present a larger interface area and negative solvation free energy values for the “alternative dimer”, indicating that this dimer is more stable in solution [19]. Small angle X-ray scattering experiments and molecular dynamics simulations with BthTX-I also indicate that the “alternative dimer” is the most probable assembly in solution [68].

Furthermore, when Lys49-PLA₂s are bound to a ligand, a conformational change occurs that induces the following: i) the reorganization of their C-termini (usually unorganized for unbound proteins), ii) the formation of interchain hydrogen bonding between the Tyr119 residues, and iii) the alignment of basic clusters from both monomers, which may be responsible for the docking of the protein into the membrane (see Section 6) [70]. This allosteric change can be measured by two angles between the monomers: the aperture and torsional angles. Interestingly, this model was able to classify the oligomeric crystal structures into two groups: unbound and bound structures [70]. More recently, a “hydrophobic site” was found in the crystal structures of PLA₂-like proteins and was associated as part of a myotoxic mechanism for these proteins [72]. This site is only exposed to solvent by both monomers when the “alternative” assembly is adopted for bound structures (Section 6).

Differing from the “alternative” oligomeric conformation proposal described above, some studies have raised contrasting explanations: i) Delatorre and colleagues [59], in a study on the structure of BnIV myotoxin complexed with myristic acid, found both “alternative” and “conventional” oligomeric structures in its asymmetric unit and argued that dimer formation would not be relevant to the myotoxic activity; ii) in the crystal structure of a Lys49-PLA₂ from *Bothrops brazili*, only the “conventional dimer” possibility was observed [73]; and iii) spectroscopic studies of BthTX-I showed an interaction between two residues of the “conventional” interface (Trp77) [66]. As discussed by Fernandes et al. [72], the crystal structure of Bn IV/myristic acid has all the indications to be considered a dimer (alternative or conventional), but the authors [59] proposed that the biologically relevant unit would be a monomer and deposited their coordinates in the PDB data bank as a

dimer (PDB ID: 3MLM). The crystal structure of Lys49-PLA₂ from *B. brazili* [73] did not present the “alternative dimer” conformation in its asymmetric unit; however, the high values of refinement statistics may indicate that the space group would be incorrect, consequently affecting the oligomeric possibilities in the asymmetric unit (PDB ID: 4DCF). Finally, spectroscopic studies with BthTX-I indicated that the “conventional dimer” is likely to occur in solution [66]. However, this observation does not exclude the possibility that both forms (conventional and alternative dimer) could be present in solution when the protein is in the unbound (inactive) state because these experiments were not performed in the presence of ligands.

In summary, the mode of oligomeric assembly seems to be a key to understanding the myotoxic function of Lys49-PLA₂s. Despite the extensive experiments performed in the last few years, a consensus has not been reached. Nevertheless, the myotoxic mechanism based on the assembly proposed by Fernandes et al. [72] is the most comprehensive hypothesis for the myotoxic activity of Lys49-PLA₂s presented to date because it takes into account the majority of available data. This hypothesis is based on the “alternative conformation” for bound proteins (active form), and it does not exclude the possible presence of populations of monomeric or “conventional dimer” assemblies when the proteins are in an unbound (or inactive) state.

4.2. Non-bothropic Lys49-PLA₂s

As discussed in Section 4.1, Lys49-PLA₂s isolated from snakes of the *Bothrops* genus exist as dimers, and their conformation appears to be essential to their myotoxic activity. However, this dimeric quaternary structure is not always observed in non-bothropic Lys49-PLA₂s crystallographic studies. The crystal structures of ACL myotoxin from *Agkistrodon contortrix laticinctus* [74], AppK-49 from *A. piscivorus piscivorus* [53], myotoxin II from *Atropoides nummifer* [75], godMT-II from *Cerrophidion godmani* [67], and Acutohaemolysin from *Agkistrodon acutus* [76] present only one molecule in an asymmetric unit. The inspection of non-bothropic Lys49-PLA₂ unit cell packing using the PISA program [71] shows that these proteins do not form stable oligomeric complexes with the exception of myotoxin II from *A. nummifer* [75]. However, it is important to note that all non-bothropic Lys49-PLA₂s were crystallized under high concentrations of ammonium sulfate or acidic conditions that could have prevented dimer formation. In Acutohaemolysin [76] and ACL myotoxin [74], the dimer can be generated by the crystallographic 2-fold axes of the unit cell of the space groups, C2 and P4₁2₁2, respectively. Consequently, further biophysical and biochemical experiments are needed to establish their active oligomeric conformations. However, as discussed in Section 6, these proteins conserve several features (e.g., hydrophobic residues in the C-termini) that lead to the conservation of the main steps of the integrative myotoxic mechanism proposed here (Section 6).

4.3. Ser49 and Arg49-PLA₂

The crystal structure of Ecarpholin S, a Ser49-PLA₂ from *Echis carinatus* venom, with lauric acid presented two monomers in its asymmetric unit [62]. The inspection of the cell unit by the PISA program [71] shows that the dimer is stable in solution with a high complexation significance score (CSS; 1.0). Moreover, the dimer interface of Ecarpholin S is formed by hydrophobic surfaces surrounding the entrance to the “active site”, which is similar to the alternative dimer assembly of Lys49-PLA₂s. In addition, this protein has similar hydrophobic and charged residue exposure to solvent compared to Lys49-PLA₂s, which suggests a similar myotoxic mechanism for both classes of PLA₂-like proteins (see Section 6). Thus, despite the lack of any other experimental evidence of its dimeric conformation, crystallographic data suggest that Ser49-PLA₂s may assemble as a homodimer.

Zhaoermiatoxin is an Arg49-PLA₂ from *Zhaoermia mangshanensis* venom that migrates in SDS-PAGE at a relative mass of ~14.8 kDa with

a weaker band at ~27.6 kDa [77]. Biochemical experiments with Promutoxin (an Arg49-PLA₂ from *Protobothrops mucrosquamatus* venom) also showed the presence of dimers in solution [78]. The crystal structure of Zhaoermiatoxin was obtained with two monomers in its asymmetric unit and displayed a PEG molecule in the hydrophobic channel for both monomers (PDB ID: 2PH4). The inspection of the cell unit by the PISA program [71] shows that the dimer is stable in solution but presents a low CSS (0.112). Despite that the dimer interface of the Zhaoermiatoxin is quite different from Ser49 and bothropic Lys49-PLA₂s, it is possible to identify similar charged and hydrophobic residues exposed to solvent for both Arg49 and Lys49-PLA₂s, suggesting a similar myotoxic mechanism for all classes of PLA₂-like proteins (see Section 6).

5. Can myotoxic basic Asp49-PLA₂s form a new PLA₂-like protein class?

As mentioned in Section 2, Lys49-PLA₂s are the most studied group of snake venom PLA₂-like toxins, and there are a few reports of Ser49, Arg49, Asn49 and Gln49-PLA₂s. However, more recently, it was reported that two basic Asp49-PLA₂s could form a new PLA₂-like protein class. BthTX-II from *B. jararacussu* [79,80] and PrTX-III from *B. pirajai* [81,82] are basic Asp49-PLA₂s that exhibit high calcium-independent myotoxic activity and display none or lower catalytic activity compared to other snake venom Asp49-PLA₂s [82–84]. To study these particular characteristics observed for BthTX-II and PrTX-III, structural studies have been performed by different research groups [85,86]. Correa and colleagues [85] suggested that the absence of (or low) catalytic activity in BthTX-II and PrTX-III structures is due to Ca²⁺-binding loop distortion, which impairs co-factor coordination [86]. Subsequently, BthTX-II was co-crystallized using a high concentration of Ca²⁺ [87]. This structure demonstrated that the Ca²⁺-binding loop conserved the same conformation observed for apo BthTX-II, which strengthens the hypothesis that the absence of catalytic activity is based on the Ca²⁺-binding loop distortion. Furthermore, based on phylogenetic analysis, these authors [87] also showed that BthTX-II is more closely related to Lys49-PLA₂s than to catalytic PLA₂s, thus allowing PLA₂s from snakes of Crotalinae subfamily to be classified into two main branches. The first branch includes proteins that display Ca²⁺-independent myotoxic activity (PLA₂-like), whereas the second contains the catalytic PLA₂s. This finding led to the hypothesis that the myotoxic mechanism for these proteins (BthTX-II and PrTX-III) would be analogous to Lys49-PLA₂s.

The dimeric conformation of BthTX-II was observed in solution using non-reducing SDS-PAGE, DLS [87], and SAXS [88], and for PrTX-III, it was observed by non-reducing SDS-PAGE and analytical size-exclusion chromatography [89]. Crystal structures of BthTX-II [85] and PrTX-III [86] revealed a common dimeric structure, which was also observed by dos Santos and colleagues [87]. However, these authors also found a new possibility of a dimeric conformation for BthTX-II using bioinformatic tools based on the more favorable energetic state of the protein. This conformation presents the interaction between Trp31 from both monomers and explains why Ca²⁺-binding loops are distorted. As a consequence, the Tyr28 is distorted in comparison to catalytic PLA₂s, impairing the hydrogen bond formation between the O_γ atom of Tyr28 and the Gly35 amino group, which is, as noted in Section 3, an important stability feature for the Ca²⁺-binding loop region [58]. In catalytic Asp49-PLA₂s, the distance between the C α atoms of Tyr28 and Gly33 residues, which reflects the aperture of this loop, is approximately 4.3 Å, whereas for BthTX-II and PrTX-III structures, this distance is greater than 7 Å. Thus, this Ca²⁺-binding loop distortion impairs the coordination of Ca²⁺ and may be responsible for the absence of catalytic activity in this special class of myotoxic Asp49-PLA₂s [87]. Furthermore, based on the analysis of the dimeric conformation found for BthTX-II and PrTX-III structures, it can be observed that their C-termini are conveniently located to interact with membranes (side-by-side and in the same face of the toxin) and present charged

and hydrophobic residues exposed to solvent. Although this dimeric conformation is different from that found for Lys49-PLA₂s (Section 4.1), the presence of conveniently positioned charged and hydrophobic residues may lead to a myotoxic mechanism similar to that of Lys49-PLA₂s and other PLA₂-like myotoxins, as discussed in Section 6. All the results presented in this section indicate that BthTX-II and PrTX-III are a special class of Asp49-PLA₂s that display calcium-independent myotoxic activity similar to Lys49-PLA₂s, thus representing a new class of PLA₂-like myotoxins.

6. An integrated hypothesis for the myotoxic mechanism of PLA₂-like myotoxins

As mentioned in previous sections, all PLA₂-like proteins for which crystallographic structures are available (Lys49; Ser49; Arg49 and basic Asp49-PLA₂ myotoxins) have produced biochemical and crystallographic data that support an integrated hypothesis for their mechanism of myotoxicity that will be discussed in this section.

Several hypotheses have been proposed to describe the activity of Lys49-PLA₂s toward membranes. Initially, Lomonte et al. [50] showed that a synthetic peptide composed of the C-terminal region (115–129) from *B. asper* myotoxin II displays cytolytic activities, and subsequently, that the equivalent synthetic peptide of AppK49 myotoxin (from *A. piscivorus piscivorus*) induces myonecrosis in mice [51]. These data led to the conclusion that this region, formed by cationic and hydrophobic residues, is primarily responsible for the toxicity of Lys49-PLA₂s [50,51]. The molecular modeling of ACL myotoxin (from *A. contortix laticinctus*) suggested the relevance of cationic residues in Lys49-PLA₂s and proposed that partially conserved residues (Lys7, Glu12, Thr13, Lys16, and Asn17) and specific basic residues (Lys78, Lys80, Lys116, and Lys117) would constitute a site responsible for their myotoxicity [90]. Subsequently, da Silva Giotto et al. [66] assumed that changes in the oligomeric conformation of Lys49-PLA₂ dimers played a role in their myotoxic mechanism in addition to the role of the C-termini. These authors solved the crystal structure of BthTX-I from *B. jararacussu* in open and closed dimeric states. Consequently, combining crystallographic and spectroscopic data, they proposed that the transition between the open and closed states would occur on the membrane, leading to the disruption of the phospholipid bilayer with a consequent loss of membrane integrity [66].

Subsequently, Lomonte and colleagues [16] combined several aspects of the former models to suggest a hypothetical myotoxic mechanism for Lys49-PLA₂s. In this proposal, positively charged residues from the C- and N-termini would make initially weak electrostatic interactions with negatively charged sites of the membrane, and these would be strengthened by the contribution of aromatic and hydrophobic residues in the C-terminal region. In this model, two additional factors would enhance the membrane perturbation: i) the oligomeric conformation changes through a molecular hinge [66] and; ii) the potential acylation of these toxins, either via autocatalysis [91] or via an interrupted catalytic cycle that fails to release a free fatty acid [56]. Site-directed mutagenesis supported the importance of the C-termini in the myotoxic mechanism and further narrowed this toxic site to the Tyr117–Lys122 segment [52,61]. Additionally, the removal of the N-terminal octapeptide by cyanogen bromide strongly reduced the myotoxic activity of bothropic Lys49-PLA₂s [92,93] with no significant conformational change of the protein [98], also suggesting a relevant role of the N-terminal region in myotoxicity.

Subsequently, Bortoletto-Bugs et al. [94], based on studies of the interaction of sodium dodecyl sulfate molecules to BthTX-I, proposed a micelle nucleation model in which Lys49-PLA₂s extract the phospholipids after electrostatic interactions with the membrane, which would lead to conformational changes in the protein, the reorganization of the phospholipids, and the loss of phospholipid bilayer integrity [94].

Importantly, all these previous hypotheses were based on the conventional dimer assembly as the quaternary structure of bothropic

Lys49-PLA₂s. Murakami et al. [69] and dos Santos et al. [70] proposed an alternative dimer assembly in the structure of Lys49-PLA₂s (see Section 4.1). Based on this oligomeric conformation, a new myotoxic mechanism has been hypothesized [70] that is supported by the observation that positively charged residues from the N- and C-termini (Lys20, Lys115 and Arg118) interact with sulfate ions in bothropic Lys49-PLA₂s structures. Indeed, X-ray crystallographic studies with the bovine pancreatic PLA₂s showed that the interactions of cationic ions could indicate the regions of PLA₂s that interact with membranes because these ions can simulate the phospholipid polar head group [95]. Therefore, dos Santos et al. [70] proposed that these three residues (and eventually other basic residues close to this particular region) would constitute the myotoxic site of bothropic Lys49-PLA₂s. More recently, Fernandes et al. [72] suggested that this basic cluster is responsible for toxin docking with the phosphatidyl group of anionic lipid membrane bilayers. Consequently, they called the basic cluster formed by the strictly conserved C-terminal residues (Lys115 and Arg118), eventually aided by other cationic and exposed residues such as Lys20, Lys80, Lys122 and Lys127, the “cationic membrane-docking site” (MDoS) (Fig. 1; Table 2). It was also observed that the presence of a hydrophobic molecule in the hydrophobic channel leads to an oligomeric change, which induces the alignment of the MDoS from both monomers and increases the efficacy of this docking process [70].

Another important functional site was also observed [72] for bothropic Lys49-PLA₂s when the “alternative conformation” was considered. Leu121 and Phe125 are conserved residues in the majority (98%) of bothropic Lys49-PLA₂s, and these hydrophobic residues are more exposed to solvent and have a strong reduction in their buried surface area for complexed structures when the alternative dimer is considered as their quaternary structure. Furthermore, after ligand binding at the hydrophobic channel, the side chains of these residues became aligned for both monomers. Taking into account that these residues (Leu and Phe) have high hydrophobicity and permeability indices [96–98] and the previous structural observations, these authors [72] proposed that this hydrophobic site is responsible for membrane disruption, naming it the “hydrophobic membrane-disruption site” (MDiS). A similar site was also observed for ACL myotoxin by Ambrosio and colleagues [74], who observed an increased hydrophobic residue exposure (Phe121 and Phe124) when a ligand was bound to the hydrophobic channel in this toxin.

Thus, by integrating all previous data, a comprehensive myotoxic mechanism for Lys49-PLA₂s was proposed, which identifies the critical residues involved [72]. This mechanism involves an allosteric transition and the participation of two independent interaction sites with the target membrane and includes the following steps (Fig. 2):

- I) Hydrophobic molecule binding at the hydrophobic channel;
- II) Allosteric activation;
- III) Protein-membrane docking (MDoS);
- IV) Protein penetration (MDiS) and membrane disorganization;
- V) Cell death.

As previously discussed, the key step for protein activation is the binding of a fatty acid at the hydrophobic channel, which leads to allosteric transition and structure stabilization (see Section 4.1), exposing MDoS and MDiS to the solvent (Fig. 1). In addition, Pedersen et al. [91] showed that Lys49-PLA₂s were able to bind to isolated muscle membranes and to the surface of liposomes with the fatty acid moiety inserted into the lipid bilayer acting as an anchor. Fatty acids may come from membrane phospholipid hydrolysis by catalytic PLA₂s, highlighting the synergism between PLA₂s and PLA₂-like proteins, as shown by Cintra-Francischini and colleagues [47]. These authors demonstrated that membrane damage occurs even with low amounts of Lys49-PLA₂s when the process occurs in the presence of PLA₂s. Furthermore, the fatty acid binding event justifies the conservation of fundamental residues from the hydrophobic and catalytic sites of

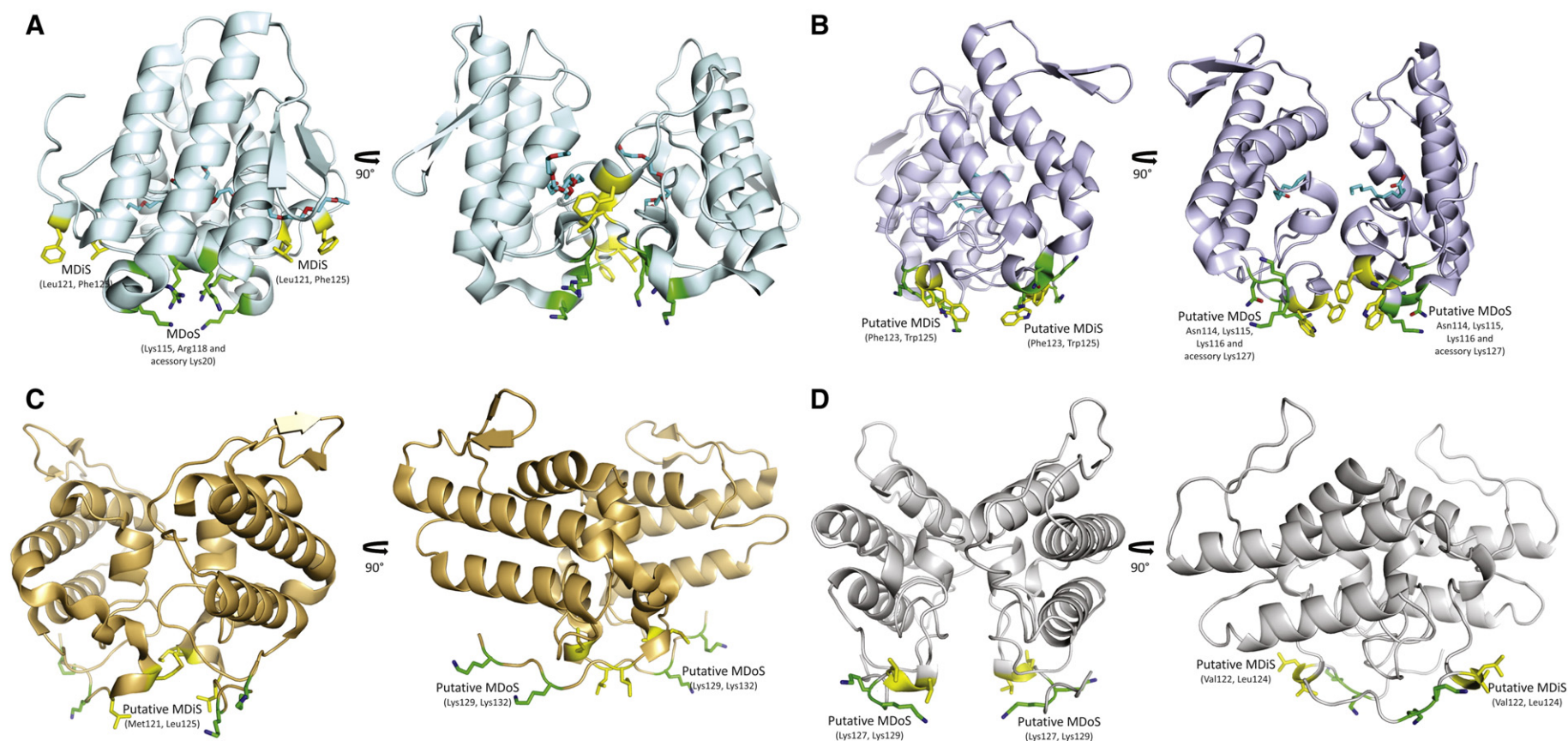


Fig. 1. Localization of MDoS (cationic membrane-docking site) (green sticks) and MDiS (hydrophobic membrane-disruption site) (yellow sticks) on crystallographic structures of PLA₂-like proteins from viperid snake venoms. (A) Bothropstoxin-I (BthTX-I), a Lys49-PLA₂ from *Bothrops jararacussu* venom, complexed with polyethylene glycol 4000 (PDB ID: 3IQ3); (B) Ecarpholin S, a Ser49-PLA₂ from *Echis carinatus* venom, complexed with lauric acid (PDB ID: 2QHD); (C) Zhaoermiatoxin, an Arg49-PLA₂ from *Zhafermia mangshanensis* venom (PDB ID: 2PH4); (D) Bothropstoxin-II (BthTX-II), a Asp49-PLA₂ basic and myotoxic from *Bothrops jararacussu* venom (PDB ID: 3JR8). PEG and lauric acid molecules are represented as sticks on hydrophobic channel of BthTX-I and Ecarpholin S, respectively. Figure drawn using PyMOL program [106].

Table 2Cationic membrane-docking site (MDoS) and hydrophobic membrane disruption site (MDiS) of the crystallographic structures of PLA₂-like proteins.

Class/protein	Species	MDoS	MDiS
<i>Lys49-PLA₂</i>			
PrTX-I	<i>Bothrops pirajai</i>	K20, K115 and R118	L121 and F125
PrTX-II		K20, K115 and R118	L121 and F125
BthTX-I	<i>Bothrops jararacussu</i>	K20, K115 and R118	L121 and F125
Myoll	<i>Bothrops asper</i>	K19, K115 and R118	L121 and L124
BnSP-7	<i>Bothrops neuwiedi pauloensis</i>	K20, K115 and R118	L121 and F125
BnSP-6		K20, K115 and R118	L121 and F125
BnIV		K20, K115 and R118	L121 and F125
BbTX-II	<i>Bothrops brazili</i>	K20, K115 and R118	L121 and L125
MTX-II		K115 and R118	L121 and F125
MjTX-II	<i>Bothrops moojeni</i>	K20, K115 and R118	L121 and F125
GodMT-II	<i>Cerrophidion godmani</i>	K115 and K118	I119 and L125
Myoll	<i>Atropoides nummifer</i>	K115 and K118	I119 and L125
ACL myotoxin	<i>Agkistrodon contortrix laticinctus</i>	K115 and K118	F121 and F124
AppK49	<i>Agkistrodon piscivorus piscivorus</i>	K115 and K118	F121 and L124
Acutohaemonlysin	<i>Deinagkistrodon acutus</i>	K20, K115 and R118	L121 and S125
<i>Basic Asp49-PLA₂</i>			
PrTX-III	<i>Bothrops pirajai</i>	Putative K127 and K128	L121 and L124
BthTX-II	<i>Bothrops jararacussu</i>	Putative K127 and K128	V124 and L125
<i>Arg49-PLA₂</i>			
Zhaoermiatoxin	<i>Zhaoermia mangshanensis</i>	Putative K129 and K132	M121 and L125
<i>Ser49-PLA₂</i>			
Ecarpholin	<i>Echis carinatus</i>	Putative N114, K115 and K116. Accessory K127	M121 and L124

PLA₂s in PLA₂-like proteins because these enzymes are their ancestral forms [87].

The last two steps are caused by the insertion of the MDiS region from both monomers into the target membrane. This penetration disrupts the lipid bilayer, causing a loss of membrane permeability, a prominent influx of ions (i.e., Ca²⁺ and Na⁺), and eventually, irreversible intracellular alterations and cell death [37,44]. Moreover, the effects of these toxins can be rapidly amplified in muscle cells because damaged cells release ATP molecules into the extracellular environment that then bind to muscle P2X purinergic receptors and induce Ca²⁺ and Na⁺ influx and K⁺ efflux in cells that have not been directly injured by the proteins [48].

Regarding other PLA₂-like proteins from viperid snake venoms, such as Ser49 and Arg49-PLA₂s, a similar myotoxic mechanism is possible. The crystal structure of Ecarpholin S also presents a hydrophobic molecule (lauric acid) in the hydrophobic channel [62]. Furthermore, in this structure it is possible to identify putative MDoS and MDiS regions, as previously noted [72]. Phe123 and Trp125 may form a putative MDiS, and the basic cluster formed by Asn114, Lys115 and Lys116 (and possibly Lys127) residues may form a putative MDoS (Fig. 1; Table 2). Finally, the buried surface areas of Phe123 and Trp125 are very small, showing high exposure to the solvent, similarly as the MDiS region in Lys49-PLA₂s. Thus, the three steps of the proposed myotoxic mechanism in bothropic Lys49-PLA₂s may also be conserved in Ser49-PLA₂s.

Murakami et al. [99] solved the crystal structure of Arg49-PLA₂ Zhaoermiatoxin, which has PEG molecules in its hydrophobic channels. They suggested that Arg34 and Arg49 residues form the anion-binding site of this protein because these residues interact with sulfate ions in the structure. However, it is difficult to identify this region as a putative MDoS because these two arginine residues are not completely exposed to solvent. Moreover, these residues are involved in the dimer interface in one monomer. A more accurate analysis of the Zhaoermiatoxin crystal structure shows that two hydrophobic (Met121 and Leu125) and two charged residues (Lys129 and Lys132) are exposed to solvent on the same plane of the C-terminal region and may constitute putative MDiS and MDoS regions, respectively (Fig. 1; Table 2). Despite this proposition, it is important to note that the myotoxic mechanism of action of Arg49-PLA₂ remains quite speculative because the dimer of Zhaoermiatoxin crystal structure has a low CCS value according to

PISA analysis, and its interface is different from other PLA₂-like dimeric structures.

Finally, in basic myotoxic Asp49-PLA₂s it is possible to identify putative MDoS and MDiS regions by the inspection of hydrophobic and charged residues exposed to solvent in the dimeric conformation suggested by dos Santos et al. [87]. In BthTX-II, Lys127, Lys129 and Lys132 would form a putative MDoS, and Val122 and Leu124 would form a putative MDiS (Fig. 1; Table 2). In PrTX-III, these sites would be formed by Lys127 and Lys128 for MDoS, and Leu121 and Leu124 for MDiS.

7. Complexes of PLA₂-like myotoxins with ligands lead to hypotheses for their activation and inhibition

In several PLA₂-like crystal structures ligands bound to the protein were found; however, the majority of these ligands were present in the crystallization solutions or in the protein sample as impurities. Continuous electron density maps have been found in the hydrophobic channel for different Lys49-PLA₂ structures and have been attributed to fatty acids or polyethylene glycol molecules [56,58,60,72,74,100]. Some authors took advantage of the presence of these ligands and hypothesized functional mechanisms based on their binding regions (refer to Sections 3 and 6) [56,58,60,72,74,100]. Table 3 summarizes the ligands found in PLA₂-like crystal structures and their roles in myotoxic activity.

Salvador and colleagues [60] solved MjTX-II/PEG 4000 in the “alternative dimer” conformation, and its comparison to other Lys49-PLA₂s revealed that this protein presents particular features due to an exclusive insertion of Asn120 residue and Leu32Gly and His121Tyr substitutions. They suggested that these differences compared to other bothropic Lys49-PLA₂ complexed to PEG [58,70] lead to a distinct mechanism of ligand binding at the toxin's hydrophobic channel and also allow the presence of an additional ligand in this region. Consequently, these data suggested that MjTX-II may require different ligands for its complete inhibition, despite functional studies indicating that MjTX-II has similar neuromuscular blockage activities as the majority of other Lys49-PLA₂s [60].

Aiming to understand the myotoxic mechanism of the PLA₂-like proteins and to search for specific inhibitors, structural studies of

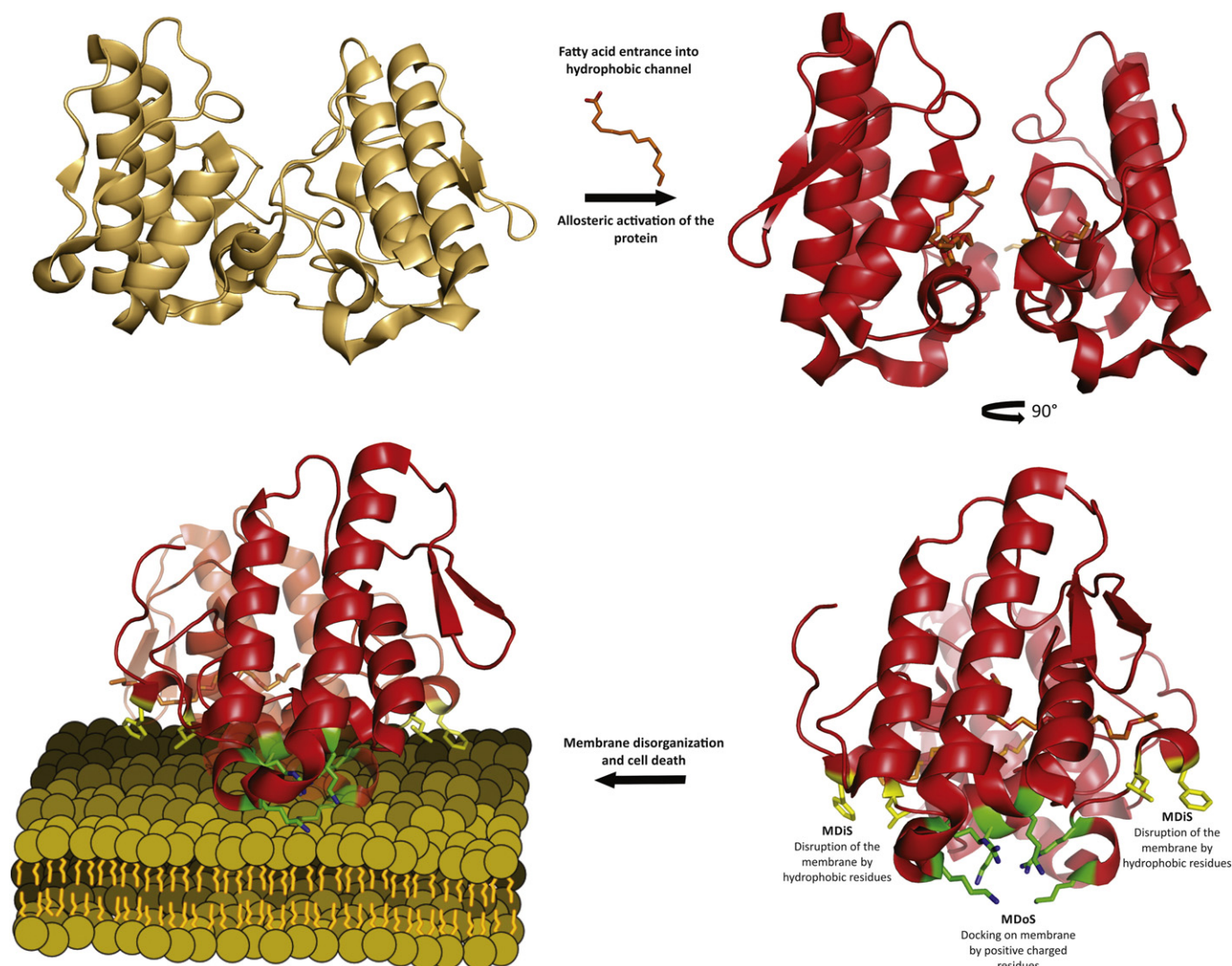


Fig. 2. Myotoxic mechanism of Lys49-PLA₂s. The entrance of a fatty acid at the hydrophobic channel of the protein leads to an allosteric activation by the dimer reorientation. In the active form, the cationic membrane-docking site (MDoS) and the hydrophobic membrane-disruption site (MDiS) became aligned on the same plane, exposed to solvent and in symmetric position on both monomers. The MDoS region stabilizes the protein on membrane by interaction of charged residues with phospholipid head group. Subsequently, the MDiS region by penetration of hydrophobic residues destabilizes the membrane causing cell death. Other PLA₂-like proteins may have similar myotoxic mechanism due to the presence of fatty acids in their hydrophobic channels and putative MDoS and MDiS on their crystallographic structures.

complexes between these toxins and ligands have been performed by co-crystallization assays and by the crystallization of chemically modified proteins. PrTX-I from *B. pirajai* and BthTX-I from *B. jararacussu* were co-crystallized with α -tocopherol [70]. Both PrTX-I/ α -tocopherol and BthTX-I/ α -tocopherol have very similar structures with ligands bound in the hydrophobic channel. Interestingly, α -tocopherol molecules do not present the same interaction with the residues of the monomers, and the quality of the electron density maps indicates that the ligand occupancy in one monomer is higher compared to the other. This non-symmetric binding behavior of the ligands may be due to the asymmetric structures of the monomers prior to ligand binding, as observed by dos Santos et al. [70].

A comparative structural study between native BthTX-I (apo) and BthTX-I chemically modified by *p*-bromophenacyl bromide (BPB) was published [58]. Interestingly, the authors crystallized each version of the toxin (apo and chemically modified BthTX-I) in two different physicochemical conditions, leading to different crystal packing (three different space groups) with the presence of one or two monomers in the asymmetric units. The analysis of their unit cells revealed that all structures most likely adopt the “alternative dimer” quaternary assembly. BPB molecules were found covalently bound to His48 in BthTX-I/

BPB complexes, similarly to catalytic PLA₂s structures [49,101,102]. In the case of catalytically active PLA₂s, the presence of the BPB molecules in their catalytic site abolishes their enzymatic activity. Surprisingly, the binding of BPB ligands to a “catalytic-related” residue (His48) causes the partial inhibition of myotoxic activity for Lys49-PLA₂s, demonstrating that the steric hindrance of this “pseudo-catalytic site” is fundamental to the inhibition of the myotoxic activity of these toxins. An obvious explanation for this phenomenon is that the presence of the BPB molecule prevents the binding of hydrophobic molecules (e.g., fatty acids) at the hydrophobic channel, thus precluding the toxin from acquiring its “active state”—the first step of the proposed myotoxic mechanism (Section 6). In contrast to this possible explanation, the analysis of BthTX-I/BPB structure revealed an oligomeric conformation similar to other complexed Lys49-PLA₂s, i.e., its conformation is in the so-called “active state”. Thus, two hypotheses can explain the BPB inhibition process: i) BPB prevents the binding of fatty acids to the toxin, avoiding conventional protein–membrane docking. Due to the presence of the MDoS of the toxin, it could still bind weakly to the membrane, explaining why BPB-complexed toxins have partially inhibited activity; and ii) despite the observation that BPB-complexed proteins have an oligomeric conformation similar to “active-state toxins”, the lack of

Table 3Ligands modeled in crystallographic structures of PLA₂-like proteins involved in activation or inhibition of their myotoxic activity.

Ligands	Structures found	PDB IDs	Main localization site	Function
Sulfate ions	BthTX-I; BnIV; MjTX-II; MTX-II; Myotoxin from <i>A. contortix laticinctus</i> ; Myoll from <i>A. nummifer</i> ; PrTX-I; Zhaoermiatoxin	3IQ3; 3CXI; 3MLM; 1XXS; 4K06; 1S8G; 1S8H; 1S8I; 2A0Z; 3CYL; 2PH4	Positively charged residues exposed to solvent	Indication of regions that PLA ₂ could interact with membranes [95]
Polyethylene glycol (PEG)	BthTX-I; MjTX-II; MTX-II; Zhaoermiatoxin	3IQ3; 4KF3; 4K06; 2PH4	Hydrophobic channel	Allosteric activator [58,72]
Fatty acids ^a	BnIV; Ecarpholin S; MjTX-II; myotoxin from <i>A. contortix laticinctus</i>	3MLM; 2QHD; 1XXS; 1S8G checar	Hydrophobic channel	Allosteric activator [57,62,74]
Alpha-tocopherol	BthTX-I and PrTX-I	3CXI; 3CYL	Hydrophobic channel	Putative allosteric activator [70]
p-Bromophenacyl bromide (BPB)	BthTX-I and PrTX-I	3HZW; 3IQ3; 2OK9	Covalent bond with His48	50% inhibition of creatine kinase release in vivo and 50% inhibition liposome disruption activity [58,105]
Suramin	Ecarpholin S and Myoll from <i>B. asper</i>	3BJW; 1Y4L	Interactions with N and C-terminal from both monomers; all hydrophobic channel (Ecarpholin S), entrance of hydrophobic channel (Myoll)	85% inhibition of creatine kinase release on in vitro and in vivo assays [62,69]
Rosmarinic acid	PrTX-I	3QNL	Entrance of hydrophobic channel	90% reduction of the blockade of indirectly evoked contractions induced by the protein [103]
Caffeic acid	PrTX-I	Preliminary structure ^b	Lys20, Lys115 and Arg118 (MDoS)	Putative inhibitor

^a Despite of crystal structure of PrTX-II was modeled with a fatty acid (N-tridecanoic acid) on hydrophobic channel, this structure it was not included in this list due to evidences that this fatty acid could be a PEG molecule, as pointed in Section 3 and by Fernandes et al. [58].

^b See Shimabuku et al. [104].

interactions between hydrophobic molecules and residues from the hydrophobic channel could create a more unstable conformation that is less likely to act efficiently in the membrane. Both events may occur simultaneously, but these hypotheses need to be tested using other biochemical, biophysical, or functional assays.

The structure of myotoxin II from *Bothrops asper* co-crystallized with suramin (an anti-trypanosomal drug that neutralizes its myotoxic activity) presented a very interesting conformation in which the ligand interacts simultaneously with the two monomers of the dimeric structure [69]. Two important observations could be obtained with this structure: i) only the “alternative dimer” is possible because if the “conventional dimer” is chosen, half of the ligand would be in contact with the solvent, which would be energetically unfavorable; ii) a new inhibition mode is observed by which the ligand restricts access to the hydrophobic channel. The complexation of the Ecarpholin S with suramin was also able to reduce the myotoxic activity of this toxin drastically [62]. In the crystal structure of this complex, the inhibitor induces toxin oligomerization and blocks the access to the hydrophobic channel [62], as observed in the BaspTX-II-suramin complex [69]. However, the binding of suramin to Ecarpholin S establishes a wide range of interactions along the hydrophobic channels that are not observed in the BaspTX-II-suramin structure.

More recently, the crystal structure of PrTX-I from *B. pirajai* co-crystallized with rosmarinic acid (RA—a plant component that neutralizes its neuromuscular blocking activity and its myotoxicity) was solved [103]. This structure revealed that the ligand interacts with the toxin at the entrance of its hydrophobic channel. The authors hypothesized that the inhibition occurs because RA impairs the binding of hydrophobic molecules at the hydrophobic channel. In addition, this structure presents just one RA molecule bound with one monomer, and a PEG molecule interacts with the other monomer. This observation is in agreement with the asymmetry observed for the monomers of Lys49-PLA₂s [70].

Finally, PrTX-I from *B. pirajai* was co-crystallized with caffeic acid [104]. This study showed electron density maps corresponding to caffeic acid molecules that were found in the C-terminal region of the toxin. This result shows that the ligand is bound to a similar region where sulfate ions were found in other Lys49-PLA₂ structures [70] which is proposed region for the “cationic membrane-docking site (MDoS)” (Section 5) [72].

These structural and functional studies with Lys49-PLA₂s and ligands allow us to classify the latter into at least three “classes” (Table 3): i) ligands bound into the hydrophobic channel (e.g., BPB,

polyethylene glycol, and α-tocopherol); ii) ligands which block or restrict access to the hydrophobic channel (e.g., rosmarinic acid and suramin); and iii) ligands which bind to C-termini or the “membrane-docking site” (e.g., caffeic acid and sulfate or phosphate ions). All of the structural evidence obtained from the study of Lys49 myotoxin/ligand complexes is in agreement with the integrated mechanism of toxicity proposed here.

8. Conclusions and future perspectives

Recently, an intense data expansion regarding the structural features of PLA₂-like proteins has provided significant insights into the mechanisms by which these proteins induce sarcolemma disorganization. All these data strongly support the alternative dimer assembly as the active quaternary structure of these proteins, and they offer a solid basis for a revised myotoxic mechanism that is able to integrate all the available biochemical and structural data. Structural studies with different inhibitors also corroborate this integrated mechanism of action and show different inhibition modes of PLA₂-like proteins. This knowledge should provide a useful molecular basis for the development of novel neutralizing strategies to improve the treatment of viperid snakebites.

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