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Review

Modulation of phospholipase A_2 activity generated by molecular evolution

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This work is dedicated to Prof. Dr. Wolfram Saenger on the occasion of his 60th birthday.

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Abstract. Snake venom oligomeric neurotoxins offer several unique examples of modulation of phospholipase A_2 (PLA_2) activity generated by molecular evolution. This phenomenon was found in evolutionary younger snakes and is probably common for representatives of the genus *Vipera*. At present, the best-studied example is the heterodimeric neurotoxin vipoxin from the venom of the southeast European snake *Vipera ammodytes meridionalis*. It is a complex between a basic strongly toxic PLA_2 and an acidic and catalytically inactive PLA_2-like component (Inh). This is the first reported example of a high degree of structural homology (62%) between an enzyme and its natural protein inhibitor. The inhibitor is a product of the divergent evolution of the unstable PLA_2 in order to stabilize it and to preserve the pharmacological activity/toxicity for a long time. Inh reduces both the catalytic activity and toxicity of PLA_2. Vipoxin also illustrates evolution of the catalytic into a inhibitory function. Vipoxin analogues have been found in the venom of viperid snakes inhabiting diverse regions of the world. An attempt is made to explain modulation of the toxic function by the three-dimensional structure of vipoxin.

Key words. Phospholipase A_2; molecular evolution; inhibitor; pharmacological sites; enzyme activity; enzyme toxicity.

Introduction

Phospholipases A_2 (PLA_2s) (phosphatide 2-acylhydrolase, EC 3.1.14) are widespread in living organisms as both intracellular and extracellular enzymes. They are among the smallest enzymes performing various vital physiological functions and have been isolated from a number of sources: snake venom, mammalian pancreas, lung, gastric mucosa, liver, spleen, alveolar macrophages, intestine, membranes, heart, placenta, and brain [1]. The PLA_2 enzyme specifically hydrolyzes the 2-acyl ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides releasing fatty acids and lysophospholipids. The enzyme catalyzes reactions at a lipid-aqueous interface and the phospholipase activity is much higher on aggregated substrates such as monolayers, bilayers, micelles, membranes, and vesicles than with monomolecular dispersed soluble substrates [2]. This phenomenon has been termed 'interfacial activation' and includes 'interfacial binding' of the enzyme and 'activation' steps.

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Structural changes were observed in PLA_2 upon its binding to membranes and were explained as a prerequisite for enzyme activation [3]. Thus it is evident that modulation of membrane-associated PLA_2 activity will influence cellular functions such as chemotaxis, cytotoxicity, and cell differentiation, and it has been established that the interfacial adsorption of PLA_2 on membranes is driven by electrostatic forces. Electrostatic interactions between positive charges from the PLA_2 recognition site and negatively charged anionic headgroups of phospholipids optimize the catalysis and are important for the adsorption and orientation of the enzyme at the lipid-water interface. Thus, two lysyl residues, Lys 7 and Lys 10, mediate the adsorption of the *Agkistrodon piscivorus piscivorus* PLA_2 to anionic interfaces [4]. Enzyme penetrability into the phospholipid membrane can be improved by positively charged residues flanking hydrophobic segments of the recognition site, which will facilitate hydrolysis of phospholipids [5]. In addition, hydrophobic side chains, such as tryptophyl residues, may penetrate into membranes enhancing PLA_2 binding [6]. Therefore, the hydrophobicity of the enzyme recognition site is important: increased hydrophobicity will improve enzyme binding to the respective target sites on the membrane substrate.

The most important active sites, such as the catalytic site, the Ca^{2+}-binding site, and the hydrophobic sub-

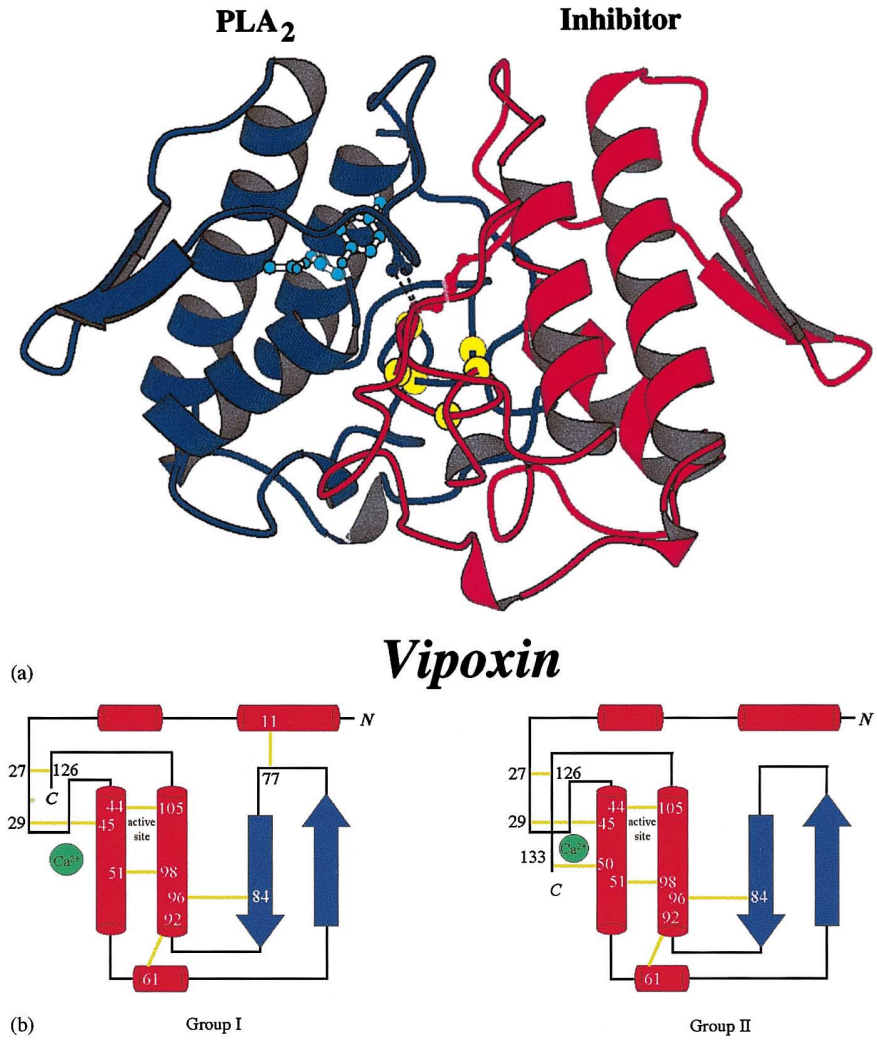


Figure 1. (*a*) Cartoon view of heterodimeric vipoxin complex as represenative of the group II PLA_2s, showing the active PLA_2 in blue and the inhibitor in red. The disulfide bridges for both molecules and the active-site residue side chains are shown for the PLA_2. The intermolecular interaction (PLA_2) Asp 49-Lys 69 (Inh) reducing vipoxin calcium-binding ability is included. The Cα positions of residues of the potential calcium-binding residues inside each calcium-binding loop are indicated as yellow circles. (*b*) Schematic representation of group I and group II PLA_2s. α-Helices are indicated as red cylinders, β-strands by blue arrows, disulfide bridges by yellow bars and the Ca^{2+}-binding site by a green circle.

strate-binding site [7–9] are conserved in the PLA_2 molecule. The structure of vipoxin, as representative for a dimeric PLA_2 complex is shown in figure 1a. The active-site configuration is similar to that of serine proteinases but the attacking nucleophile is a water molecule instead of a serine hydroxyl group [10]. The water molecule is activated through the abstraction of a proton by His 48 and attacks the scissile bond [7]. Ca^{2+} is essential for PLA_2 catalysis and facilitates substrate binding to group II enzymes. However, substrate binding to group I PLA_2s is independent of calcium binding [11]. The snake venom enzymes, although they share structural and catalytic similarities with the crude PLA_2 enzymes cause, in addition, enormous destruction by interfering in the normal physiological processes of the victim, including a variety of pharmacological effects. So far, some progress has been made in understanding the complex structure-function relationship of this protein family, because the pharmaceutical effects, in principle, are independent of the enzymatic activity which, however, can potentiate and induce these effects.

Classification of PLA_2s

PLA_2s form a superfamily of specific hydrolases which includes two major classes of enzymes: secretory/extracelular and intracellular. The classification of these biocatalysts was based mainly on sequence homology and the position of disulfide bridges. Recently, a large number of new enzymes hydrolyzing of glycerophospholipids at the *sn*-2 position have been characterized and the traditional classification into three main groups and several subgroups [12, 13] was extended. Thus Dennis [14] extended the group numbering system and classified secretory and intracellular PLA_2s into nine groups [14]. A new 13.6-kDa acidic PLA_2, structurally different from the other members of the superfamily, has been isolated from human fetal lung [15]. It was proposed as a first member of a new group X. Some groups and subgroups of PLA_2s include: (a) PLA_2 enzymes from Elapidae and Hidrophiidae snake venoms and mammalian pancreas; (b) neurotoxins from Viperinae and Crotalinae snake venoms and mammalian platelets; (c) PLA_2s from bee venom and related glycoproteins from *Heloderma suspectum* and *H. horridum horridum* venoms [16, 17], and (d) PLA_2 enzyme from the *Conus magus* venom [5].

On the basis of disulfide bridge positions, PLA_2s were further subclassified into two groups, as indicated in figure 1b: group I contains enzymes from mammalian pancreatic juices and venom of Elapidae and Hydrophiidae snakes; PLA_2s from the venom of Viperinae and Crotalinae snakes and of mammalian non-pancreatic origin belong to group II [12]. The members of the first group possess a S-S bridge between the side chains of Cys 11 and Cys 80. The enzymes from the second group have a C-terminal extension of five to seven residues including a C-terminal Cys residue linked to the SH group of residue 50. The other six disulfide bridges are conserved in the structure of the proteins from both groups. Group I has been additionally subdivided into five subgroups. Two main subgroups include venom PLA_2s from Elapidae and Hydrophiidae snakes (subgroup IA), and mammalian pancreatic PLA_2s, possessing the so-called 'pancreatic loop,' form subgroup IB. Proteins, enzymatically active or inactive, from group II were subdivided into six subgroups. Again, two main groups have been formed: group IIA includes proteins with seven S-S bridges and group IIB contains proteins with eight disulfide linkages [1] [for more details on PLA_2 classification see refs 1, 5, 14, 18].

The secondary and three-dimensional structures of the group I/II PLA_2s are illustrated in figure 1a, b. The N-terminal α-helix is followed by a short α-helix of only one-and-a-half turns. This is followed by an almost random coil region up to residue 38. This region incorporates the Ca^{2+}-binding loop, residues 26–34. The following α-helix is extended for the pancreatic enzymes by the region called the elaptic loop also incorporating a short α-helix turn motif. Residues 74–85 form the antiparallel β-wing structure, which is again followed by a loop connecting this part of the structure with the final α-helix. This is then followed by a extended stretch of random coil ending at the C terminus. This coil is reduced by six to seven amino acids for group I PLA_2s, and for group II enzymes, the C terminus is additionally tightly attached to the main part of the protein by a disulfide bridge.

Pharmacological activities of PLA_2

Although PLA_2s are small proteins comprising about 120 amino acid residues they display a wide variety of pharmacological activities such as presynaptic/postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, antiplatelet, convulsant, hypotensive, hemolytic, hemorrhagic, and edema-inducing effects [19]. Snake venom PLA_2 toxins act on the presynaptic and/or postsynaptic sites of the neuromuscular junctions. The presynaptic neurotoxins cause rapid death due to blockade of transmission across the neuromuscular junctions of the breathing muscles [20]. The modulation of neurotransmitter release on nerve-muscle preparations is triphasic: initial depression, facilitation, and final blockade of neurotransmission [21]. Postsynaptic toxins act on the postsynaptic site of the neuromuscular junctions preventing the binding of acetylcholine to its receptor [22].

Although the phospholipase activities of the enzymes are well understood, the toxic function has not been

satisfactorily explained in structural terms. Because the three-dimensional structures of PLA_2s are very similar, the reason for the different toxic effects must be hidden in discriminating differences between their structures. A comprehensive model to explain the extensive variety of toxic effects has been proposed [23], introducing theories and models for toxic sites, where the catalytic machinery is located, which should be situated at the surface of the molecule. This region has to be complementary in charge distribution, hydrophobicity, and conformation to a target receptor site, which can be a specific protein rather than a lipid domain, on the surface of the target cells.

The different types of toxicity are linked to the existence of high-affinity receptors for PLA_2s. Two types of receptor have been identified: N (neuronal) and M (muscle) type [24]. The N receptors are located on 36- to 51-kDa and 85- to 88-kDa proteins and were initially found in rat brain membranes. They recognize toxic secretory PLA_2s from snake and bee venoms. These sites have a very low affinity for non-toxic-venom PLA_2 enzymes [25]. The M receptor recognizes with a high specificity porcine pancreatic and human inflammatory secretory PLA_2s as well as non-toxic-venom PLA_2s [26]. This receptor does not bind toxic-venom PLA_2s and was initially characterized as a 180-kDa protein in rabbit skeletal muscle [27]. The so-called 'pancreatic loop' at residues 62–66 is not essential for PLA_2 affinity for the M-type receptor. Gly 30, Leu 31, and Asp 49 are essential for the binding activity of pancreatic phospholipase A_2. Ca^{2+} is not important for the association and this process is even more efficient in the absence of this ion [24].

PLA_2 participates in cellular processes including inflammation. A human secretory phospholipase has been found at high concentrations in the synovial fluid of patients with rheumatoid arthritis and in the plasma of patients with septic shock [28 and references therein]. The enzyme releases arachidonic acid from the *sn*-2 position of the plasma membrane phospholipids. In this way, PLA_2 is involved in inflammatory processes and diseases, such as rheumatoid arthritis and asthma, because arachidonate is a precursor of eicosanoid mediators of inflammation including leukotrienes, prostaglandins and thromboxanes [29]. Thus, PLA_2s are regulators of eicosanoid synthesis. Free fatty acids, eicosanoids and lysophospholipids released by this hydrolase are regulators of inflammation, reproduction, and neurotoxicity [30–33]. Cytosolic PLA_2 has been shown to be important for macrophage production of inflammatory mediators, fertility, and in the pathophysiology of neuronal death after local cerebral ischemia [34]. Inhibition of PLA_2 is of medicinal interest because this will reduce inflammation, and can be used to design therapeutic agents. This possibility stimulated the synthesis of specific inhibitors [7]. Structural information about the binding of inhibitors to the PLA_2 active site was used for a structure-based design of a potent and selective inhibitor of human non-pancreatic secretory PLA_2 [35].

PLA_2s can affect platelet aggregation which maintains the integrity of the blood vessel walls [36] and influences the activation of coagulant factors and clot retraction. Diversions in these processes result in clot formation which can create cardiac infarction and stroke due to a restricted supply of blood to the heart and brain. Investigations into the mechanism of the phospholipase inhibition can be used to design antiplatelet agents, useful tools for preventing/treating atherosclerosis [37].

In general, basic PLA_2s show cardiotoxicity. Recently, an acidic phospholipase A_2, inducing such an effect after intravenous injection to rat or incubation with rat heart preparations, was isolated from the venom of *Ophiophagus hannah* (king cobra) [30]. This enzyme also inhibits platelet aggregation and shows myotoxicity. It is the first elapid venom PLA_2 containing a 'pancreatic loop,' typical for the pancreatic secretory PLA_2s. This loop is absent in the other snake venom enzymes isolated so far. The literature contains contradictory opinions about the significance of the enzymatic activity for the pharmacological action of PLA_2. The specific pharmacological effects of phospholipase enzymes are most likely due to high-affinity interactions between the PLA_2 protein and a membrane protein in the tissue. Phospholipid hydrolysis at the target site is important for the induction of pharmacological effects [5]. The PLA_2 enzymatic activity of β-neurotoxins is not directly responsible for the neurotoxicity but is implicated in the toxin action on neuromuscular transmission. There is evidence that the neurotoxic site is usually separated from the catalytic site [38, 39]. According to Rosenberg [40], PLA_2 activity alone cannot explain the lethal potency. He found no relationship between the enzymatic activity of PLA_2 enzymes and their LD_{50} values and the catalytic activity does not appear to be essential for the lethal action or for the other pharmacological effects.

The three isotoxins isolated from the venom of *Vipera ammodytes ammodytes* (ammodytoxins) offer good examples for studying the structural basis of toxicity. Ammodytoxins are highly homologous proteins and differ in their amino acid sequences in only two to three residues. These changes result in 30-fold differences in lethal action and allow elucidation of the PLA_2 toxic site. The difference in toxicity of ammodytoxins A and C was explained by the substitution of Lys 128 for Glu 128, creating a substantial change in the charge distri-

bution. This substitution probably leads to differences in the electrostatic interactions between ammodytoxin and the receptor environment [41].

Oligomeric snake venom neurotoxins

Oligomeric snake venom neurotoxins represent protein complexes in which at least one subunit possesses PLA_2 activity. The non-toxic components can have inhibitory and/or chaperonic action preventing non-specific adsorption to membranes. PLA_2 is usually a basic protein while the second subunit is acidic. The polypeptide chains are associated in the complex through non-covalent interactions. However, there are exceptions to these rules. Thus, the two subunits in bungarotoxins are bound covalently by a disulfide bridge. In the complex, a 7.0-kDa homologue of a Kunitz-type trypsin inhibitor is associated with a toxic PLA_2. The non-toxic chain has no inhibitory effect on trypsin, chymotrypsin, or elastase [42]. The Kunitz-type subunit of the complex shows considerable structural differences when compared to the bovine trypsin inhibitor. This explains the lack of inhibitory activity. The crystallographic structure of the β_2-bungarotoxin [43] revealed that the substrate-binding surface of PLA_2 is partially hidden, in agreement with its weak enzymatic activity. The two-component toxin from the venom of *V. palaestinae* comprises of an acidic PLA_2 and a basic protein with a molecular mass of 15 kDa. When separated, the components are non-toxic and only their mixture has a lethal effect [44]. This is an example of synergistic toxicity.

Several neurotoxins such as crotoxin (*Crotalus durissus terrificus*), Mojave toxin (*Crotalus s. scutulatus*), concolor toxin (*Crotalus viridis concolor*), vegrandis toxin (*Crotalus vegrandis*), and canebrake toxin (*Crotalus horridus atricaudatus*) are complexes between a PLA_2 enzyme and a polypeptide which is a proteolysed part of PLA_2. Crotoxin is a heterodimeric presynaptic neurotoxin isolated from the venom of the South American rattlesnake *C. d. terrificus*. The complex is composed of a basic and weakly toxic PLA_2 and an acidic non-enzymatic and non-toxic subunit. The second subunit is made of three disulfide-linked polypeptide chains. More than 15 isoforms of crotoxin, derived from a precursor by posttranslational events, have been found. The isoforms differ by only a few amino acid residues [45]. During the interactions with synaptic membranes, the complex dissociates to subunits: the enzyme binds to a membrane, while the acidic subunit is released in solution. The non-toxic component acts as a chaperon preventing non-specific adsorption of PLA_2 to inefficient binding sites [46]. It has been proposed that the 'chaperonic' subunit may participate in the formation of a transient ternary complex with PLA_2 and the acceptor targeting the enzyme onto the membrane acceptor and increasing its pharmacological activity [47]. The covalent linkage of the subunits by a bifunctional reagent does not reduce the enzymatic activity but completely abolishes the lethal potency of crotoxin [48]. Immunological studies with monoclonal antibodies allowed characterization of epitopic regions on both subunits with respect to 'toxic' and 'catalytic' sites in PLA_2 [49].

Taipoxin from the venom of Australian Taipan snake (*Oxyuranus scutellatus scutellatus*) is a trimeric neurotoxin. It consists of three non-covalently bound distinct PLA_2 subunits: α, β, and γ. The α-subunit is a basic toxic protein with PLA_2 activity. The second 'neutral' component is devoid of enzymatic activity and the γ-subunit is an acidic protein similar to porcine pancreatic PLA_2 with weak enzymatic activity [50–52]. Homodimeric snake venom neurotoxins are also known. The neurotoxin from the venom of *Trimeresurus flavoviridis* is composed of two identical PLA_2 subunits. Each subunit contains 122 amino acid residues [53]. A homotrimeric crystallographic structure of PLA_2 from *Naja naja naja* was recently published [54]. This is impressive because a monomeric X-ray structure of the closely related enzyme from *N. n. atra* was reported 8 years ago [29]. The amino acid sequences of the two PLA_2s differ by only a few residues. The observed oligomeric structure is probably a result of the conditions used for crystallization rather than demonstrating the natural occurrence of a homotrimeric complex.

Vipoxin: a unique example of modulation of the toxic function generated by molecular evolution

General characterization of vipoxin

Three subspecies of the genus *Vipera* inhabit the central part of the Balkan peninsula (southeast Europe): *V. a. ammodytes*, *V. a. montandoni*, and *V. a. meridionalis*. These vipers are the most lethal snakes in Europe. The unique heterodimeric neurotoxin vipoxin has only been found in the venom of *V. a. meridionalis* [55]. The venom of the other two snakes, which are evolutionary older, contains monomeric PLA_2s.

Vipoxin is an ion-type complex between a basic strongly toxic PLA_2 with a pI of 10.4 and an acidic non-toxic and catalytically inactive component (Inh) with a pI of 4.6 [56]. Vipoxin PLA_2 is a group IIA enzyme which loses its toxicity and enzymatic activity a few days after fractionation of the complex. The original toxicity and stability of the neurotoxin are fully recovered by mixing the freshly fractionated components. The enzyme exhibits a maximal catalytic activity at pH 10.0 with phosphatidylcholine as a substrate. Calcium is necessary for the activity and the optimal concentration is 3×10^{-3} M. Toxicity of the vipoxin PLA_2 increases

fivefold after separation from the inhibitor. The vipoxin Inh lacks enzymatic activity and reduces phospholipase activity in vitro by up to 60% [57]. It is a natural inhibitor of the vipoxin PLA_2.

The two components of vipoxin, PLA_2 and Inh, are closely related proteins with 62% sequence identity [58]. This is the first reported example of a high degree of structural homology between an enzyme and its natural protein inhibitor. The inhibitor lacks catalytic activity due to the substitution of Gln 48 in place of the active-site His 48. It appears that the inhibitor is a product of divergent evolution of the unstable PLA_2 in order to stabilize it and in this way preserve the pharmacological activity (toxicity) for a long period. In contrast to the separated components, the complex is stable and toxic for years. The inhibitor reduces both the catalytic activity and toxicity considerably and in this respect vipoxin is an example of regulation of a toxic function generated by molecular evolution. This is also confirmed by the fact that the snake whose venom contains vipoxin is evolutionarily the youngest among the three types of vipers inhabiting the Balkan peninsula. Vipoxin also demonstrates the evolution of a catalytic function into an inhibitory one. The inhibitor is a unique example of a snake venom PLA_2 acquiring an inhibitory function during evolution. The modulation of PLA_2 activity, realized in the vipoxin complex, is of pharmacological interest and can be used for structure-based drug design [59].

Vipoxin is different from the other toxic complexes probably as a result of different evolutionary events. In contrast to Inh, the non-toxic components of the multichain neurotoxins potentiate the toxicity. In all other known oligomeric neurotoxins, the toxic subunit has a lower lethality than the complex [60]. The heterodimeric toxins usually have a presynaptic action, while vipoxin is a postsynaptic neurotoxin. However, vipoxin PLA_2 separated from the complex exhibits presynaptic activity. X-ray studies have demonstrated that the complex formation in vipoxin is significantly different to that seen in the known structures of snake venom neurotoxins [59]. The conformation is mainly supported by the ionic interaction of the Inh Lys 69 toward Asp 49 of the PLA_2, distorting the Ca^{2+}-binding region and, furthermore, the carbonyl oxygens of the potential calcium-binding ligands Tyr 28 and Gly 32 are turned away to be involved in intermolecular H bonds.

The crystallographic structure of Inh has been published [61], and it shows almost the same homodimeric conformation as found for other snake PLA_2 homodimers, demonstrating that vipoxin is a unique complex.

Stabilization role of the inhibitor

The most obvious function of the inhibitor is to stabilize the vipoxin PLA_2 and to preserve its activity for a long period. We have determined the free energy of stabilization in water, $\Delta G_D^H 2^O$, for the unfolding reaction of the complex and the two components [62]. $\Delta G_D^H 2^O$ is a measure of the protein conformational stability in water solutions. Values of 13.4, 17.2, and 23.5 kJ mol^{-1} were calculated for PLA_2, Inh, and vipoxin, respectively. The enzyme is less stable than the inhibitor, and complex formation increases the free energy of stabilization. These observations also demonstrate that vipoxin is more stable than the separated subunits. However, the functional relevance of the vipoxin inhibitor is not completely clear. It could also be a 'chaperonic' subunit for selective binding of the toxic PLA_2 to membranes.

Vipoxin analogues in the venom of other viperid snakes

Surprisingly, two structural analogues of vipoxin have been found in the venoms of viperid snakes inhabiting widely separated regions of the world. A heterodimeric neurotoxin was isolated from the venom of the Taiwan viper (Asia) *V. russelli formosensis*. It consists of two PLA_2s, designated RV4 and RV7, which show a high 92% sequence identity to the vipoxin PLA_2 and Inh, respectively. RV4 is enzymatically active and neurotoxic while RV7 possesses low catalytic activity and is non-toxic. The components of the Taiwan viper toxin are closely related proteins with 65% homology [9]. Comparison with vipoxin (table 1) revealed differences which are impressive given the high sequence homology between the respective subunits of the two neurotoxins. Despite these similarities, there are remarkable differences in the biochemical and pharmacological properties of the RV4/RV7 complex and vipoxin. For example, vipoxin is a postsynaptic neurotoxin while the RV4/RV7 complex acts on presynaptic sites of the neuromuscular junction. Inh decreases the toxicity of the vipoxin complex about fivefold while RV7 potentiates the toxicity of the RV4/RV7 complex. The Inh in vipoxin lacks the active site residue His 48 and has no enzymatic activity, while RV7 has His 48 and possesses low PLA_2 activity. The RV7 subunit inhibits the enzymatic activity of the toxic RV4 and also seems to play a 'chaperonic' role [9]. Moreover, vipoxin is readily soluble at neutral pH while the RV4/RV7 complex is not. Hence, the RV4/RV7 complex appears like a natural variant of the vipoxin complex with altered biochemical and pharmacological properties. The three-dimensional structure of the RV4/RV7 complex will provide a structural basis for these differences. Preliminary results from crystallization and other structural analyses are about to be published [63]: the structural information

Table 1. Comparison of heterodimeric snake venom neurotoxins.

Heterodimeric neurotoxin	Neurotoxicity	Type of complex	Effect of the non-toxic chain on enzymatic activity	Effect of the non-toxic chain on toxicity	Catalytic activity of the non-toxic chain	Sequential homology between the subunits (%)	Role of the non-toxic chain
Vipoxin from *Vipera a. meridionalis* [57, 58]	postsynaptic	ionic	inhibitor, 2.5-fold decrease of enzymatic activity	5-fold decrease of PLA_2 toxicity	inactive (Gln 48)	62	stabilizes the toxic PLA_2 'chaperon'?
Neurotoxin from *Vipera r. formosensis* [9]	presynaptic	ionic	inhibitor	potentiates PLA_2 toxicity	active (His 48)	65	'chaperon'
Neurotoxin from *Vipera a. zinnikeri* [64]		ionic		potentiates PLA_2 toxicity	inactive (Gln 48)	64	'chaperon'
Crotoxin from *Crotalus du-rissus terrificus* [90]	presynaptic	ionic	inhibitor	potentiates PLA_2 toxicity	inactive		'chaperon'

provided by these studies will further improve our understanding of oligomerization and regulation of PLA_2 activities.

Another vipoxin analogue was obtained from *V. aspis zinnikeri*, inhabiting southwestern France [64]. It is a heterodimer of an acidic and basic subunit with 64% sequence identity. Both subunits consist of 122 amino acid residues like the components of vipoxin. As with Inh, the acidic subunit lacks enzymatic and pharmacological activities. Again, the active site His 48 is substituted with Gln 48. It can be concluded that the two subunits of the *V. a. zinnikeri* neurotoxin are essential for the pharmacological activity.

Table 1 summarizes data about vipoxin and its analogues as well as for crotoxin, one of the best-studied oligomeric neurotoxins. The sequence homology between the subunits of the members of the 'vipoxin family' is practically the same, 62–65%. The non-toxic component is usually an inhibitor of the enzymatic activity, plays a role as 'chaperon' and potentiates the toxicity of the catalytically active subunit. The only exception is the vipoxin inhibitor which decreases the PLA_2 toxicity considerably. The non-toxic acidic subunit can be catalytically active, containing a histidyl residue in position 48, or inactive, with Gln 48 instead of His 48 in the polypeptide chain. It seems very probable that vipoxin is a phenomenon common for viperid snakes. Isolation of new analogues from other representatives of this genus can be expected, especially from the venom of evolutionarily younger vipers.

Evolutionary relationships between vipoxin and other snake venom PLA_2s

The amino acid sequence of vipoxin PLA_2 has been compared with those of related enzymes and PLA_2-like proteins to reveal evolutionary relationships in this important family of hydrolytic enzymes. The vipoxin PLA_2 possesses a C-terminal extension of six residues and a disulfide bridge between the C-terminal half-cystine and the SH group of Cys 50. For this reason, it should be classified as a member of the subgroup IIA. Comparison of the vipoxin PLA_2 sequence with those of approximately 200 related proteins sequenced to date [1] demonstrated a high degree of conservation of structurally and functionally important residues in the phospholipase from the venom of *V. a. meridionalis*. Alignment of the sequences revealed 41 highly conserved residues (fig. 2), 25 of them, i.e., more than 50%, being absolutely or almost absolutely conserved (fig. 3). These residues include the active site His 48, Tyr 52, Tyr 74, and Asp 99. His 48 is substituted by Asn 48 in the protein from *Laticauda colubrina* [65] and by Gln 48 in both the highly homologous protein inhibitor of the vipoxin PLA_2 [51] and the protein from *Xenopus laevis* [66]. The three PLA_2-like proteins are non-toxic.

Seven tyrosyl residues are highly conserved in all the PLA_2 sequences and are probably functionally important. Tyrosines are important for the lethal toxicity of notexin [67]. The phenolic hydroxyl groups of Tyr 52 and Tyr 74 participate in the active-site hydrogen bond network [8]. Tyr 74 is the only tyrosyl residue out of 14 which can be modified by *p*-nitrobenzenesulfonyl fluoride [68]. This side chain is involved in the pharmacologically active site of β_1-bungarotoxin. Most of the residues mentioned in figure 2 legend are more than 90 or even 95% conserved. Exceptions are Leu 2, Tyr 76, and Met 8 which are approximately 80% conserved. Most of these residues participate in structurally and functionally important sites as the catalytic, substrate-binding and calcium-binding sites. The last site includes the segment of the polypeptide chain between Tyr 25 and Gly 35 and Asp 49 which is also well conserved in the vipoxin PLA_2.

The basicity of PLA_2s is usually correlated with their toxicity. It appears that the positively charged residues increase the enzyme penetrability into the membranes which is important for further hydrolysis of phospholipids and, thus, for the pharmacological potency [5]. The vipoxin PLA_2 is a basic toxic protein with clusters of basic amino acids: e.g., Lys 82, Lys 83, Lys 92, Arg 101, Arg 108, Lys 118, Lys 123, Lys 127. However, the available experimental data show that the charge effect alone cannot explain the PLA_2 toxicity. Furthermore, not all toxic enzymes are basic [69].

Studies have indicated that the N-terminal region of PLA_2s can play a functional/mechanistical role in catalysis. A hypothesis has been introduced that this region can surve as a 'recognition site' for micellar substrates [70]. The surface properties of the segment are suitable for protein-lipid interactions. Thus, as a hydrolytic product, the N-terminal 15-residue peptide of the *Crotalus atrox* PLA_2 forms a stable monolayer at the air-water interface. This fragment binds to a single lecithin

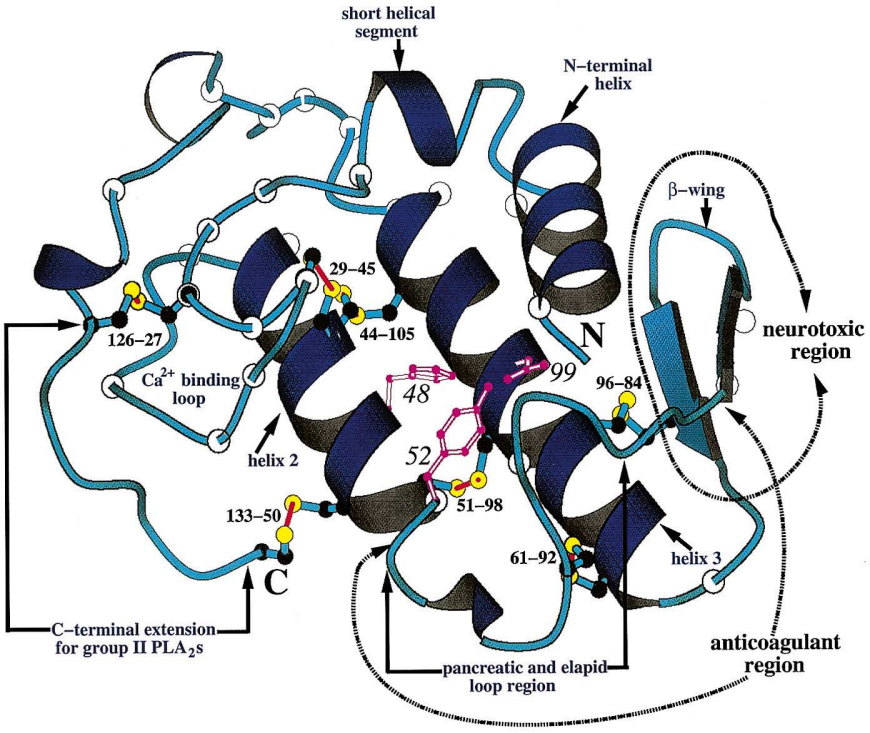
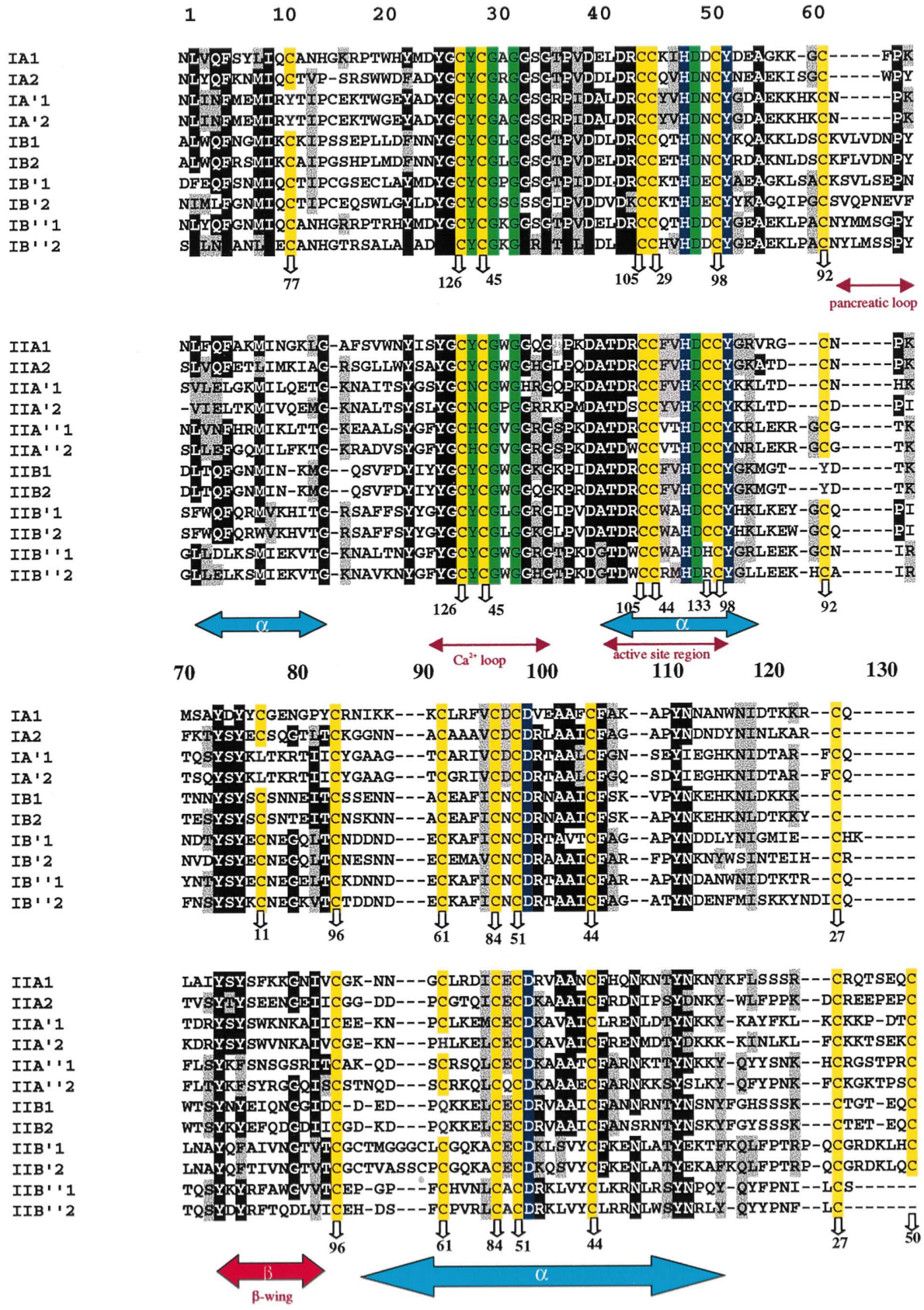


Figure 2. Ribbon representation of a monomeric group II PLA_2. The bound Ca^{2+}, the disulfide bridges and the active-site residues are included as ball-and-stick models. Secondary-structure regions are labeled as are regions recognized for an associated pharmaceutical function. Conserved and invariant residues, as summarized below using the one-letter code, found in approximately 200 sequences of PLA_2s [1] are inscribed at their Cα position.

2		*5*	*8*	*9*	*15*	*22*	*25*	*26*	*27*	*28*	*29*	*30*	*32*	*33*	
L/V,I,F		**F/L**	**M/L**	**I/V**	**G/C**	**Y**	**Y**	**G**	**C**	**Y/H,N,F**	**C**	**G**	**G**	**G**	
35	*37*	*39*	*41*	*42*	*44*	*45*	*48*	*49*	*51*	*52*	*61*	*69*		*74*	
G	**P**	**D/P**	**T/L**	**D/L**	**C**	**C**	**H**	**D/K,I**	**C**	**Y**	**C**	**P/T,I,V**		**Y**	
76		*89*	*99*	*104*	*106*	*107*	*108*		*110*	*111*		*113*	*114*	*121*	*136*
Y/F,W		**C**	**C**	**C**	**C**	**D**	**R/K/V,L**		**A/V,L,S**	**A/V**		**C**	**F/L**	**Y**	**C**


1 10 20 30 40 50 60
IA1
IA2
IA'1
IA'2
IB1
IB2
IB'1
IB'2
IB''1
IB''2
77
126
45
105
29
98
92
pancreatic loop
IIA1
IIA2
IIA'1
IIA'2
IIA''1
IIA''2
IIB1
IIB2
IIB'1
IIB'2
IIB''1
IIB''2
126
45
105
44
133
98
92
α
α
Ca2+ loop
active site region
70 80 90 100 110 120 130
IA1
IA2
IA'1
IA'2
IB1
IB2
IB'1
IB'2
IB''1
IB''2
11
96
61
84
51
44
27
IIA1
IIA2
IIA'1
IIA'2
IIA''1
IIA''2
IIB1
IIB2
IIB'1
IIB'2
IIB''1
IIB''2
96
61
84
51
44
27
50
β
α
β-wing

vesicle [70]. The authors concluded that the peptide constitutes a 'surface-active component' of the catalytic site. The N-terminal α-helix of the vipoxin PLA_2 (residues 2–14) contains the lipophilic residues Leu 2, Phe 3, Phe 5, Ile 9, and Leu 13. Three of them are conserved in all PLA_2 structures. Some of the N-terminal fragment residues, namely Leu 2, Phe 5, Met 8, and Ile 9, are part of the substrate-binding site which also includes residues 22, 29, 45, 110, 111, and 114 [69]. All these residues are highly conserved (fig. 3).

Antibody to the peptide segment between residues 114–121 [numbering according to ref. 1] completely abolished the toxicity of ammodytoxin A. However, a comparison of all the approximately 200 PLA_2 sequences in this region did not produce definite conclusions about the structural basis of toxicity. No regular substitutions of general validity were found when toxic and non-toxic PLA_2s and related proteins were compared. The comparative investigations described here show that the vipoxin PLA_2 contains highly conserved amino acid residues and sequences characteristic for all PLA_2s. This is comprehensible because the members of the PLA_2 family share definite functional characteristics which require common structural features. Evidently, a specific structural scaffold was selected during the evolution of these proteins. Thus, the polypeptide chain contains 118–133 amino acids and 6–8 disulfide bonds. However, a large diversity in quaternary structures has been observed: monomeric, homodimeric, heterodimeric, and more complex structures are known.

Evolutionary relationships of PLA_2s have been studied using both cDNA and protein sequences. Evolutionary trees of different groups of PLA_2s were derived from amino acid sequences available before 1990 [13] demonstrating the subdivision of these proteins into groups I and II. 'Trees' of Viperidae snake venom PLA_2, constructed using cDNA sequences, revealed separation of Crotalinae and Viperinae enzymes [71]. Data on the molecular evolution of the venom PLA_2 genes are summarized by Gubensek and Kordis [72]. We have compared the amino acid sequences of 22 PLA_2s or related proteins including two representatives each from the known 11 subgroups (fig. 3). Evidently, some regions of the PLA_2 structure, like those between residues 10–21, 56–67, 85–93, and the C terminus, have undergone more substitutive mutations than the others. The respective identity scores are shown in figure 4. Values near to or higher than 50% predominate for the members of group I and they can be classified as closely related proteins. An average identity score of 51% was calculated for this group. Considerably lower homology was observed for the group II PLA_2s, where values between 30 and 40% prevail. These proteins are more distantly related than their counterparts from group I, with an average identity score of 42%. Comparison of groups I and II showed considerably less homology and an average identity of 29.6% was calculated for the members of the two groups. This is characteristic for distantly related proteins. The same value, 30%, was calculated for the amino acid residues conserved in all 200 PLA_2s sequenced so far. This means that the basic scaffold, shown in figures 2 and 3, is the only common structure of the compared proteins. The 'framework' is probably associated with the toxicity but is not enough for the expression of the toxic function because it is preserved in both toxic and non-toxic proteins. A hypothesis was proposed that all PLA_2s probably diverged from a common ancestor and the genes encoding the two groups have derived from duplication of a common gene [1]. Further duplication may have generated genes encoding proteins from the subgroups. However, there is another possibility distantly related proteins: they could elaborate a common catalytic 'machinery' during convergent evolution. The low homology observed after comparison of PLA_2s and related proteins from groups I and II suggests that this possibility cannot be completely rejected.

The crystallographic model of vipoxin: an attempt to explain the modulated PLA_2 activity

The three-dimensional structure of vipoxin differs to some extent from that of the other PLA_2s. The efficiency of interfacial catalysis depends on the adsorption of PLA_2 to the lipid-water interface [8]. Both electrostatic and hydrophobic interactions are important for the binding of neurotoxins to membranes. The complex formation between the vipoxin components results in a decrease in the hydrophobic accessible surface area of the toxic PLA_2 by almost 3900 $Å^2$. Most probably, the

Figure 3. Comparison of PLA_2 sequences. The sequences of group I/II are highly homologous and have been aligned using the common numbering system [1]. Residues involved in coordination of Ca^{2+} are indicated as squares, catalytic residues are shown as closed ovals and supporting residues as open ovals. IA, IB, and so on indicate subgroup IA, subgroup IB accordingly. The PLA_2 sources are as follow: IA1, *Notechis scutatus scutatus* (notexin Np) [73]; IA2, *Naja naja atra* [74]; IA′1, *Bungarus multicinctus* A4 chain [75]; IA′2, *Bungarus multicinctus* A1 chain [76]; IB1, *Bos taurus*, bovine pancreas [77]; IB2, *Sus scrofa*, porcine pancreas [78]; IB′1, *Oxyuranus scutellatus scutellatus* (taipoxin) [52]; IB′2, *Pseudonaja textilis* [79]; IB″1, *Notechis scutatus scutatus* (Hte hypotension) [80]; IB″2, *Oxyuranus scutellatus scutellatus* [24]. IIA1, *Vipera ammodites meridionalis* (vipoxin) [58]; IIA2, *Crotalus atrox* [81]; IIA′1, *Agkistrodon piscivorus piscivorus* [81]; IIA′2, *Trimeresurus gramineus* [82]; IIA″1, human synovial fluid [83]; IIA″2, *Rattus norvegicus* [84]; IIB1, *Bitis gabonica* [85]; IIB2, *Bitis nasicornis* [86]; IIB′1, *Rattus rattus* [87]; IIB′2, *Mus musculus* [87]; IIB″1, *Homo sapiens* (brain) [88]; IIB″2, *Rattus rattus* (heart) [89].

		I												II								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
		IA2	IA'1	IA'2	IB1	IB2	IB'1	IB'2	IB"1	IB"2	IIA1	IIA2	IIA'1	IIA'2	IIA"1	IIA"2	IIB1	IIB2	IIB'1	IIB'2	IIB"1	IIB"2	
1	IA1	51	45	46	44	47	47	42	63	48	37	34	25	22	34	30	29	28	27	27	27	27	
2	IA2		50	49	55	54	57	55	63	55	44	37	27	29	34	33	38	36	32	28	31	29	
3	IA'1			95	42	45	41	40	45	43	39	35	28	30	34	27	29	32	26	25	28	30	
4	IA'2				42	44	41	40	45	42	40	35	28	30	33	27	28	31	27	26	27	29	
5	IB1					83	50	44	54	49	40	34	33	26	32	25	31	30	30	28	26	23	I
6	IB2						48	44	56	48	36	34	28	27	27	25	29	28	29	28	27	24	
7	IB'1							55	63	58	28	30	23	24	28	28	37	36	25	25	26	24	
8	IB'2								52	44	34	30	24	23	31	25	37	35	24	23	26	25	
9	IB"1									70	37	31	25	23	29	34	36	36	26	26	27	28	
10	IB"2										32	34	27	25	30	28	33	33	25	25	29	28	
11	IIA1											49	46	43	47	42	52	53	39	38	40	38	
12	IIA2												47	45	45	42	42	44	40	40	38	39	
13	IIA'1													70	45	45	40	42	38	37	42	43	
14	IIA'2														40	39	38	38	37	36	33	32	
15	IIA"1															71	36	39	39	38	46	43	
16	IIA"2																34	35	37	34	46	44	II
17	IIB1																	88	33	33	28	31	
18	IIB2																		31	31	32	33	
19	IIB'1																			89	39	33	
20	IIB'2																				38	33	
21	IIB"1																					76	

Figure 4. Identity scores of 22 PLA_2 and related proteins including two representatives of the known 11 subgroups. For the abbreviations see the legend to figure 3.

'blocked' area includes, at least in part, the interfacial recognition site. Residues 6, 12, 76–81 and 119–125 were identified as important for the neurotoxicity of the dimeric PLA_2s from *V. r. formosensis* [9]. This toxin is 92% identical to vipoxin. Inspection of the X-ray model showed that the segment 119–125 of the vipoxin PLA_2 is partially blocked by the inhibitor. Decreased hydrophobicity in the region of substrate binding and steric hindrance can explain the reduced toxicity of PLA_2 in the vipoxin complex.

Concluding remarks

The snake venom dimeric neurotoxin vipoxin is a product of molecular evolution of a toxic but unstable PLA_2 into an inhibitor which stabilizes the enzyme and preserves its pharmacological activity. It is found in evolutionarily younger snakes. The non-toxic component of the complex partially inhibits the enzymatic activity and considerably reduces the toxicity of PLA_2. In this respect, vipoxin is an example of modulation of PLA_2 activity generated by molecular evolution. This is the first reported example of high structural identity between an enzyme and its natural inhibitor. The unique complex demonstrates the evolution of an enzymatic and toxic function into an inhibitory and non-toxic one. The evolution of the enzymatic and toxic functions of the viper PLA_2 into a non-enzymatic/inhibitory and non-toxic one is probably a process occurring throughout the world: it was first found in southeast Europe (southeast Bulgaria, then southwest France) and was recently observed in Taiwan (Asia). The X-ray model of vipoxin indicates that the reduced toxicity is due to a decrease in the hydrophobicity of the substrate-binding surface area and to steric hindrance.

PLA_2s and related proteins of group II are more distantly related than their counterparts from group I. The intergroup homology between the members of groups I and II is considerably lower than that within the groups. The two PLA_2 groups are distantly related proteins with an average identity score of 29.6%.

A basic scaffold containing 30% of the amino acid residues is conserved in approximately 200 PLA_2 sequences. This is the most important part of the molecule which is necessary for the hydrolytic activity and is preserved in all catalytically active proteins. The scaffold is preserved in both toxic and non-toxic proteins. It is probably associated with the expression of the toxic function but its presence alone is not sufficient for the toxicity. No basic residues, with the exception of Lys/Arg 108, are identically conserved in all PLA_2s.

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