ELSEVIER

Toxicon 42 (2003) 869–883

TOXICON

www.elsevier.com/locate/toxicon

Mapping structural determinants of biological activities in snake venom phospholipases A_2 by sequence analysis and site directed mutagenesis

Lucimara Chioato[a], Richard J. Ward[b,*]

[a]*Department of Biochemistry and Immunology, FMRP-USP, Universidade de São Paulo, Brazil*
[b]*Department of Chemistry, FFCLRP-USP, Universidade de São Paulo, Avenida Bandeirantes 3900, Monte Alegre, CEP 14040-901, Ribeirão Preto-SP, Brazil*

Abstract

In addition to their catalytic activity, snake venom phospholipases A_2 (vPLA$_2$) present remarkable diversity in their biological effects. Sequence alignment analyses of functionally related PLA$_2$ are frequently used to predict the structural determinants of these effects, and the predictions are subsequently evaluated by site directed mutagenesis experiments and functional assays. In order to improve the predictive potential of computer-based analysis, a simple method for scanning amino acid variation analysis (SAVANA) has been developed and included in the analysis of the lysine 49 PLA$_2$ myotoxins (Lys49-PLA$_2$). The SAVANA analysis identified positions in the C-terminal loop region of the protein, which were not identified using previously available sequence analysis tools. Site directed mutagenesis experiments of bothropstoxin-I, a Lys49-PLA$_2$ isolated from the venom of *Bothrops jararacussu*, reveals that these residues are exactly those involved in the determination of myotoxic and membrane damaging activities. The SAVANA method has been used to analyse presynaptic neurotoxic and anti-coagulant vPLA$_2$s, and the predicted structural determinants of these activities are in excellent agreement with the available results of site directed mutagenesis experiments. The positions of residues involved in the myotoxic and neurotoxic determinants demonstrate significant overlap, suggesting that the multiple biological effects observed in many snake vPLA$_2$s are a consequence of superposed structural determinants on the protein surface.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Lys49-PLA2; Myotoxin; Neurotoxin; Anti-coagulant; Bioinformatics

1. Introduction

The secreted group I/II phospholipases A_2 (PLA$_2$, EC 3.1.1.4) are small (~14 kDa), stable enzymes that are encountered in a wide variety of biological fluids and cells. PLA$_2$s catalyse the hydrolysis of the *sn*-2 ester bond of *sn*-3 glycerophospholipids to release lysophospholipids and fatty acids (van Deenen and de Haas, 1963). The rate of hydrolysis is considerably enhanced against the phospholipid substrate in an aggregated form such as a micelle or a bilayer, and the enzymatic mechanism by which this process of interfacial activation occurs has become a paradigm for interfacial catalysis (Berg et al., 2001). The products of phospholipid hydrolysis may themselves be bioactive, or may serve as precursors for the synthesis of other bioactive compounds (Dennis, 1994; Dessen, 2000). Due to their central role in many cellular processes, PLA$_2$s from a variety of sources have been extensively studied, not only to understand the molecular bases of the catalytic mechanism and interfacial binding (Scott and Sigler, 1994), but also with a view to understanding their regulatory functions within the cell (Murakami et al., 1997; Murakami and Kudo, 2001). Snake venoms are abundant sources of group I/II PLA$_2$, and although these venom derived PLA$_2$s (vPLA$_2$s) show a high level degree of structural conservation with mammalian secreted PLA$_2$s, they present remarkable diversity in terms of biological activities, and a given vPLA$_2$ may demonstrate multiple biological effects.

* Corresponding author. Tel.: +55-16-602-3484; fax: +55-16-633-8151.
E-mail address: rjward@fmrp.usp.br (R.J. Ward).

0041-0101/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2003.11.027

It has been established that catalytic activity plays a key role in certain pharmacological effects of vPLA$_2$s (Chang and Su, 1982; Díaz-Oreiro and Gutiérrez, 1997), however, it is now well proven that many pharmacological effects are at least partially independent of hydrolytic activity (Kini and Evans, 1995; Rufini et al., 1996; Páramo et al., 1998; Soares et al., 2001). The absence of a clear correlation between catalysis and pharmacological activity together with the diversity of biological effects raises the question as to the structural bases of these biological functions. A growing body of evidence suggests that these activities may be mediated by interactions between vPLA$_2$s and acceptors for endogenous PLA$_2$s on the membranes of the target cell (Lambeau and Lazdunski, 1999; Hanasaki and Arita, 1999; Valentin and Lambeau, 2000). The identification of additional endogenous mammalian PLA$_2$s (Six and Dennis, 2000), and the discovery of their protein acceptors in human cells (Sribar et al., 2001; Higashino et al., 2002; Boilard et al., 2003), has expanded the number of potential targets and possible mechanisms of action for vPLA$_2$s.

Acceptor mediated responses may explain many of the observed effects of vPLA$_2$s, however, other mechanisms of PLA$_2$/membrane interactions may also contribute to toxicity of these proteins. For example, the role of quaternary structure is highlighted by the high level of toxicity of the crotoxin heterodimer in comparison with each individual sub-unit (Habermann and Breithaupt, 1978; Faure et al., 1993). Furthermore, it is noteworthy that two different types of dimeric interaction (Brunie et al., 1985; Arni et al., 1995), and a trimeric association (Fremont et al., 1993) have been observed for vPLA$_2$s in the crystalline state (Arni and Ward, 1996; Ward et al., 1998a). Although the effect of quaternary structure on catalytic and biological activities remains unclear, a mechanism involving quaternary structural change in the Lys49-PLA$_2$ homodimer has been proposed to explain the increased permeability of artificial membranes after treatment with the protein, and this mechanism is independent of both catalytic activity and acceptor binding (da Silva Giotto et al., 1998).

In addition to their pharmacological functions, vPLA$_2$s also present a wide range of substrate preferences, which must be determined by the specific surface topology of the region of the protein that interacts with the membrane surface. In group I/II PLA$_2$s, this region is defined by a highly conserved hydrophobic surface cleft that binds the fatty acyl chain of the phospholipid substrate, together with a surrounding ring of more variable polar and charged residues, and which is called either the interfacial recognition site (IRS; Pieterson et al., 1974) or the i-face (Ramirez and Jain, 1991). Variation in the topology of the i-face determines the specificity of phospholipid head-group binding (Snitko et al., 1999; Yu et al., 2000), and may give rise to additional and unexpected catalytic effects. For example, the neutral PLA$_2$ from *Naja n. atra* venom exhibits the phenomenon of 'PC activation' (Adamich and Dennis, 1978), in which the hydrolysis of phospholipids containing ethanolamine head groups is enhanced in membranes that include phosphatidycholine (PC). This effect is determined by the binding of the PC head-group to a cluster of surface residues close to the active site region in the i-face (Lefkowitz et al., 1999).

These examples demonstrate the remarkable diversity displayed by vPLA$_2$s in their surface recognition properties. This raises the question as to how the surface of small proteins such as the group I/II PLA$_2$s can determine such functional complexity. In the group I/II PLA$_2$s, between 35 and 40% of the residues are exposed to solvent at the surface of the protein, therefore around 40–50 residues participate in the definition of the physico-chemical properties of the protein surface. Which of these residues participate in the formation of the specific surface topologies that determine a given biological function? In the face of such complexity, what strategies can be employed to study these structural determinants? Here we review amino acid sequence comparison methods that have been applied to map the structural determinants of vPLA$_2$s. A sequence analysis of the lysine 49 phospholipase A$_2$ (Lys49-PLA$_2$) myotoxins is presented which uses a simple method to evaluate amino acid substitutions, and the results are discussed in the light of recent site-directed mutagenesis studies. We have also applied the method to study the structural determinants of pre-synaptic neurotoxic and anticoagulant activities observed in other vPLA$_2$s. The method is robust and may be applicable to the investigation of the surface properties of other families of homologous proteins.

2. Lysine 49 phospholipase A$_2$ (Lys49-PLA$_2$) myotoxins

Lys49-PLA$_2$s have been identified as abundant components of the venoms from New World *Bothrops* and *Agkistrodon* snake species (Gutiérrez and Lomonte, 1995), in the Asiatic *Trimeresurus* species (Liu et al., 1990) and have been discovered more recently in additional New World viperid species, although in lesser quantities (Tsai et al., 2001). Not only is the distribution of the Lys49-PLA$_2$s more widespread than previously thought, but also the range of known biological effects is broader. In addition to myotoxic (Gutiérrez and Lomonte, 1995), cytotoxic (Fletcher and Jiang, 1998) and edema inducing (Liu et al., 1991; Landucci et al., 2000) effects, the Lys49-PLA$_2$s show pre-synaptic neurotoxicity (Dhillon et al., 1987), stimulate the degranulation of mast cells (Landucci et al., 1998) and directly influence leukocyte mobility (de Castro et al., 2000).

The Lys49-PLA$_2$s are characterized as a sub-family of group IIA PLA$_2$s (using the nomenclature of Six and Dennis, 2000) in which the aspartic acid residue at position 49 (Asp49) in the active site region is substituted by lysine (Lys49). In the Asp49-PLA$_2$s, the carboxyl group oxygens of Asp49 contribute to the coordination of the catalytically

essential Ca^{2+}co-factor, and the substitution by lysine at this position results in steric hindrace of Ca^{2+}binding by the ε-amino group of the Lys49. It has been suggested that the loss of Ca^{2+}binding in the Lys49-PLA_2s results in the observed lack of hydrolytic activity against both synthetic and natural phospholipids (Maraganore and Heinrikson, 1986; van den Bergh et al., 1988). Despite their lack of catalytic activity, the Lys49-PLA_2s demonstrate membrane damaging activity via a Ca^{2+}-independent, non-hydrolytic mechanism (Díaz et al., 1991; Rufini et al., 1992; de Oliveira et al., 2001).

As new members of the Lys49-PLA_2s sub-family are discovered, the number of amino acid sequences of deposited in the protein databases has steadily increased. Due to the range of biological effects and the unusual mechanism of membrane damage induced by these toxins, the Lys49-PLA_2 family represents an interesting example to study by protein surface mapping in order to identify the structural determinants of biological activities. This strategy involves, using a series of bioinformatic tools to analyze amino acid sequences and to predict the residues that are structural determinants for a given biological effect. These residues are subsequently targeted by site directed mutagenesis experiments.

3. Amino acid sequence alignment analysis

3.1. Initial sequence alignment and visual comparison

Studies comparing a limited number of similar amino acid sequences can be made using manual sequence alignment. Apart from being time consuming, this simple method has the principal disadvantage that the positions of amino acid insertions and deletions are not reliably determined. This is a major drawback, since a reliable sequence alignment is critical for the correct interpretation of the results. With increased access via internet to sequence alignment tools such as CLUSTAL (Higgins et al., 1992; Thompson et al., 1994), the automated alignment of greater numbers of amino acid sequences has become widespread. It should be noted that although automated sequence alignment programs speed up the process of obtaining an initial 'draft' alignment, the algorithms employed are not infallible, and where possible alignments made by computer should always be checked against superposed three-dimensional protein structures in order to verify the positions of insertions and deletions. The quality of a sequence alignment is crucial for subsequent analyses, and the importance of a reliable sequence alignment should be emphasized.

The most straightforward method to analyze a sequence alignment is by visual evaluation, and several programs are available on the internet that aid this type of analysis by coloring each amino acid residue symbol according to its physicochemical properties (Beitz, 2000). Key differences in the active site and substrate binding cleft regions of the Lys49-PLA_2 from *Agkistrodon p. piscivorus* were identified using visual analysis of a manual sequence alignment (Maraganore and Heinrikson, 1986). In a subsequent study using similar techniques, sequence comparison of the Lys49-PLA_2 myotoxin-II from *Bothrops asper* with a limited set of other Lys49-PLA_2 confirmed these differences between the Lys49 and Asp49-PLA_2s, and in addition drew attention to the cationic/aromatic residues which were grouped in the C-terminal loop region of the proteins (Francis et al., 1991). Based on a visual analysis of a computer generated Lys49-PLA_2 sequence alignment, an extended surface including polar and cationic residues primarily on helix 3 was suggested to be involved in the determination of myotoxic activity (Selistre de Araujo et al., 1996). This analysis may be compared with a previous prediction that residues 78–87 in the β-wing region of the PLA_2s are determinants of the myotoxic effect (Kini and Iwanaga, 1986a). Taken together, these studies highlight the ambiguities that arise from attempts to identify the structural determinants of biological activity by visual analysis, and suggest the need for more sophisticated analyses to extract useful information from the increasingly large datasets used in automated sequence alignments.

3.2. Residue mapping using the 'SequenceSpace' analysis

Automated amino acid sequence alignments commonly use progressive pairwise sequence comparison algorithms, which results in the clustering of the most similar sequences within the alignment (Feng and Doolittle, 1987). This sequence pairing is based on the optimization of a numeric value, or score, and although a comparison of all residues contributes to the score value, the exact information as to which residues are conserved within a particular sequence pair is lost. This information is unfortunately the most interesting for defining conserved sequence motifs in protein families with common functions. To recover this information, computational methods have been developed which identify the specific residues that define sub-groups of amino acid sequences within multiple sequence alignments (Casari et al., 1995; Lichtarge et al., 1996; Andrade et al., 1997). In these methods, all sequences in an alignment are mapped as vectors in a multi-dimensional 'SequenceSpace'. Vectors pointing to conserved amino acid residues and patterns in this sequence space become clustered, and these clusters will therefore be comprised of specific amino acid residues that 'define' sub-groups of proteins.

When applied to a data set of 72 PLA_2 sequences this analysis yielded a list of 12 residues that were highly specific to the Lys49-PLA_2s sub-family (Ward, 1998b). Surface mapping of these residues revealed that 10 out of these 12 residues mapped to three amino acid clusters located in the active site region, the hydrophobic fatty acyl chain binding pocket and the tip of the β-wing. The cluster of residues in the active site region includes Lys49, Leu32

and Asn28 and has been the focus of a site directed mutagenesis study using bothropstoxin-I (BthTx-I), a Lys49-PLA_2 from the venom of *Bothops jararacussu* (Ward et al., 2002). Recombinant BthTx-I shows no detectable hydrolytic activity against natural phospholipid substrates, and mutation of Lys49Asp did not restore catalytic activity. This implies that the lack of catalytic activity observed in the Lys49-PLA_2 is not simply a consequence of the presence of the Lys49, and mutagenesis of other residues in the active site region is currently underway in order to investigate this result. The mutagenesis study of the active site region also included residues that are highly conserved in the active site of the Asp49-PLA_2s. None of the active site mutants, including substitution of the nucleophile His48 by glutamine, influenced the myotoxic activity which provides strong evidence against the involvement of phospholipid hydrolysis in the myotoxic effect (Ward et al., 2002). The second residue cluster in the hydrophobic and fatty acyl binding site of Lys49-PLA_2s is comprised of Leu5/Val102/Leu106, which substitute the Phe5/Ala102/Phe106 triad found in the Asp49-PLA_2s. Although the total volume of the amino acid triad remains virtually unchanged, the topology of the binding site region is altered, and the consequence of these alterations is currently unknown.

The substitutions in the active site and hydrophobic fatty acyl chain binding pocket regions of the Lys49-PLA_2s had already been noted by visual comparison (Maraganore and Heinrikson, 1986; Francis et al., 1991), however, the β-wing cluster was not detected. Analysis of the crystal structures of several Lys49-PLA_2s demonstrated that the Glu12, Trp77 and Lys80 residues in the β-wing cluster participate in intermolecular contacts resulting in the formation of a homodimer (Arni et al., 1995; Arni and Ward, 1996). The Glu12 and Lys80 from each monomer in the homodimer form two salt-bridges, and this interaction is weakened at reduced pH resulting in separation of the two monomers. In the BthTx-I, the separation of the monomers is concomitant with the reduction of the Ca^{2+}-independent membrane damaging activity (de Oliveira et al., 2001).

The 'SequenceSpace' analysis is a useful tool for identifying amino acid clusters that are specific to a protein family, however, the question remains as to the significance of the result. A drawback of the technique is that the sequences become grouped based on increasingly subtle similarities, and at a certain level the similarities due to the high amino acid identity in PLA_2s from related species mask the similarities arising from shared biological function. In order to minimize this effect, an amino acid sequence selection procedure is used which eliminates sequences that are considered to be highly similar, such as isoforms. This sequence selection procedure is common practice in many comparative studies, and unfortunately may result in the loss of relevant information from the alignment under analysis. Furthermore, those amino acids that may determine common functional properties between otherwise dissimilar proteins will have a decreased significance. For example, the aromatic/cationic C-terminal loop cluster is conserved in all Lys49-PLA_2s, however, the 'SequenceSpace' analysis identified only one cationic residue the C-terminal loop region (Ward, 1998b), which is a consequence of this aromatic/cationic motif being common to other families of PLA_2s.

3.3. Scanning amino acid variability analysis (SAVANA) of sequence alignments

Although a powerful analytic tool, the 'SequenceSpace' program failed to extract subtle yet important sequence information, and this has prompted us to develop a new analysis specifically designed for alignments of highly similar (>95% identity) amino acid sequences. The analysis is simple and is derived from a comparison of the amino acid sequences of isoforms with differing levels of a given activity. Any differences in the sequences must be responsible for the observed modulation of the effect of interest, and surface mapping of these positions will therefore indicate the location of the structural determinant of the effect. Initially the amino acid sequence database is searched in order to identify the most closely related sequences to the protein of interest. Typically, an alignment of the 8–10 most closely related sequences is performed, and although the sequences are closely related, subsequent manual adjustments may still be required.

The results of the database search and sequence alignments of three vPLA$_2$s with different biological activities are presented in Fig. 1. These sequence alignments serve as the input for the SAVANA program, which initially derives the consensus amino acid sequence shown for each sequence block in Fig. 1. Subsequently, the program scans all positions in the alignment, identifies every amino acid variation from the consensus sequence, and calculates a position score based on a point accepted mutation matrix (PAM, Dayhoff et al., 1978). We have chosen the PAM5 matrix since the sequences that are routinely included in the analysis differ by less than 5%. The result of SAVANA is a score for each position in the sequence alignment that is directly related to the number and type of amino acid substitutions found at that position. This score is a numerical value that can be represented using a color scale in a solid surface representation of a protein structure hosen from the PLA_2 family that is under analysis. In this way the positions and significance of dissimilarities between otherwise highly similar amino acid sequences may be identified and mapped onto the protein surface.

The SAVANA program treats the insertion or deletion of an amino acid residue as a highly significant event which is assigned a high score. In order to represent these insertion events in a surface map, the 3D structure of the protein with the longest amino acid sequence must therefore be used. In the case of the Lys49-PLA_2 myotoxins, the longest sequence is that of the myotoxin-II from *Bothrops*

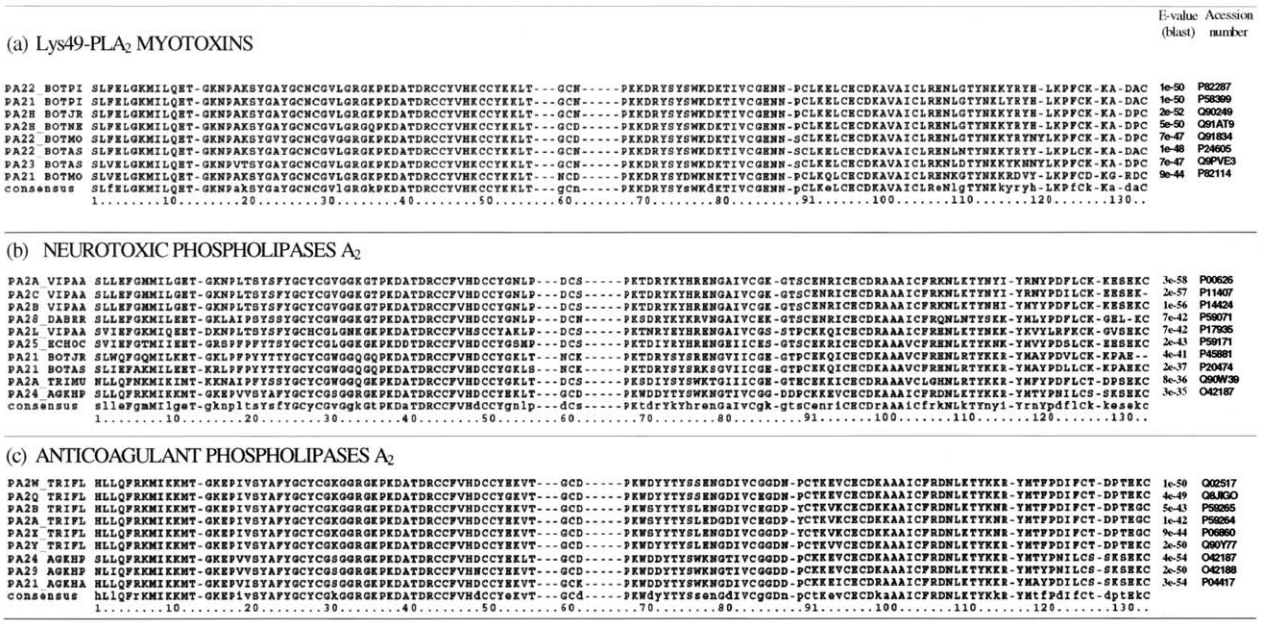

Fig. 1. Amino acid sequence alignment of snake venom phospholipases A_2. **(a) Myotoxic Lys49 PLA2 s from *Bothrops* species.** MjTX-I: myotoxin I *Bothrops moojeni* (Soares et al., 2000a); MjTX-II: myotoxin II *B. moojeni* (Soares et al., 1998); PrTX-I: piratoxin-I *B. pirajai* (Toyama et al., 1998); PrTX-II: piratoxin-II *B. pirajai* (Toyama et al., 2000); BnSP-7: Lys49-PLA2 *B. neuwiedi* (Soares et al., 2000b); BthTX-I: bothropstoxin-I *B. jararacussu* (Cintra et al., 1993); BaspII: myotoxin II *B. asper* (Francis et al., 1991); and M1-3-3: myotoxin II iosoform *B. asper* (Pescatori et al., 1998). **(b) Pre-synaptic neurotoxic PLA_2 s**, PA2C_VIPAA from *Vipera ammodytes ammodytes* (Pungercar et al., 1989); PA2A_VIPAA from *Vipera a. ammodytes* (Pungercar et al., 1991); PA2B_VIPAA from *Vipera a. ammodytes* (Kordis et al., 1990); PA28 DBRR from *Daboia russellii russellii* (Gowda et al., 1994); PA2L_VIPAA from *Vipera a. ammodytes* (Pungercar et al., 1990); PA25_ECHOC from *Echis ocellatus* (Harrison et al., 2002); PA21_BOTAS from *Bothrops asper* (Kaiser et al., 1990); PA21_BOTJR from *Bothrops jararacussu* (Moura-da-Silva et al., 1995); PA24_AGKHP from *Gloydius halys* (Pan et al., 1998) and PA2A_TRIMU from *Protobothrops mucrosquamatus* (Guo et al., 2001). **(c) Anti-coagulant PLA_2 s.** PA2A_TRIFL from *Trimeresurus flavoviridis* (Yamaguchi et al., 2001); PA2B_TRIFL from *T. flavoviridis*(Yamaguchi et al., 2001); PA2X_TRIFL from *T. flavoviridis* (Kini et al., 1986); PA2Q_TRIFL from *T. flavoviridis* (Chijiwa et al., 2003); PA2W_TRIFL from *T. flavoviridis* (Ogawa et al., 1992); PA2Y_TRIFL from *T. flavoviridis* (Chijiwa et al., 2003); PA29_AGKHP from *Gloydius halys pallas* (Pan et al., 1998); PA24_AGKHP from *Gloydius h. pallas* (Pan et al., 1998); and PA21_AGKHA from *Gloydius h. blomhoffi* (Forst et al., 1986). The consensus sequence is shown in the last line for each alignment block. The first sequence in each block was used as the query sequence in the database searches using BLAST (Altschul et al., 1990), and the penultimate column shows the expectation values for each sequence.

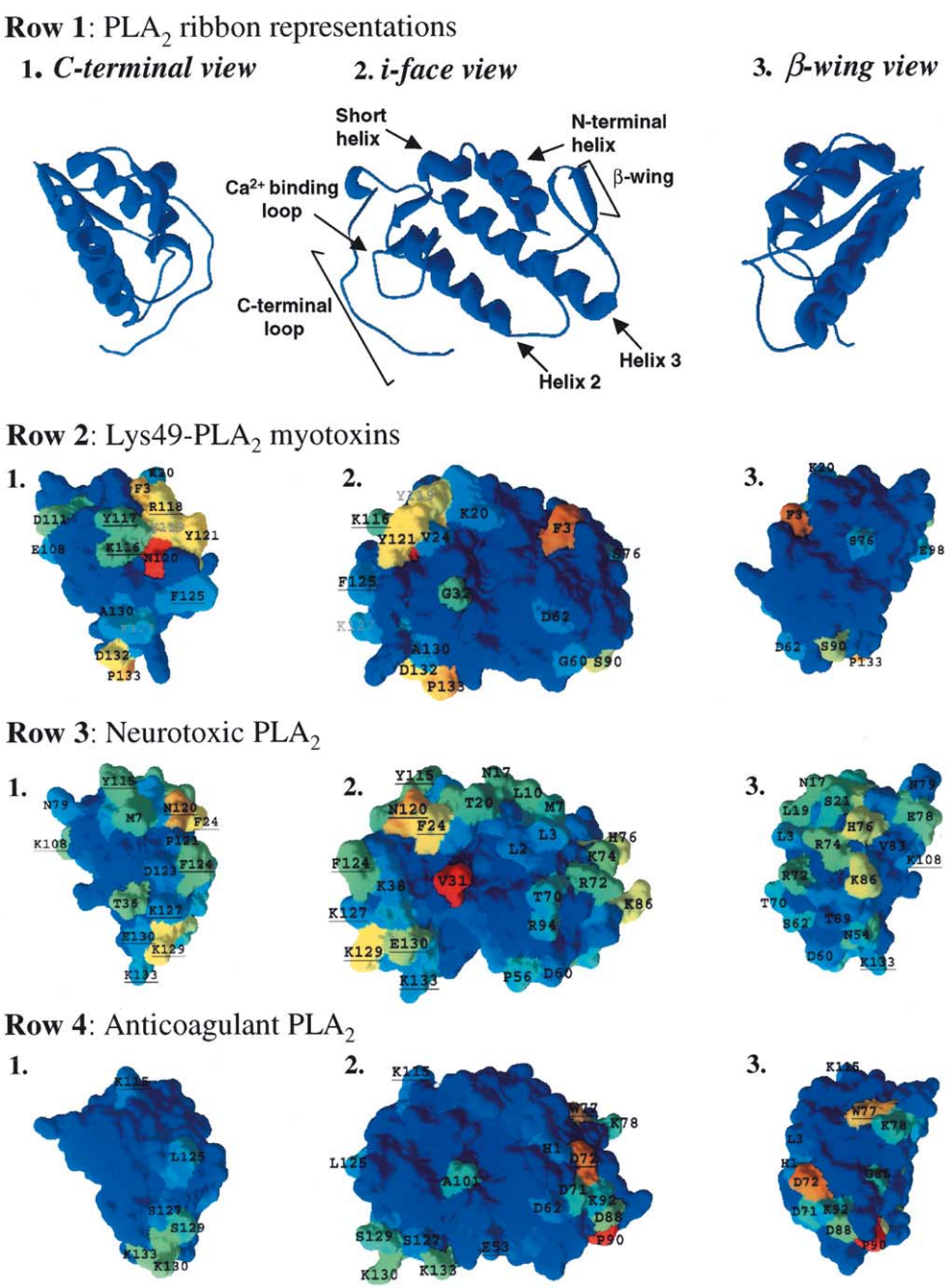

Fig. 2. Solid surface representations of SAVANA results. Each column presents the view of the chosen PLA_2 structure looking towards: (1) the C-terminal loop region (left column), (2) the i-face (centre column) and (3) the β-wing region (right column). The first row presents ribbon representations of the PLA_2 molecules in each of the three orientations, with the principal structural features labelled in the case of the i-face view. The second, third and fouth rows present solid surface representations of: myotoxin II, a Lys49-PLA_2 myotoxin from *Bothrops moojeni* (de Azevedo et al., 1997); Ammodytoxin A, a pre-synaptic neurotoxin from *Vipera a. ammodytes* (computer generated molecular model—see text for details); Basic PLA_2, an anticoagulant PLA_2 from *Gloydius (Agkistrodon) halys Pallas* (Zhao et al., 1998). The surfaces are coloured according to the position scores derived from the SAVANA of each corresponding sequence block shown in Fig. 1. Fully conserved positions are shown in dark blue, the highest scored (i.e. the most significant) positions are shown in red, and intermediate scores lie on a colour scale between these two extremes. All non-conserved positions are labelled in black, and those positions which have been targeted by site directed mutagenesis and which influenced the biological activity are underlined. Those positions, which have been targeted by site directed mutagenesis and which did not influence the biological activity are shown in grey. The figure was prepared using the Swiss-Pdb Viewer program (Guex and Peitsch, 1997).

moojeni, and the surface map representation of the SAVANA analysis is presented in Fig. 2 using the crystal structure of this protein (de Azevedo et al., 1997). High scores were observed for residues in the C-terminal loop region of the protein, which are therefore identified as candidates for being the structural determinants of the biological effects of Lys49-PLA_2 myotoxins. This prediction is supported by the observation that a synthetic peptide of residues 115–129 in the C-terminal region of myotoxin II from *Bothrops asper* demonstrates biological activity, although at reduced levels when compared to the whole protein (Lomonte et al., 1994). When the prediction made by SAVANA is correlated with results of site directed mutagenesis experiments in BthTx-I, the result is quite striking. The positions 117–123 identified in the SAVANA superpose exactly with the BthTx-I mutants that influence the myotoxic activity in the C-terminal loop region (Chioato et al., 2002). Furthermore, SAVANA identified additional positions 115–123 in the C-terminal loop region which show a strong correlation with those positions that influence membrane damaging activity as identified by site directed mutagenesis experiments (Chioato et al., 2002). The SAVANA analysis therefore successfully identified the structural determinants involved in both the membrane damaging and myotoxic effects. It should be noted, however, that additional positions (Phe3, Asp62, Ser90, Glu82, Lys20 and Ser76) are also identified in the analysis, and these remain to be evaluated experimentally using site directed mutagenesis. We are currently using a surface walking strategy centered on the C-terminal loop region that will encompass these additional positions.

4. Expression of recombinant $vPLA_2$s

The value of sequence analysis lies in the reduction of a vast range of possibilities to a manageable number of site-directed mutagenesis experiments. In order to use this strategy an efficient heterologous protein expression system, together with a purification protocol, which yields native protein, is essential. The successful expression of group I pancreatic PLA_2s as heterologous proteins in *Escherichia coli* or yeast when coupled with site-directed mutagenesis have proven to be powerful and reliable tools in the study of the structural bases of basic enzymatic properties such as catalysis, lipid specificity and interfacial activation (Yuan and Tsai, 1999). The use of these tools to study of the structural bases of biological activities of $vPLA_2$s depends on the development of adequate expression systems, and as shown in Table 1, the last decade has witnessed a steady increase in the number and diversity of $vPLA_2$s that have been expressed and studied using site directed mutagenesis.

Venom PLA_2s frequently express well in *E. coli*, and are generally non-toxic to the host cells. However, in most cases the major hurdle is the purification of the recombinant material in the native conformation, since without exception, group I/II PLA_2s form inclusion bodies when expressed in *E. coli*. Although easily purified by centrifugation, the protein in inclusion bodies must be solubilized with chemical denaturants and refolded to their native conformation by slowly decreasing the denaturant concentration. Furthermore, since native group I/II PLA_2s contain 6–8 disulphide bonds, the protein refolding must be performed in the presence of an oxidation/reduction buffer. Native recombinant $vPLA_2$s have been refolded from inclusion bodies in high yields by changing the concentration of the chemical denaturant using dialysis (see Table 1). More recently, protein refolding protocols using commonly available gel filtration resins have been reported (Batas and Chaudhuri, 1996; 1999a,b) and these methods have been successfully used to refold BthTx-I expressed as inclusion bodies *E. coli* (Ward et al., 2001). Successful attempts to circumvent the refolding problem in *E. coli* through the use of fusion protein constructs have also been reported (Liang et al., 1993; Hodgson et al., 1993; Pan et al., 1994; Giuliani et al., 2001; Yang et al., 2003). In these cases, although heterologous protein is expressed in the refolded state, enzymatic or chemical treatment is required to liberate the native PLA_2 from the fusion protein. Although the use of eukaryotic cells for the production of native correctly folded mammalian class I/II PLA_2s has been reported (Bekkers et al., 1991), to date the neutral PLA_2 from *Naja n. naja* expressed in *Pichia pastoris* is the only $vPLA_2$ produced using a eukaryotic host cell system (Lefkowitz et al., 1999). These expression systems have been used to produce site directed mutants, which allows the correlation of amino acid prediction studies with experimental surface mapping results.

5. Comparison of $vPLA_2$ protein surfaces

In the case of the Lys49-PLA_2 myotoxins, preliminary surface mapping results have revealed that the structural determinants of the myotoxic and membrane damaging activities are localized in the C-terminal region of the protein. Although the residues involved in each of these activities localizes to the same region in the protein structure, the specific residues involved in each activity are clearly independent (Chioato et al., 2002). This provides clear and direct evidence that specific biological effects are determined by defined residue clusters on the protein surface. The strategy used in the Lys49-PLA_2 surface mapping project was alanine scanning mutagenesis, in which all target residues in a given surface region are substituted by alanine residues. This strategy has proven to be successful in the mapping and study of protein surfaces

Table 1
Snake venom phospholipases A_2 expressed in *E. coli*

Phospholipase A_2	Species	Activity	Comments	Referenece
Acidic PLA_2	*Naja naja naja*	Non-toxic	Expression in *E. coli*, refolded from inclusion bodies	Kelley et al., 1992
			Mutation of positive charge reduces manoalide binding	Bianco et al., 1995
Acidic PLA_2	*Naja naja naja*	Non-toxic	Expression of native protein in *Pichia pastoris*	Lefkowitz et al., 1999
			Mutant D23N reduced 'PC activation' effect	
Acidic PLA_2	*Naja naja naja*	Non-Toxic	Expression of synthetic gene in *E.coli*, refolded from inclusion bodies.	Sumandea et al., 1999
			Mutation of aromatic residues in IRS influences binding to lipid surfaces.	
Acidic PLA_2 (and isoforms)	*Naja naja atra*	Non-toxic	Expression in *E. coli*, His-tag fusion protein refolded from inclusion bodies.	Pan et al., 1994
			Active site mutagenesis abolished catalytic activity in two isoforms (PLA_{2-1}, and PLA_{2-2}).	Pan et al., 1998
PLA_2	*Bungarus multicinctus* (Taiwan banded krait)	Non-toxic	Expression in *E. coli*, refolded from inclusion bodies	Chang et al., 1996a
				Chang et al., 1997
Ammodytoxin A	*Vipera ammodytes ammodytes*	Presynaptic neurotoxin	Expression in *E. coli* as a fusion protein	Liang et al., 1993
			F24 mutant show decreased neurotoxicity	Petan et al., 2002
			Position 124 important for neurotoxicity	Pungercar et al., 1999
			Mutagenesis in the C-terminal loop region influences receptor binding and neurotoxicity	Prijatelj et al., 2000, 2002
				Ivanovski et al., 2000
Trimucrotoxin	*Trimeresurus mucrosquamatus*	Presynaptic neurotoxin	Expression in *E. coli*, His-tag fusion protein refolded from inclusion bodies.	Tsai and Wang, 1998
			- Mutation of residues in N-terminal helix reduced activity	
β-bungarotoxin	*Bungarus multicinctus* (Taiwan banded krait)	Presynaptic neurotoxin	Expression in *E. coli*, refolded from inclusion bodies.	Kuo et al., 1995; Chang et al., 1996b
(A-chain)			Alternative engineered disulphides had no effect on catalytic activity	Chang et al., 1996c
APP-D-49	*Agkistrodon piscivorus piscivorus*	Anti-coagulant	Expression in *E. coli*, refolded from inclusion bodies	
			Mutation N1S had no effect on catalytic activity.	Lathrop et al., 1992
			Cationic residues in N-terminal and active site regions mediate protein binding to the lipid interface.	Han et al., 1997
Acidic/basic PLA_2 ($ABPLA_2$)	*Agkistrodon halys pallas*	Anti-coagulant	Expression in *E. coli*, refolded from inclusion bodies	Liu et al., 1999
Acidic PLA_2	*Agkistrodon halys Pallas*	Anti-coagulant	Expression in *E. coli*, refolded from inclusion bodies	Pan et al., 1998
($APLA_2$)			Mutation of residues in N- and C-termini reduced activity	Liu et al., 2001
Acidic-PLA_2 ($APLA_2$ isoform)	*Agkistrodon halys Pallas*	Anti-coagulant	Expression in *E. coli*, refolded from inclusion bodies	Zhong et al., 2001
PLA_2-9	*Lapemis hardwickii*	Anti-coagulant	Expression in *E. coli* as a fusion protein	Yang et al., 2003
			Active site mutants do not affect anti-coagulant activity.	
Notechis 11'2	*Notechis scutatus scutatus*	Myotoxin	Expression in *E. coli* as a fusion protein	Hodgson et al., 1993
			Mutant M8L had no effect on catalysis or myotoxicity	
ACL myotoxin	*Agkistrodon contortrix laticinctus*	Myotoxin	Expression in *E. coli* as a fusion protein	Giuliani et al., 2001

(*Continued on next page*)

Table 1 (*continued*)

Phospholipase A_2	Species	Activity	Comments	Referenece
(Lys49-PLA_2)				
Bothropstoxin-I	*Bothrops jararacussu*	Myotoxin	Expression in *E. coli*, refolded from inclusion bodies.	Ward et al., 2001
(Lys49-PLA_2)			Active site mutants do not influence myotoxic activity	Ward et al., 2002
			Mutagenesis in the C-terminal loop region influences myotoxic activity	Chioato et al., 2002

involved in protein/protein interactions (Bogan and Thorn, 1998; Raffa, 2002) and preliminary results have shown that this strategy is equally successful in the study of the myotoxic and membrane damaging activities of the Lys49-PLA_2 (Chioato et al., 2002).

We have extended the SAVANA analysis to predict residues that may be determinants of pre-synaptic neurotoxicity using the ammodytoxin A from *Vipera a. ammodytes* (Pungercar et al., 1991) as the reference sequence for the database searches (see Fig. 1). Pre-synaptic neurotoxic PLA_2s have been extensively studied, and the influence of site-directed mutants on the neurotoxic activity of ammodytoxin A has been reported (Pungercar et al., 1999; Prijatelj et al., 2000; 2002; Ivanovski et al., 2000; Petan et al., 2002). The three-dimensional structure of ammodytoxin A has not been experimentally determined, therefore the surface mapping representation of the SAVANA analysis of this protein is presented in Fig. 2 using a molecular model of ammodytoxin A (SWISSPROT Acc. No: PA2A_VIPAA, Pungercar et al., 1991a) as generated by the program MODELLER (Sali et al., 1995) using the structure of the presynaptic neurotoxic PLA_2 from *Daboia russelli pulchella* (PDB code 1FB2, Chandra et al., 2001) as the protein structure template.

In contrast to the Lys49-PLA_2 myotoxins, the presynaptic neurotoxinic PLA_2s show a more extensive biologically active surface that extends from the C-terminal loop region through the calcium binding loop and short helical turn and includes positions in the N-terminal helix and β-wing (see Fig. 2). A previous prediction based on the visual analysis of a manual amino acid sequence alignment suggested that residues 80–110 on helix 3 were involved in the structural determinant of neurotoxicity (Kini and Iwanaga, 1986b; Tsai et al., 1987). However, the current analysis clearly demonstrates that the protein surface involved in this activity is more extensive, and comparison of those regions of the ammodytoxin A molecule identified in the SAVANA with the results site directed mutagenesis experiments reveals a strong correlation. For example, position 24 is located on the short helix (Fig. 2) scores highly in SAVANA, and has been demonstrated by site directed mutagenesis to be a critical residue involved in neurotoxic activity (Petan et al., 2002). Furthermore, many of the positions identified by SAVANA and mapped by site directed mutagenesis are located on the i-face of the protein, and not only modulate neurotoxicity but also may result in alterations in the catalytic properties of these PLA_2s (Prijatelj et al., 2000, 2002; Ivanovski et al., 2000; Petan et al., 2002), perhaps as a consequence of the modification of interfacial binding properties. Finally, comparison of the surface maps in Fig. 2 reveals that the C-terminal loop region is a structural determinant of both presynaptic neurotoxic and myotoxic activities. This is a highly significant result, since it suggests that a shared structural motif may explain the mixed neurotoxic and myotoxic activities observed in many of $vPLA_2$s.

Site directed mutagenesis has recently been used to probe the structural determinants of the anti-coagulant effect induced by $vPLA_2$s, and we have compared the available mutagenesis data with the results of SAVANA. The sequence of the basic PLA_2 from *Gloydius halys Pallas* (previously *Agkistrodon halys Pallas*; Pan et al., 1998) was used to search the amino acid database, and Fig. 2 presents the surface mapping representation of the analysis using the structure of the basic anti-coagulant PLA_2 from *Gloydius halys Pallas* (PDB code; 1B4W, Zhao et al., 1998, SWISSPROT; PA24_AGKHP, Pan et al., 1998) as the protein structure template. The results reveal that a cluster of positions between 71 and 95 at the base of the beta wing and residues 115–133 in the C-terminal loop region may be important structural determinants of the anticoagulant activity.

A previous visual analysis of a manual amino acid sequence alignment predicted that residues at 55–77 at the end of helix 2 and the first strand of the β-wing define a region that is the anticoagulant determinant (Kini and Evans, 1987), and this prediction therefore correlates well with the results of the current computer-based analysis. Furthermore, the results of site directed mutagenesis experiments are in good agreement with these predictions, which have identified positions 72 and 77 as significant determinants of activity (positions 67 and 70 using the numbering scheme adopted by the authors (Zhong et al., 2001), together with residues in the C-terminal loop region

(Liu et al., 2001). As is the case in the pre-synaptic neurotoxins, several of the positions identified as anticoagulant determinants are located on the i-face of the protein, and site directed mutagenesis in these regions reduce both the catalytic and anticoagulant activities (Liu et al., 2001). However, these studies show no clear correlation between lipid hydrolysis and anticoagulant potency, which suggests a more subtle interplay between the two activities. Indeed, the regulation of prothrombinase activity by human secreted group IIA PLA_2 involves both catalytic and non-catalytic mechanisms, and position 56 is a structural determinant for the non-catalytic mechanism (Inada et al., 1994).

6. Interpretation of surface mapping predictions

Comparative analyses of amino acid sequences make the inherent assumption that the PLA_2s used in the sequence alignment not only show the same biological function (e.g. myotoxicity), but also have the same mechanism of action (e.g. association with the same acceptor). However, the correlation of structure with function may not be as straightforward as is it might first appear. For example, myotoxic PLA_2s as a group include toxins which act at the pre-synaptic membrane of the neuromuscular junction (Fatehi et al., 1994) that may be distinct from the Lys49-PLA_2 mechanism of action, and which may be have a discrete structural determinant. Furthermore, the Lys49-PLA_2 myotoxins demonstrate various additional biological effects (see Section 2), and so caution should be exercised when assigning a surface feature as a structural determinant of a given biological function. In a final example, neurotoxic PLA_2s as a group have been shown to target diverse membrane acceptors (Krizaj et al., 1994; 1997), which raises the question as to whether a unique 'neurotoxic' structural determinant exists.

These considerations pose challenges for the reliable interpretation of comparative analyses of amino acid sequences that are selected and grouped according to biological function, and raise the question as to how can information with respect to the structural determinants of a given function be derived from grouped protein sequences. The answer to this problem lies in the careful selection of sequences, and the use of adequate bioinformatics tools to search and analyze the protein sequence databases. Predictions derived from these studies may serve to focus site-directed mutagenesis experiments on specific residues or to indicate regions on the protein surface that are of potential interest. In the preceeding sections we have described how Lys49-PLA_2 amino acid sequences may be analyzed, which highlights the strengths and weaknesses of computer based analyses. A sequence analysis can be used to make predictions, but the ultimate proof of the involvement of a surface region as a determinant of biological activity is experimental evidence derived from site directed mutagenesis.

7. Conclusions and perspectives

Computer-based structural prediction in conjunction with site directed mutagenesis is proving to be a powerful combination for the mapping of protein surfaces. Although no single computer program has proven to be adequate to extract all the information from a sequence alignment, the use of several programs in conjunction, each of which analyzes the same data in a different way, is proving to be an effective strategy for sequence analysis. Comparisons of computer predictions with site-directed mutagenesis studies provide insights as to the structural bases of pharmacological effects of $vPLA_2$s, and highlight some basic concepts that are useful to understand the relation between structure and function of these proteins.

The weight of evidence suggests that a given pharmacological activity is determined by a defined surface region on the $vPLA_2$, which may reflect the conservation of a specific protein surface topology for a specific receptor. This is in accord with the concept of a surface 'hot-spot', which is a localized region on a protein surface that determines a specific protein/protein interaction (Bogan and Thorn, 1998). Studies with $vPLA_2$ isoforms indicate that amino acid substitutions within the surface regions of likely to alter the affinity for these receptors, and thereby modulate the activity of any given PLA_2. Since the surface topology of a protein determines the structural basis of a given biological activity, overlap between the surface regions that determine specific activities will give rise to PLA_2s with mixed pharmacological properties. Since PLA_2s are relatively small proteins, the surface area is limited and therefore overlap between structural determinants of different pharmacological activities is likely to be a frequent event, and may be the structural basis that underlies the rich diversity of $vPLA_2$ effects. SAVANA results indicate a significant variation in the areas of the protein surfaces that contribute to the structural determinants of biological activities. This may reflect the intrinsic variations of the interactions of different $vPLA_2$s with their specific receptors, and is in accord with the wide range of buried protein surface areas observed for different protein/protein interactions (Jones and Thornton, 1996).

The study of $vPLA_2$s has found applications in the production and improvement of anti-venoms, and computer prediction together with surface mapping may provide a useful strategy for the selection or improvement of venom components that are used as antigens in this process. More recently, the focus of $vPLA_2$ research has shifted to the identification and characterization of protein/ligand interactions, and the study of the mechanism of action of $vPLA_2$s has led to the identification of many cell surface proteins which act as acceptors for endogenous secreted mammalian PLA_2, providing important insights as to the molecular and cellular biology of this important class of regulatory proteins. Furthermore, in recent years the complexity of the regulation of $vPLA_2$ activity through interaction with

specific protein inhibitors has become apparent, and the techniques described here may be applied to study the structural bases of the interaction between PLA_2s and these novel inhibitors. Such insights are fundamental in the design of novel PLA_2 inhibitors with improved specificities.

Acknowledgements

We are grateful to Dr Jerônimo Ruiz de Conceição for help with the protein modeling. The financial support of FAPESP genome program (SmolBNet Proc. No. 01/07537-2), FAPESP Proc. No. 02/04367-1 and CNPq is acknowledged. LC is the recipient of FAPESP doctorate fellowship 01/00279-8.

References

Adamich, M., Dennis, E.A., 1978. Specificity reversal in phospholipase A_2 hydrolysis of lipid mixtures. Biochem. Biophys. Res. Commun. 80, 424.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.

Andrade, M.A., Casari, G., Sander, C., Valencia, A., 1997. Classification of protein families and detection of the determinant residues with an improved self-organizing map. Biol. Cybern. 76 (6), 441–450.

Arni, R.K., Ward, R.J., 1996. Phospholipase A_2–a structural review. Toxicon 34 (8), 827–841.

Arni, R.K., Ward, R.J., Cintra, A.C., Giglio, J.R., 1995. Crystallization and preliminary diffraction data of bothropstoxin I isolated from the venom of Bothrops jararacussu. Toxicon 33 (3), 383–386.

de Azevedo, W.F., Ward, R.J., Lombardi, F.R., Giglio, J.R., Fontes, M.R.M., 1997. Crystal structure of myotoxin-II: a myotoxic phospholipase A_2 homologue from Bothrops moojeni venom. Prot. Peptide Lett. 4, 329–334.

Batas, B., Chaudhuri, J.B., 1996. Improving protein refolding yields by minimizing aggregation. Ann. NY Acad. Sci. 782, 495–505.

Batas, B., Chaudhuri, J.B., 1999a. Inclusion body purification and protein refolding using microfiltration and size exclusion chromatography. J. Biotechnol. 68 (2–3), 149–158.

Batas, B., Chaudhuri, J.B., 1999b. Considerations of sample application and elution during size-exclusion chromatography-based protein refolding. J. Chromatogr. A 864 (2), 229–236.

Beitz, E., 2000. TEXshade: shading and labeling of multiple sequence alignments using LATEX2 epsilon. Bioinformatics 16 (2), 135–139.

Bekkers, A.C., Franken, P.A., van den Bergh, C.J., Verbakel, J.M., Verheij, H.M., de Haas, G.H., 1991. The use of genetic engineering to obtain efficient production of porcine pancreatic phospholipase A_2 by *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. 1089 (3), 345–351.

Berg, O.G., Gelb, M.H., Tsai, M.D., Jain, M.K., 2001. Interfacial enzymology: the secreted phospholipases A_2-paradigm. Chem. Rev. 101, 2613–2653.

van den Bergh, C.J., Slotboom, A.J., Verheij, H.M., de Haas, G.H., 1988. The role of aspartic acid-49 in the active site of phospholipase A_2. A site-specific mutagenesis study of porcine pancreatic phospholipase A_2 and the rationale of the enzymatic activity of [lysine49]phospholipase A_2 from Agkistrodon piscivorus piscivorus venom. Eur. J. Biochem. 176 (2), 353–357.

Bianco, I.D., Kelley, M.J., Crowl, R.M., Dennis, E.A., 1995. Identification of two specific lysines responsible for the inhibition of phospholipase A_2 by manoalide. Biochim. Biophys. Acta 1250 (2), 197–203.

Bogan, A.A., Thorn, K.S., 1998. Anatomy of hot spots in protein interfaces. J. Mol. Biol. 280 (1), 1–9.

Boilard, E., Bourgoin, S.G., Bernatchez, C., Surette, M.E., 2003. Identification of an autoantigen on the surface of apoptotic human T-cells as a new protein interacting with inflammatory group IIA phospholipase A_2. Blood (Epub ahead of print).

Brunie, S., Bolin, J., Gerwith, D., Sigler, P.B., 1985. The refined crystal structure of dimeric phospholipase A_2 at 2.5 Å. Access to a shielded catalytic site. J. Biol. Chem. 260, 9742–9749.

Casari, G., Sander, C., Valencia, A., 1995. A method to predict functional residues in proteins. Nat. Struct. Biol. 2 (2), 171–178.

de Castro, R.C., Landucci, E.C., Toyama, M.H., Giglio, J.R., Marangoni, S., De Nucci, G., Antunes, E., 2000. Leucocyte recruitment induced by type II phospholipases A(2) into the rat pleural cavity. Toxicon 38 (12), 1773–1785.

Chandra, V., Kaur, P., Jasti, J., Betzel, C., Singh, T.P., 2001. Regulation of catalytic function by molecular association: structure of phospholipase A_2 from Daboia russelli pulchella (DPLA2) at 1.9 A resolution. Acta Cryst. D Biol. Crystal 57 (Pt 12), 1793–1798.

Chang, C.C., Su, M.J., 1982. Presynaptic toxicity of the histidine-modified, phospholipase A_2-inactive, beta-bungarotoxin, crotoxin and notexin. Toxicon 20 (5), 895–905.

Chang, L.S., Wu, P.F., Chang, C.C., 1996a. cDNA sequence analysis and mutagenesis studies on the A chain of beta-bungarotoxin from Taiwan banded krait. J. Prot. Chem. 15 (8), 755–761.

Chang, L.S., Wu, P.F., Chang, C.C., 1996b. Expression of Taiwan banded krait phospholipase A_2 in *Escherichia coli*, a fully active enzyme generated by hydrolyzing with aminopeptidase. Biochem. Biophys. Res. Commun. 225 (3), 990–996.

Chang, L.S., Wu, P.F., Chang, C.C., 1996c. cDNA sequence analysis and expression of the a chain of beta-bungarotoxin from *Bungarus multicinctus* (Taiwan banded krait). Biochem. Biophys. Res. Commun. 221 (2), 328–332.

Chang, L.S., Chang, C.C., Wu, P.F., 1997. The structural and functional essentiality of the N-terminal alpha-helix in the phospholipase A_2 of the Taiwan banded krait. Biochem. Mol. Biol. Int. 41 (6), 1247–1253.

Chijiwa, T., Yamaguchi, Y., Ogawa, T., Deshimaru, M., Nobuhisa, I., Nakashima, K., Oda-Ueda, N., Fukumaki, Y., Hattori, S., Ohno, M., 2003. Interisland evolution of *Trimeresurus flavoviridis* venom phospholipase A(2) isozymes. J. Mol. Evol. 56 (3), 286–293.

Chioato, L., de Oiveira, A.H.C., Ruller, R., Sá, J.M., Ward, R.J., 2002. Distinct sites for myotoxic and membrane-damaging activities in the C-terminal region of a Lys49 phospholipase A_2. Biochem. J. 366, 971–976.

Cintra, A.C.O., Marangoni, S., Oliveira, B., Giglio, J.R., 1993. Bothropstoxin-I: amino acid sequence and function. J. Prot. Chem. 12, 57–64.

van Deenen, L.L.M., de Haas, G.H., 1963. The substrate specificity of phospholipase A_2. Biochem. Biophys. Acta 70, 538–553.

Dayhoff, M.O., Schawartz, R.M., Arcutt, 1978. A model of evolutionary change in proteins. In: Dayhoff, M.O., (Ed.), tlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington, DC, pp. 345–352.

Dennis, E.A., 1994. Diversity of group types, regulation, and function of phospholipase A_2. J. Biol. Chem. 269 (18), 13057–13060.

Dessen, A., 2000. Phospholipase A_2 enzymes: structural diversity in lipid messenger metabolism. Structure, r15–r22.

Dhillon, D.S., Condrea, E., Maraganore, J.M., Heinrikson, R.L., Benjamin, S., Rosenberg, P., 1987. Comparison of enzymatic and pharmacological activities of lysine-49 and aspartate-49 phospholipases A_2 from *Agkistrodon piscivorus* piscivorus snake venom. Biochem. Pharmacol. 36, 1723–1730.

Díaz-Oreiro, C., Gutiérrez, J.M., 1997. Chemical modification of histidine and lysine residues of myotoxic phospholipases A_2 isolated from *Bothrops asper* and *Bothrops godmani* snake venoms: effects on enzymatic and pharmacological properties. Toxicon 35 (2), 241–252.

Díaz, C., Gutiérrez, J.M., Lomonte, B., Gené, J.A., 1991. The effect of myotoxins isolated from Bothrops snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities. Biochem. Biophys. Acta 1070, 455–460.

Fatehi, M., Rowan, E.G., Harvey, A.L., Harris, J.B., 1994. The effects of five phospholipases A_2 from venom of king brown snake. *Pseudechis australis*, on nerve and muscle. Toxicon 32 (12), 1559–1572.

Faure, G., Harvey, A.L., Thomson, E., Saliou, B., Radvanyi, F., Bon, C., 1993. Comparison of crotoxin isoforms reveals that stability of complex plays a major role in its pharmacological action. Eur. J. Biochem. 214, 491–496.

Feng, D.F., Doolittle, R.F., 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. 25 (4), 351–360.

Fletcher, J.E., Jiang, M.S., 1998. Lys49 phospholipase A_2 myotoxins lyse cell cultures by two distinct mechanisms. Toxicon 36 (11), 1549–1555.

Forst, S., Weiss, J., Blackburn, P., Frangione, B., Goni, F., Elsbach, P., 1986. Amino acid sequence of a basic Agkistrodon halys blomhoffii phospholipase A_2. Possible role of NH_2-terminal lysines in action on phospholipids of *Escherichia coli*. Biochemistry 25 (15), 4309–4314.

Francis, B., Gutiérrez, J.M., Lomonte, B., Kaiser, I.I., 1991. Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A_2. Arch. Biochem. Biophys. 284, 352–359.

Fremont, D.H., Anderson, D.H., Wilson, I.A., Dennis, E.A., Xuong, N.H., 1993. Crystal structure of phospholipase A_2 from Indian cobra reveals a trimeric association. Proc. Natl Acad. Sci. USA 90 (1), 342–346.

Giuliani, C.D., Iemma, M.R., Bondioli, A.C., Souza, D.H., Ferreira, L.L., Amaral, A.C., Salvini, T.F., Selistre-de-Araujo, H.S., 2001. Expression of an active recombinant lysine 49 phospholipase A(2) myotoxin as a fusion protein in bacteria. Toxicon 39 (10), 1595–1600.

Gowda, V.T., Schmidt, J., Middlebrook, J.L., 1994. Primary sequence determination of the most basic myonecrotic phospholipase A_2 from the venom of *Vipera russelli*. Toxicon 32 (6), 665–673.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18 (15), 2714–2723.

Guo, Y.W., Chen, C.T., Chang, T.Y., 2001. Sequence Direct Submission, Accession number Q90W39,.

Gutiérrez, J.M., Lomonte, B., 1995. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. Toxicon 33 (11), 1405–1424.

Habermann, E., Breithaupt, H., 1978. Mini-review. The crotoxin complex—an example of biochemical and pharmacological protein complementation. Toxicon 16 (1), 19–30.

Han, S.K., Yoon, E.T., Scott, D.L., Sigler, P.B., Cho, W., 1997. Structural aspects of interfacial adsorption. A crystallographic and site-directed mutagenesis study of the phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. J. Biol. Chem. 272 (6), 3573–3582.

Hanasaki, K., Arita, H., 1999. Biological and pathological functions of phospholipase A(2) receptor. Arch. Biochem. Biophys. 372 (2), 215–223.

Harrison, R.A., Hasson, S., Bharati, K., 2002. Sequence direct submission. Acession number, P59171.

Higashino, K, 2002. Identification of a soluble form of phospholipase A_2 receptor as a circulation endogenous inhibitor for secretorry phospholipase A_2. J. Biol. Chem. 277 (16), 13583–13588.

Higgins, D.G., Bleasby, A.J., Fuchs, R., 1992. CLUSTAL V: improved software for multiple sequence alignment. CABIOS 8 (2), 189–191.

Hodgson, D., Gasparini, S., Drevet, P., Ducancel, F., Bouet, F., Boulain, J.C., Harris, J.B., Menez, A., 1993. Production of recombinant notechis II'2L, an enzymatically active mutant of a phospholipase A_2 from *Notechis scutatus* scutatus venom, as directly generated by cleavage of a fusion protein produced in *Escherichia coli*. Eur. J. Biochem. 212 (2), 441–446.

Inada, M., Crowl, R.M., Bekkers, A.C., Verheij, H., Weiss, J., 1994. Determinants of the inhibitory action of purified 14-kDa phospholipase A_2 on cell-free prothrombinase complex. J. Biol. Chem. 269 (42), 26338–26343.

Ivanovski, G., Copic, A., Krizaj, I., Gubensek, F., Pungercar, J., 2000. The amino acid region 115–119 of ammodytoxins plays an important role in neurotoxicity. Biochem. Biophys. Res. Comm. 276, 1229–1234.

Jones, S., Thornton, J.M., 1996. Principles of protein–protein interactions. Proc. Natl. Acad. Sci. USA 93, 13–20.

Kaiser, I.I., Gutiérrez, J.M., Plummer, D., Aird, S.D., Odell, G.V., 1990. The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. Arch. Biochem. Biophys. 278 (2), 319–325.

Kelley, M.J., Crowl, R.M., Dennis, E.A., 1992. Renaturation of cobra venom phospholipase A_2 expressed from a synthetic gene in *Escherichia coli*. Biochim. Biophys. Acta 1118 (2), 107–115.

Kini, R.M., Evans, H.J., 1987. Structure-function relationships of phospholipases. The anticoagulant region of phospholipases A_2. J. Biol. Chem. 262 (30), 14402–14407.

Kini, R.M., Evans, H.J., 1995. The role of enzymatic activity in inhibition of the extrinsic tenase complex by phospholipase A_2 isoenzymes from *Naja nigricollis* venom. Toxicon 33 (12), 1585–1590.

Kini, R.M., Iwanaga, S., 1986a. Structure–function relationships of phospholipases II: Charge density distribution and the myotoxicity of presynaptically neurotoxic phospholipases. Toxicon 24 (9), 895–905.

Kini, R.M., Iwanaga, S., 1986b. Structure–function relationships of phospholipases I: Prediction of presynaptic neurotoxicity. Toxicon 24 (6), 527–541.

Kini, R.M., Kawabata, S.I., Iwanaga, S., 1986. Comparison of amino terminal region of three isoenzymes of phospholipases A_2 (TFV PL-Ia, TFV PL-Ib, TFV PL-X) from *Trimeresurus flavoviridis* (habu snake) venom and the complete amino acid sequence of the basic phospholipase, TFV PL-X. Toxicon 24 (11-12), 1117–1129.

Kordis, D., Pungercar, J., Strukelj, B., Liang, N.S., Gubensek, F., 1990. Sequence of the cDNA coding for ammodytoxin B. Nucleic Acids Res. 18 (13), 4016.

Krizaj, I., Dolly, J.O., Gubensek, F., 1994. Identification of the neuronal acceptor in bovine cortex for ammodytoxin C, a presynaptically neurotoxic phospholipase A_2. Biochemistry 33 (46), 13938–13945.

Krizaj, I., Faure, G., Gubensek, F., Bon, C., 1997. Neurotoxic phospholipases A_2 ammodytoxin and crotoxin bind to distinct high-affinity protein acceptors in *Torpedo marmorata* electric organ. Biochemistry 36 (10), 2779–2787.

Kuo, K.W., Chen, Y.C., Chang, C.C., 1995. cDNA sequence analysis and expression of alpha-bungarotoxin from Taiwan banded krait *Bungarus multicinctus*. Biochem. Biophys. Res. Commun. 216 (3), 1088–1094.

Lambeau, G., Lazdunski, M., 1999. Receptors for a growing family of secreted phospholipases A_2. Tr. Pharm. Sci. 20 (4), 162–170.

Landucci, E.C., Castro, R.C., Pereira, M.F., Cintra, A.C., Giglio, J.R., Marangoni, S., Oliveira, B., Cirino, G., Antunes, E., De Nucci, G., 1998. Mast cell degranulation induced by two phospholipase A_2 homologues: dissociation between enzymatic and biological activities. Eur. J. Pharmacol. 343 (2–3), 257–263.

Landucci, E.C., de Castro, R.C., Toyama, M., Giglio, J.R., Marangoni, S., De Nucci, G., Antunes, E., 2000. Inflammatory oedema induced by the Lys-49 phospholipase A(2) homologue piratoxin-I in the rat and rabbit. Effect of polyanions and p-bromophenacylbromide. Biochem. Pharmacol. 59 (10), 1289–1294.

Lathrop, B.K., Burack, W.R., Biltonen, R.L., Rule, G.S., 1992. Expression of a group II phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus* in *Escherichia coli*: recovery and renaturation from bacterial inclusion bodies. Prot. Expr. Purif. 3 (6), 512–517.

Lefkowitz, L.J., Deems, R.A., Dennis, E.A., 1999. Expression of group IA phospholipase A_2 in *Pichia pastoris*: identification of a phosphatidylcholine activator site using site-directed mutagenesis. Biochemistry 38 (43), 14174–14184.

Liang, N.S., Pungercar, J., Krizaj, I., Strukelj, B., Gubensek, F., 1993. Expression of fully active ammodytoxin A, a potent presynaptically neurotoxic phospholipase A_2, in *Escherichia coli*. FEBS Lett. 334 (1), 55–59.

Lichtarge, O., Bourne, H.R., Cohen, F.E., 1996. An evolutionary trace method defines binding surfaces common to protein families. J. Mol. Biol. 257 (2), 342–358.

Liu, S.Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S., Kihara, H., 1990. Purification and amino acid sequence of basic protein II, a lysine-49-phospholipase A_2 with low activity, from *Trimeresurus flavoviridis* venom. J. Biochem. (Tokyo) 107 (3), 400–408.

Liu, C.S., Chen, J.M., Chang, C.H., Chen, S.W., Teng, C.M., Tsai, I.H., 1991. The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. Biochim. Biophys. Acta 1077 (3), 362–370.

Liu, X., Pan, H., Yang, G., Wu, X., Zhou, Y., 1999. Cloning, expression and biochemical characterization of a basic–acidic hybrid phospholipase A_2-II from *Agkistrodon halys Pallas*. Biochim. Biophys. Acta 1431 (1), 157–165.

Liu, X., Wu, X., Zhou, Y., 2001. Identification of key residues responsible for enzymatic and platelet-aggregation-inhibiting activities of acidic phospholipase A_2 from *Agkistrodon halys Pallas*. J. Nat. Toxins 10 (1), 43–55.

Lomonte, B., Moreno, E., Tarkowski, A., Hanson, L.A., Maccarama, M., 1994. Neutralizing interaction between heparin and myotoxin II, a Lys49 phospholipase A2 from *Bothrops asper* snake venom. Identification of a heparin-binding and cytolytic toxin region by the use of synthetic peptides and molecular modelling. J. Biol. Chem. 269, 29867–29873.

Maraganore, J.M., Heinrikson, R.L., 1986. The lysine-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. Relation of structure and function to other phospholipases A_2. J. Biol. Chem. 261 (11), 4797–4804.

Moura-da-Silva, A.M., Paine, M.J., Diniz, M.R., Theakston, R.D., Crampton, J.M., 1995. The molecular cloning of a phospholipase A_2 from *Bothrops jararacussu* snake venom: evolution of venom group II phospholipases A_2 may imply gene duplications. J. Mol. Evol. 41 (2), 174–179.

Murakami, M., Kudo, I., 2001. Diversity and regulatory functions of mammalian secretory phospholipases A_2. Adv. Immunol. 77, 163–194.

Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., Kudo, I., 1997. Regulatory functions of phospholipase A_2. Crit. Rev. Immunol. 17 (3–4), 225–283.

Ogawa, T., Oda, N., Nakashima, K., Sasaki, H., Hattori, M., Sakaki, Y., Kihara, H., Ohno, M., 1992. Unusually high conservation of untranslated sequences in cDNAs for Trimeresurus flavoviridis phospholipase A_2 isozymes. Proc. Natl. Acad. Sci. USA 89 (18), 8557–8561.

de Oliveira, A.H., Giglio, J.R., Andrião-Escarso, S.H., Ward, R.J., 2001. The effect of resonance energy homotransfer on the intrinsic tryptophan fluorescence emission of the bothropstoxin-I dimer. Biochem. Biophys. Res. Comm. 284 (4), 1011–1015.

Pan, F.M., Chao, S.C., Wu, S.H., Chang, W.C., Chiou, S.H., 1998. Characterization of phospholipase A_2 (PLA_2) from Taiwan Cobra: isoenzymes and their site-directed mutants. Biochem. Biophys. Res. Comm. 250 (1), 154–160.

Pan, F.M., Yeh, M.S., Chang, W.C., Hung, C.C., Chiou, S.H., 1994. Sequence analysis and expression of phospholipase A_2 from Taiwan cobra. Biochem. Biophys. Res. Comm. 199 (2), 969–976.

Páramo, L., Lomonte, B., Pizarro-Cerdá, J., Bengoechea, J.A., Gorvel, J.P., Moreno, P., 1998. Bactericidal activity of Lys-49 and Asp-49 myotoxic phospholipase A_2 from *Bothrops asper* snake venom: synthetic Lys49 myotoxin II-(115–129)-peptide

identifies its bactericidal region. Eur. J. Biochem. 253 (2), 452–461.

Pan, H., Liu, X.L., Ou-Yang, L.L., Yang, G.Z., Zhou, Y.C., Li, Z.P., Wu, X.F., 1998. Diversity of cDNAs encoding phospholipase A_2 from *Agkistrodon halys Pallas* venom, and its expression in *E. coli*. Toxicon 36 (8), 1155–1163.

Pescatori, M., Grasso, A., Rufini, S., 1998. Molecular cloning of a K49 PLA2-like myotoxin from the snake *Bothrops asper*, Direct Submission. Accession number AF109911_1,.

Petan, T., Krizaj, I., Gubensek, F., Pungercar, J., 2002. Phenylalanine-24 in the N-terminal region of ammodytoxins is important for both enzymatic activity and presynaptic toxicity. Biochem. J. 363, 353–358.

Pieterson, W.A., Vidal, J.C., Volwerk, J.J., de Haas, G.H., 1974. Zymogen-catalyzed hydrolysis of monomeric substrates and the presence of a recognition site for lipid–water interfaces in phospholipase A_2. Biochemistry 13 (7), 1455–1460.

Prijatelj, P., Copic, A., Krizaj, I., Gubensek, F., Pungercar, J., 2000. Charge reversal of ammodytoxin A, a phospholipase A_2-toxin, does not abolish its neurotoxicity. Biochem. J. 352, 251–255.

Prijatelj, P., Krizaj, I., Krajl, B., Gubensek, F., Pungercar, J., 2002. The C-terminal region of ammodytoxins is important but not sufficient for neurotoxicity. Eur. J. Biochem. 269, 5759–5764.

Pungercar, J., Kordis, D., Jerala, R., Trstenjak-Prebanda, M., Dolinar, M., Curin-Serbec, V., Komel, R., Gubensek, F., 1989. Amino acid sequence of ammodytoxin C as deduced from cDNA. Nucl. Acids Res. 17 (11), 4367.

Pungercar, J., Liang, N.S., Strukelj, B., Gubensek, F., 1990. Nucleotide sequence of a cDNA encoding ammodytin L. Nucl. Acids Res. 18 (15), 4601.

Pungercar, J., Kordis, D., Strukelj, B., Liang, N.S., Gubensek, F., 1991. Cloning and nucleotide sequence of a cDNA encoding ammodytoxin A, the most toxic phospholipase A_2 from the venom of long-nosed viper (*Vipera ammodytes*). Toxicon 29 (2), 269–273.

Pungercar, J., Krizaj, I., Liang, N.S., Gubensek, F., 1999. An aromatic, but not a basic, residue involved in the toxicity of group-II phospholipase A_2 neurotoxins. Biochem. J. 341, 139–145.

Ramirez, F., Jain, M.K., 1991. Phospholipase A_2 at the bilayer interface. Proteins 9 (4), 229–239.

Raffa, R., 2002. Harmonic mean relationship between affinity for wold-type receptors and alanine-scan mutants. J. Theor. Biol. 218, 207–214.

Rufini, S., Cesaroni, P., Desideri, R.F., Gubensek, F., Gutiérrez, J.M., Luly, P., Massoud, R., Morero, R., Pedersen, J.Z., 1992. Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. Biochemistry 31, 12424–12430.

Rufini, S., Cesaroni, M.P., Balestro, N., Luly, P., 1996. Proliferative effect of ammodytin L from the venom of *Vipera ammodytes* on 208F rat fibroblasts in culture. Biochem. J. 320 (Pt 2), 467–472.

Sali, A., Potterton, L., Yuan, F., van Vlijmen, H., Karplus, M., 1995. Evaluation of comparative protein modeling by MODELLER. Proteins 23 (3), 318–326.

Scott, D.L., Sigler, P.B., 1994. Structure and catalytic mechanism of secretory phospholipases A_2. Adv. Protein Chem. 45, 53–58.

Selistre de Araujo, H.S., White, S.P., Ownby, C.L., 1996. Sequence analysis of Lys49 phospholipase A_2 myotoxins: a highly conserved class of proteins. Toxicon 34 (11/12), 1237–1242.

da Silva Giotto, M.T., Garratt, R.C., Oliva, G., Mascarenhas, Y.P., Giglio, J.R., Cintra, A.C., de Azevedo, W.F., Arni, R.K., Ward, R.J., 1998. Crystallographic and spectroscopic characterization of a molecular hinge: conformational changes in bothropstoxin I, a dimeric Lys49-phospholipase A_2 homologue. Proteins 30 (4), 442–454.

Six, D.A., Dennis, E.A., 2000. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta 1488, 1–19.

Snitko, Y., Han, S.K., Lee, B.I., Cho, W., 1999. Differential interfacial and substrate binding modes of mammalian pancreatic phospholipases A_2: a comparison among human, bovine, and porcine enzymes. Biochemistry 38 (24), 7803–7810.

Soares, A.M., Rodrigues, V.M., Homsi-Brandeburgo, M.I., Toyama, M.H., Lombardi, F.R., Arni, R.K., Giglio, J.R., 1998. A rapid procedure for the isolation of the Lys-49 myotoxin II from *Bothrops moojeni* (caissaca) venom: biochemical characterization, crystallization, myotoxic and edematogenic activity. Toxicon 36 (3), 503–514.

Soares, A.M., Andrião-Escarso, S.H., Angulo, Y., Lomonte, B., Gutiérrez, J.M., Marangoni, S., Toyama, M.H., Arni, R.K., Giglio, J.R., 2000a. Structural and functional characterization of myotoxin I, a Lys49 phospholipase A_2 homologue from *Bothrops moojeni* (Caissaca) snake venom. Arch. Biochem. Biophys. 373, 7–15.

Soares, A.M., Andriao-Escarso, S.H., Bortoleto, R.K., Rodrigues-Simioni, L., Arni, R.K., Gutiérrez, J.M., Giglio, J.R., 2001. Dissociation of enzymatic and pharmacological properties of piratoxins-I and -III, two myotoxic phospholipases A_2 from *Bothrops pirajai* snake venom. Arch. Biochem. Biophys. 387 (2), 188–196.

Soares, A.M., Guerra-Sá, R., Borja-Oliveira, C.R., Rodrigues, V.M., Rodrigues-Simioni, L., Rodrigues, V., Fontes, M.R.M., Lomonte, B., Gutiérrez, J.M., Giglio, J.R., 2000b. Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A_2 homologue from Bothrops neuwiedi pauloensis venom. Arch. Biochem. Biophys. 378, 201–209.

Sribar, J., Copic, A., Paris, A., Sherman, N.E., Gubensek, F., Fox, J.W., Krizaj, I., 2001. A high affinity acceptor for phospholipase A_2 with neurotoxic activity is a calmodulin. J. Biol. Chem. 276 (16), 12493–12496.

Sumandea, M., Das, S., Sumandea, C., Cho, W., 1999. Roles of aromatic residues in high interfacial activity of *Naja naja atra* phospholipase A_2. Biochemistry 38 (49), 16290–16297.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Toyama, M.H., Soares, A.M., Vieira, C.A., Novello, J.C., Oliveira, B., Giglio, J.R., Marangoni, S., 1998. Amino acid sequence of piratoxin-I, a myotoxin from *Bothrops pirajai* snake venom and its biological activity after alkylation with p-bromophenacyl bromide. J. Prot. Chem. 17, 713–718.

Toyama, M.H., Soares, A.M., Novello, J.C., Oliveira, B., Giglio, J.R., Marangoni, S., 2000. Amino acid sequence of piratoxin-II, a Lys49 phospholipase A_2 homologue, from *Bothrops pirajai* venom. Biochimie 82, 245–250.

Tsai, I.H., Liu, H.C., Chang, T., 1987. Toxicity domain in presynaptically toxic phospholipases A_2 of snakes venom. Biochim. Biophys. Acta. 6 (1), 94–99.

Tsai, I.H., Wang, Y.M., 1998. Effect of site directed mutagenesis on the activity of recombinant trimucrotoxin, a neurotoxic phospholipase from *Trimeresurus mucrosquamatus* venom. Toxicon 36 (11), 1591–1597.

Tsai, I.H., Chen, Y.H., Wang, Y.M., Tu, M.C., Tu, A.T., 2001. Purification, sequencing, and phylogenetic analyses of novel Lys-49 phospholipases A(2) from the venoms of rattlesnakes and other pit vipers. Arch. Biochem. Biophys. 394 (2), 236–244.

Valentin, E., Lambeau, G., 2000. Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. Biochim. Biophys. Acta 1488 (1-2), 59–70.

Ward, R.J., de Azevedo, W.F. Jr, Arni, R.K., 1998a. At the interface: crystal structures of phospholipases A_2. Toxicon 36 (11), 1623–1633.

Ward, R.J., Rodrigues Alves, A., Rugiero Neto, J., Arni, R.K., Casari, J., 1998b. A SequenceSpace analysis of Lys49 phospholipases A_2: clues towards identification of residues involved in a novel mechanism of membrane damage and in myotoxicity. Prot. Engng. 11, 285–294.

Ward, R.J., de Oliveira, A.H., Bortoleto, R.K., Rosa, J.C., Faca, V.M., Greene, L.J., 2001. Refolding and purification of Bothropstoxin-I, a Lys49-phospholipase A_2 homologue, expressed as inclusion bodies in *Escherichia coli*. Prot. Expr. Purif. 21 (1), 134–140.

Ward, R.J., Chioato, L., de Oliveira, A.H., Ruller, R., Sa, J.M., 2002. Active-site mutagenesis of a Lys49-phospholipase A_2: biological and membrane-disrupting activities in the absence of catalysis. Biochem. J. 362, 89–96.

Yamaguchi, Y., Shimohigashi, Y., Chijiwa, T., Nakai, M., Ogawa, T., Hattori, S., Ohno, M., 2001. Characterization, amino acid sequence and evolution of edema-inducing, basic phospholipase A_2 from *Trimeresurus flavoviridis* venom. Toxicon 39 (7), 1069–1076.

Yang, W.L., Peng, L.S., Zhong, X.F., Wei, J.W., Jiang, X.Y., Ye, L.T., Zou, L., Tu, H.B., Wu, W.Y., Xu, A., 2003. Functional expression and characterization of a recombinant phospholipase A_2 from sea snake *Lapemis hardwickii* as a soluble protein in *E. coli*. Toxicon 41 (6), 713–721.

Yu, B.Z., Poi, M.J., Ramagopal, U.A., Jain, R., Ramakumar, S., Berg, O.G., Tsai, M.D., Sekar, K., Jain, M.K., 2000. Structural basis of the anionic interface preference and kcat* activation of pancreatic phospholipase A_2. Biochemistry 39 (40), 12312–12323.

Yuan, C., Tsai, M., 1999. Pancreatic phospholipase A(2): new views on old issues. Biochim. Biophys. Acta 1441 (2-3), 215–222.

Zhao, K., Song, S., Lin, Z., Zhou, Y., 1998. Structure of a basic phospholipase A_2 from Agkistrodon halys Pallas at 2.13 Å resolution. Acta Cryst. D Biol. Cryst. 54 (Pt 4), 510–521.

Zhong, X., Liu, J., Wu, X., Zhou, Y., 2001. Expression, purification and biochemical characterization of a recombinant phospholipase A_2, with anticoagulant activity from *Agkistrodon halys Pallas*. J. Nat. Toxins 10 (1), 17–25.