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Search for a "toxic site" in snake venom phospholipases A_2*

Localización de un "sitio tóxico" en fosfolipasas A_2 de venenos de serpientes

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Secondary structure predictions on nine snake venom phospholipases A_2 by the Chou and Fasman and Cid *et al.* prediction methods, have led to the location of two possible "toxic sites", responsible for the neurotoxic and myotoxic action of the basic snake venom PLA_2, respectively. The accessibility to the neurotoxic site is blocked by the presence of a small helical structure (helix D in the bovine PLA_2). The role of lysine residues is found to be decisive in the venom's toxicity.

All presynaptic snake venom neurotoxins show a PLA_2 activity which has been related to their specific action. In general, they present multiple polypeptide chains, with different degrees of toxicity and, at least one of these chains is a PLA_2. They are neurotoxic, myotoxic, cardiotoxic or show anticoagulant activity. Their lethality is variable, and it has been found to be dependent on the basic character of the toxin: basic presynaptic neurotoxins, notexin and notechis II-5 and the myotoxin from *Enhydrina schistosa* present a high lethality (1, 2), on the contrary, neutral or acidic phospholipases from snake venoms present a scarce or null toxicity (3). On Table 1 are listed the characteristics of nine PLAs obtained from snake venoms used in the present study, together with those of the bovine pancreas PLA_2. All of them belong to Group I of PLA according to the classification of Heinrikson *et al.* (4).

Snake-venom phospholipases show great similarities among them and with the mammal's PLAs: all have about 120 amino acid residues and seven disulfide bridges; their sequences present about 50% of homology. A remarkable feature in the snake venom and mammal's PLA_2 is the invariant position of the 7 disulfide bonds (with the only exception of the β_1-bungarotoxin which has 6), which gives a rather rigid conformation to the molecule. It is then reasonable to assume that the coarse folding of the polypeptide chains of all snake phospholipases should be, not only similar among them, but also with that of PLA_2 obtained from mammals. Results from circular dichroism measurements performed in six PLAs obtained from Elapid snake's venoms indicate a high degree of similarity in their secondary structures (5).

Chemical modification of mammalian and snake PLAs with *p*-bromophenacyl bromide shows another close relationship between their structures. The modification of one histidine residue which caused the loss of phospholipasic activity in the pig PLA_2 (6) also produced the simultaneous loss of the phospholipasic and neurotoxic activities in the PLAs obtained from the snakes *Notechis scutatus scutatus, Bungarus multicintus, Naja naja atra, Naja nigricollis* and *Hemachatus haemachatus* (7-9).

However, the relationship between toxicity and catalytic action of the toxic PLAs is by no means established, mainly due to

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Abbreviations: PLA_2, phospholipase A_2

the lack of information on the tertiary structure of these presynaptic toxins. Only the crystalline structure of a dimeric PLA_2 from the venom of *Crotalus atrox* has been determined to 2.5 Å resolution, but the details of this structure are not sufficient to locate the sites responsible for toxicity (10).

In the absence of a detailed knowledge of the tertiary structure, the secondary structure could provide a better pattern to compare the snake enzymes among themselves and with the mammal phospholipases than the primary structure. The tertiary structures of bovine and pig pancreatic PLA_2 have been determined to a high resolution (11, 12). Thus, a comparison of the structural differences at the secondary structure level could lead first, to an explanation of the remarkable differences in specific action between snake venom and mammal PLAs in spite of the great structural conservation, second, to the location of the regions of the polypeptide chain responsible for the toxic action, and finally to a better comprehension of the links between the phospholipasic and the neurotoxic activities in the snake venom enzymes.

METHODS AND RESULTS

The reversible character of denaturation-renaturation experiments on proteins, is an indication that the nature and sequence of the amino acid residues determine to a great extent both the secondary and tertiary structures of these macromolecules (13). This fact is the central idea of a great number of methods devised to attempt a prediction of the secondary structure of proteins based on the information contained in their primary structures. Two methods, which have been reported to give 80% reliability have been used in this work; these are the Chou and Fasman's method and the method by Cid *et al.* (14, 15).

The Chou and Fasman's method is based on empirical probabilities: it defines conformational parameters, P_α, P_β and P_t, for each of the 20 natural amino acids, which represent the normalized frequency of occurrence of each amino acid residue in a particular type of secondary structure, as obtained from a data base of 29 fully determined protein structures. An average $\langle P_j \rangle$ greater than 1.0, obtained for a group of amino acids taken in sequence (6 for a helix, 5 for a β-strand and 4 for a β-turn) is an indication that the structure type j is likely to occur in that region of the sequence. A modification that replaces the average of the conformational parameters by their product, in order to improve the sensitivity of the method in the vicinity of the limit value 1.0 has been used in this work (16), as well as another modification that considers 4 conformational parameters for each amino acid residue in a turn structure (17).

The method by Cid *et al.* (15) combines physicochemical measurements of solubilities of amino acids in polar and non-polar solvents, with information obtained from a data base of 21 known protein structures (18). It gives the relative position of the polypeptide chain with respect to the protein surface, since it has been shown that a linear correlation exists between this distance and a hydrophobicity coefficient defined by Ponnuswamy *et al.* (18). The "hydrophobicity profile" of a protein is simply a plot of this coefficient $\langle H_f \rangle$, versus the amino acid number in the sequence. Four basic profiles have been defined for four types of secondary structure: helix, β-turn, buried and exposed β-strands (15). The identification of these patterns in the hydrophobicity profile of the protein yields the predicted secondary structure.

The prediction of the secondary structure was done independently by both methods. For a joint prediction, when discrepancies were found, the results from the hydrophobicity profiles' method were preferred, if the profile resembled precisely one of the four typical profiles described in (15), otherwise, the Chou and Fasman prediction was accepted.

The predicted secondary structures of all the snake venom PLA_2 listed in Table 1, compared to the experimentally determined secondary structure of the bovine pancreatic PLA_2, are schematically illustrated in Fig. 1. The predicted secondary structure of the mammalian phospholipase, compared to that determined from the tertiary structure, gave an estimation of the reliability of the method: 90% in this case (15).

A comparison of the hydrophobicity profiles allows the aligning of the sequences and a detailed differentiation of the secondary structures. On Fig. 2a are shown the profiles for all the PLA_2 listed in Table 1, up to amino acid 60, displaying the similarity of these profiles in this constant region. Fig. 2b shows the rest of the sequences aligned according to the shape of the profiles. The presence of important lysine residues are marked with an asterisk in the profiles.

In order to facilitate the comparison, a common numbering for all the sequences, as proposed in (5) but considering the bovine PLA_2 without deletions has been used in Figures 1 and 2.

DISCUSSION

The predicted secondary structures for the nine PLAs schematized in Fig. 1, indicate that the secondary structure is practically

TABLE 1

Some characteristics of 9 snake-venom PLAs and of bovine pancreas PLA_2

Phospholipase A_2	AA Residues	Character	Activity	LD_{50} μg/g mouse	Reference
1. Bovine pancreas PLA_2	123	Basic	Non toxic	–	
2. Notexin	119	Basic	Neurotoxic & Myotoxic	0.017[a]	(1)
3. Notechis II-5	119	Basic	Neurotoxic & Myotoxic	0.045[a]	(1)
4. Notechis II-1	119	Neutral	Non toxic	–	(1)
5. *Enhydrina schistosa* PLA_2	119	Basic	Myotoxic & Neurotoxic	0.040[a]	(28)
6. β_1-bungarotoxin (chain A)	120	Basic	Neurotoxic	0.020-0.030[b]	(23)
7. *Naja nigricollis* PLA_2	118	Basic	Moderately toxic	0.63[a]	(9)
8. *Naja naja atra* PLA_2	119	Acidic	Weakly toxic	8.6[a]	(9)
9. Taipoxin γ-subunit	125	Acidic	Non toxic	–	(29)
10. *Hemachatus haemachatus* PLA_2	119	Neutral	Weakly toxic	8.6[a]	(9)

a Intravenous.
b Intraperitoneal.

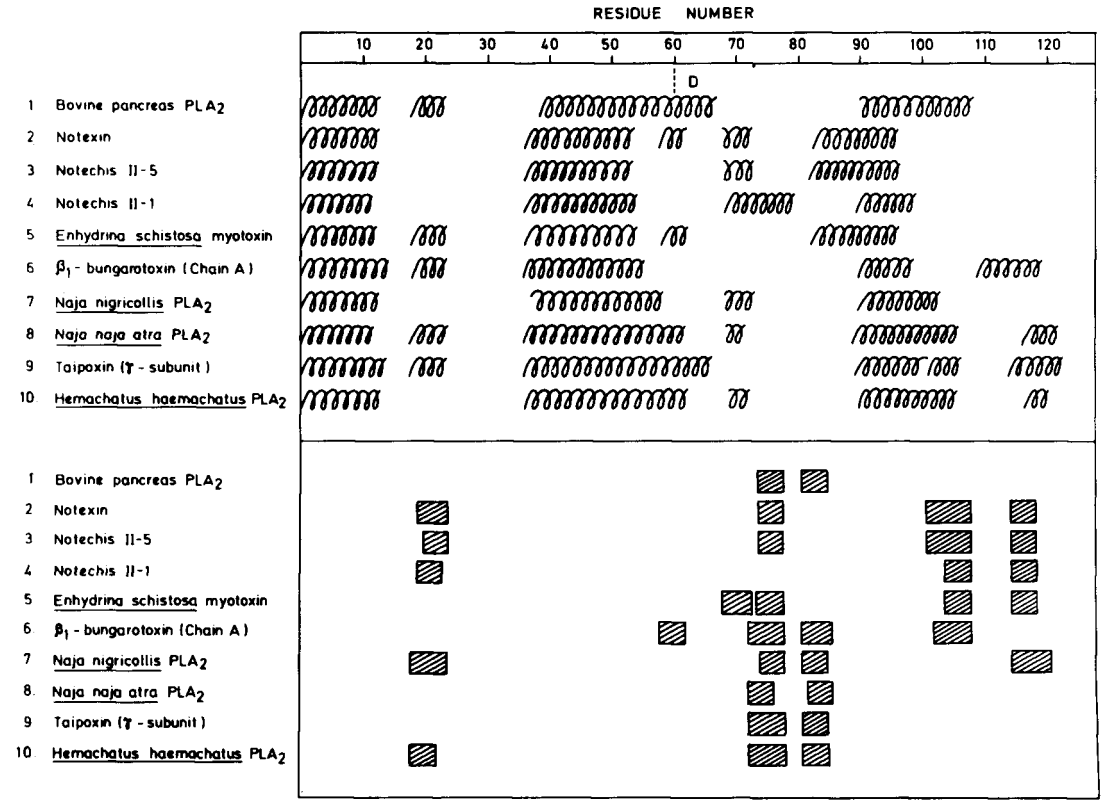


Fig. 1: Structural relationships between snake-venom and mammalian phospholipases. The predicted secondary structures of nine snake venom phospholipases A_2 are compared with the secondary structure of the bovine enzyme obtained from its known tertiary structure. Helical zones are represented by springs and regions of extended structure by stippled blocks. The position of helix D is marked in the bovine PLA_2 secondary structure.

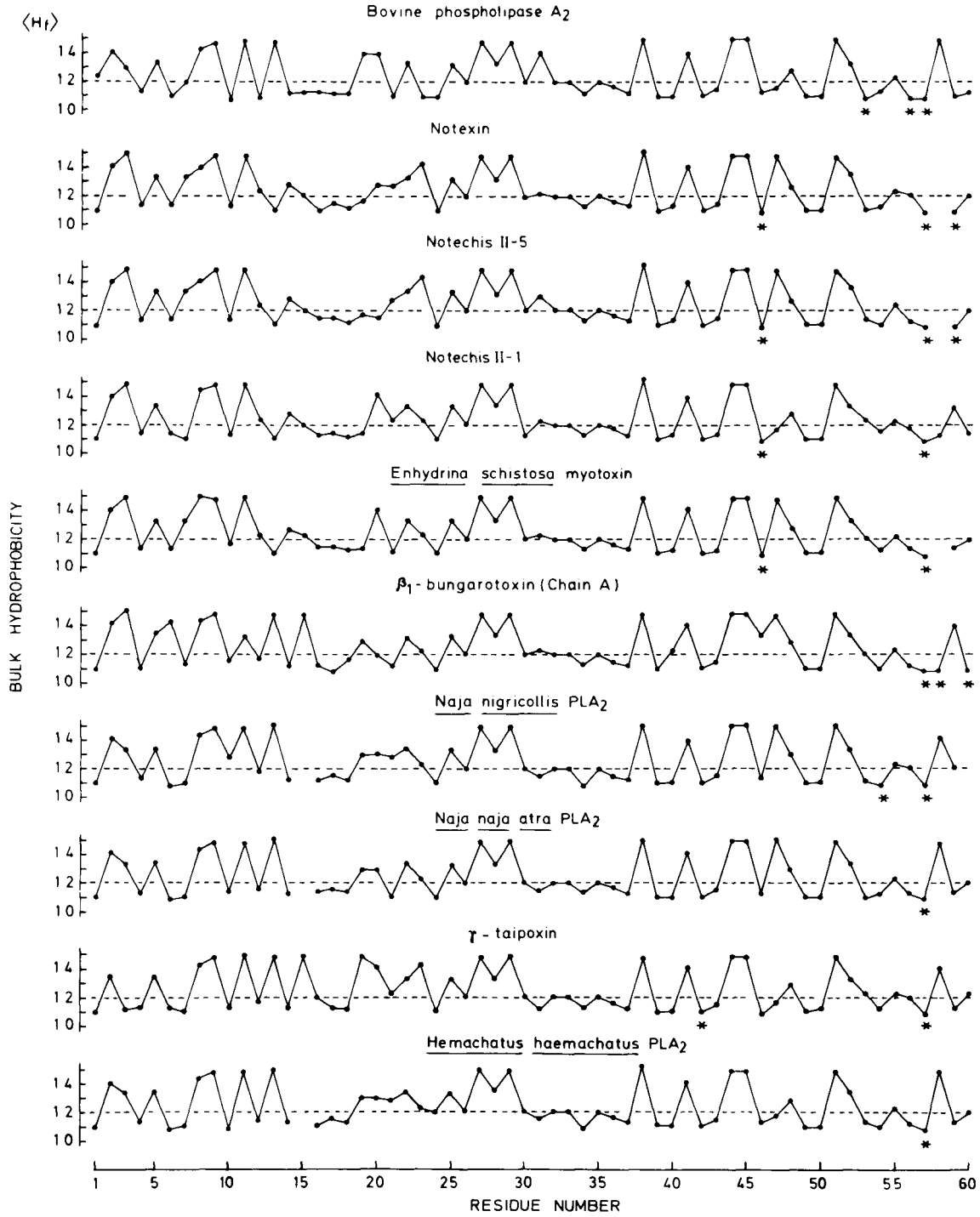
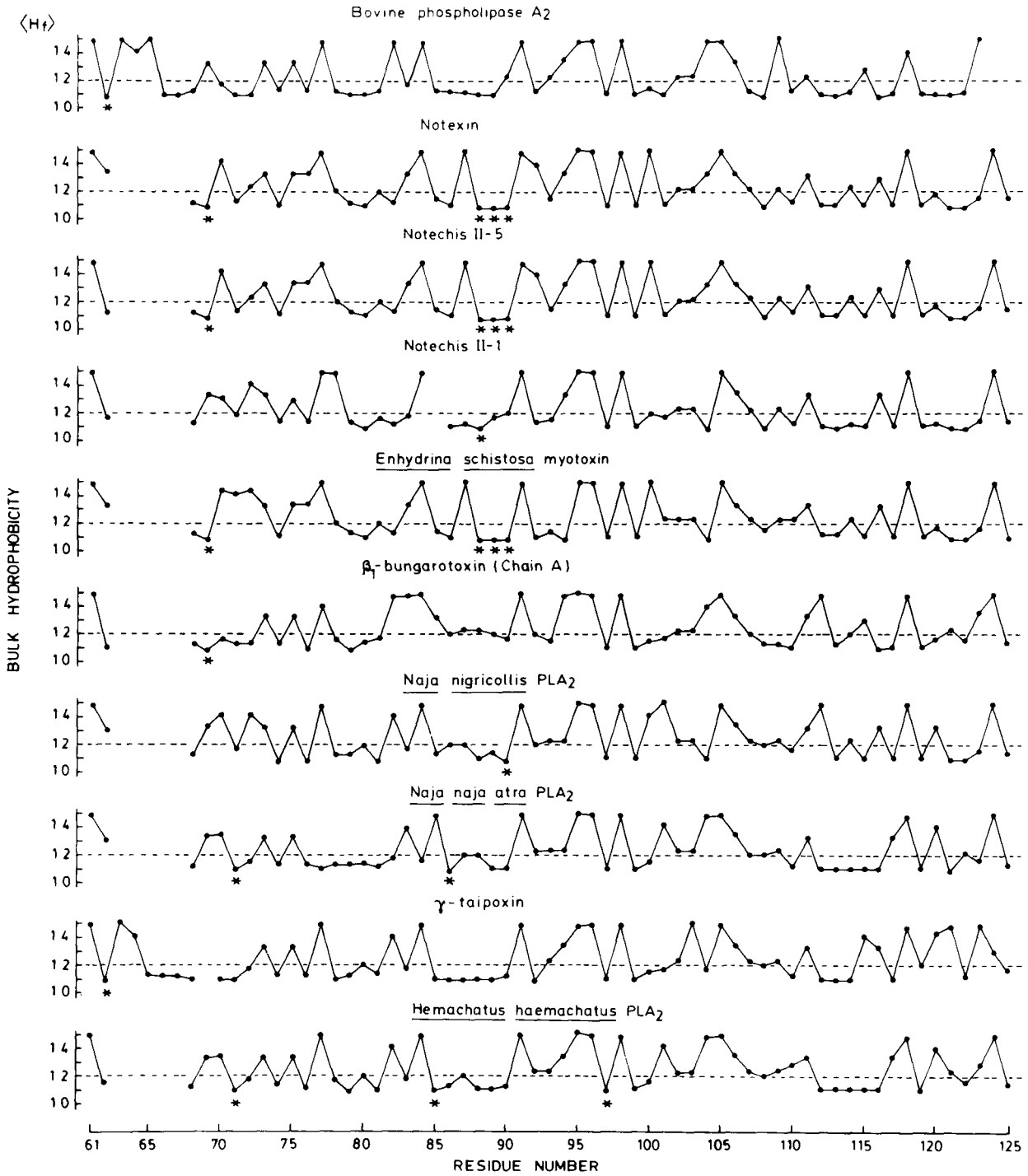


Fig. 2: Hydrophobicity profiles of bovine and snake-venom PLA_2 aligned to best fit. A five residue deletion after residue 61 is observed in the snake-venom PLA_2, with the only exception of γ-Taipoxin. The position of the lysine residues has been marked with an asterisk. Note the similarity of the profiles for the first 60 amino acid residues (Fig. 2a).


⟨Hf⟩
Bovine phospholipase A2
Notexin
Notechis II-5
Notechis II-1
Enhydrina schistosa myotoxin
β1-bungarotoxin (Chain A)
Naja nigricollis PLA2
Naja naja atra PLA2
γ-taipoxin
Hemachatus haemachatus PLA2
BULK HYDROPHOBICITY
14
12
10
61
65
70
75
80
85
90
95
100
105
110
115
120
125
RESIDUE NUMBER

invariant for the first 60 amino acid residues, a region which is involved in the phospholipasic activity of the enzymes (19, 20). If we compare these predictions to the schematic representation of the secondary structure of bovine PLA_2 as determined from X-ray diffraction methods- shown in Fig. 3 (redrawn from Fig. 3 of reference 5), helices A and B are present in all phospholipases studied, as well as the turn-turn zone stabilized by disulfide bridges corresponding to the amino acid sequence 22 to 35. The ligands of the Ca^{+2} ion, which enhances the phospholipasic activity, are located in this last invariant zone. After this zone, there is another helical region, that in mammal's phospholipases includes helices C and D (Fig. 3). In most of the phospholipases obtained from snake venoms, due to a deletion of 5 amino acid residues, helix D is absent, the only exception known so far being γ-taipoxin (5). In helix C is located His-48, an amino acid essential for the phospholipasic activity.

After amino acid residue 61, there are clear differences between the secondary structures determined for bovine PLA_2 and those predicted for the snake-venom enzymes (Figs. 1 and 2b). These differences are larger with the basic snake venom

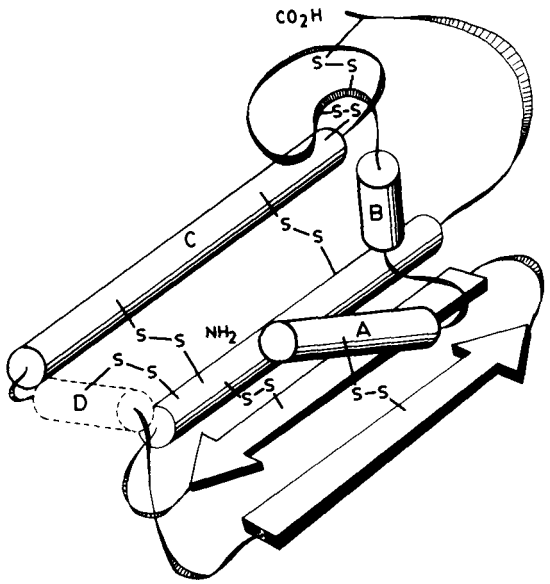


Fig. 3: Secondary structure of the bovine phospholipase A_2, in the cyllinder-and-arrow representation, redrawn from Fig. 3, reference (5). Note the location of helix D absent in most snake-venom phospholipases.

PLA_2 (row 2, 3, 6, 7) than with the acidic and neutral enzymes (rows 8 to 10). The importance of the basic residues in the phospholipasic activity has been confirmed by experiments that demonstrate that replacement of some amino acid residues by others of basic character enhances the

TABLE 2

Distribution of basic amino acid residues in two sites of the phospholipase sequences

Phospholipase A_2	60	90
Bovine pancreas PLA_2	K K L D S C K V L V	S S E N N A C E A F
Notexin	G K – K G C F P K M	R N I K K K C L R F
Notechis II-5	E K – K G C S P K M	R N I K K K C L R F
Notechis II-1	T K S Y S C T P Y W	– D S K T G C Q R F
Enhydrina schistosa PLA_2	E K – Q G C Y P K M	R N V K K K C N R K
β_1-bungarotoxin (chain A)	E K K H K C N P K T	Y G A A G T C G R I
Naja nigricollis PLA_2	G K M – G C W P Y L	S G G N S K C G A A
Naja naja atra PLA_2	E K I S G C W P Y F	K G G N N – C A A A
Taipoxin γ-subunit	G K L S A C K S V L	N D D N D E C K A F
Hemachatus haemachatus PLA_2	E K I S G C R P Y F	K E G N N E C A A F

Bars indicate deletions introduced to provide proper aligment of the sequences with respect to the position of the half-cystine residues. All snake venom PLA_2 with the exception of γ-taipoxin have a 5-residue deletion in this region after AA 63 with respecto to the bovine PLA_2, not indicated. The position of the lysine residues are shown by asterisks. The IUPAC one-letter notation for amino acids is used.

affinity of the enzyme for lipidic aggregates (21).

It has also been reported (3) that presynaptic neurotoxins from snake venoms are either basic PLA_2 or, at least contain one subunit that fullfils this requirement: for example, in the taipoxin complex, α-taipoxin is a basic phospholipase A whereas β and γ-taipoxin are neutral and acidic phospholipases respectively. Myonecrotic toxins are also basic PLAs. Table 2 shows a concentration of basic residues in the vicinity of positions 60 and 90 of the bovine PLA_2 sequence. In the first region, notexin and notechis II-5 have three lysines in a stretch of 7 amino acid residues; chain A of β_1-bungarotoxin has four lysines in the same region. The rest have one or two lysine residues, with the exception of bovine PLA_2 that has three. According to Table 1, these three toxins have the strongest neurotoxic character, between 30 to 300 fold higher than the rest.

It is interesting to analyze the toxicity and the presence of lysines in notexin, notechis II-5 and notechis II-1, three PLA_2 obtained from the venom of the same snake, *N. scutatus scutatus*. Two out of three lysines of the first site have been replaced by Tyr in notechis II-1, PLA which does not present neurotoxic or myotoxic activity (22). Another comparative study on proteins obtained from the same snake venom has been done on the bungarotoxins obtained from *B. multicintus* (23). The A chain from β_1-bungarotoxin (Table 2) differs from that of β_5-bungarotoxin in only 9 amino acid residues; of these, lysines 57, 58 and 60 have been replaced by Ala, Ile and Asp, respectively. The neurotoxic character of β_5-toxin is at least one order of magnitude smaller than that of β_1-bungarotoxin.

The second site which shows a cluster of Lys residues is in the vicinity of amino acid 90 (Table 2). Notexin, notechis II-5 and PLA_2 from *E. schistosa* present in this region a sequence of Lys-Lys-Lys, which is not present in any other of the PLAs studied here. It is interesting to point out that only these three enzymes present a strong myotoxic activity. Notechis II-1 has lost two of these lysine residues and does not present myotoxic activity.

The existence of a "toxic site" (defined as an affinity site to presynaptic membrane), different from the "phospholipasic site", has been demonstrated experimentally for the A chain of β-bungarotoxin: after the toxin was treated with ethoxyformic anhydride (which reacts with Lys and His residues), in the presence of dihexanoyl lecithin, it loses its toxic action but retains its phospholipasic activity (24). The complementary experiment was performed by Abe *et al.* (25) who showed that when β-bungarotoxin is modified with *p*-bromophenacyl bromide, it loses its phospholipasic activity without preventing its binding to the axon membrane.

Even though the enzymatic activity of snake venom PLAs is independent of the toxic action, the reverse is not true: studies of inhibition of acetylcholine release by the action of β-bungarotoxin, shows that when the toxin has been exposed to Sr^{2+} or has been modified with *p*-bromophenacyl bromide in order to suppress its enzymatic action, the inhibition is transient, contrary to what is observed with the native toxin, where the effect is irreversible.

A secondary structure study on 32 phospholipases A_2, 29 of them obtained from snake venom, has been done by Dufton *et al.* (5). The prediction method used is a modified version of the Chou and Fasman's method, and it was complemented with studies of circular dichroism and relative interface hydrophobicity. Their conclusion is that in all phospholipases the helical structure is predominant, and that there are fundamentally similar secondary and tertiary structures in all PLAs. The possibility that the snake venom enzymes could present β-strands in addition of those two described for the bovine PLA_2, is not mentioned; however, Fig. 4 of this reference shows that snake venom PLAs present a higher probability for β-structure than the mammalian PLAs. According to our prediction, snake venom phospholipases, specially the basic ones, present more zones with β-structure in the last part of their sequences, than the mammalian PLA (Figs. 1 and 2). It should be remember-

ed that the Chou and Fasman's method tends to overpredict helical zones, and that the CD method gives its best results also in the determination of helices. The role of helix D in the neurotoxicity of snake venom PLAs is also emphasized in reference (5).

A sequence alignment of seven snake-venom phospholipases, three neurotoxic and four non-toxic, made in (27), suggests that fifteen amino acid residues could have a role in the toxic properties of these PLA_2. Ten out of these fifteen residues are conserved only in the three neurotoxic PLAs. Among these residues are mentioned one lysine in the vicinity of position 60, and two out of the cluster of three lysines in the neighborhood of position 90, (see Table 2). Since the three neurotoxic phospholipases chosen by the authors are also myotoxic, no attempt is made to relate the residues mentioned with a specific function. It should be mentioned however that none of the ten conservative residues is present in the sequence of β_1-bungarotoxin (chain A), and only three of them (G 83, K 94 and A 118) are found in the *Naja nigricollis* PLA_2, two neurotoxic snake venom PLAs (Table 1).

In summary, we propose the following clues to explain the behaviour of snake venom PLAs, as compared to the non-toxic mammalian enzymes:

(1) The presynaptic neutoroxins must bind to the nerve terminal membrane by means of a special recognition site (24, 25). This site is not accessible when helix D (Fig. 3) is present, as it is in the mammalian PLA_2 (11, 12, 5). Sequence alignment (5) and the secondary structure prediction made in this work, show that helix D is absent from all snake venom PLAs, with the only exception of Taipoxin γ-subunit.

(2) The presence of the positively charged lysine residues plays a fundamental role in the neurotoxic properties of the snake venom PLAs. This fact may account for the observed lack of toxicity of some venom phospholipases which do not have such lysines, even though helix D is absent. This fact is more striking when the relation between toxicity and the replacement of lysines is observed in isoenzymes obtained from the same snake venom (22, 23).

(3) It is possible to identify two different sites related to the toxic action of snake venom PLA_2, belonging to group I (4); one of them, preferentially involved in the neurotoxic action would be located in the region 58 to 65 of the basic PLA_2 sequences; the second "toxic site" could be assigned to the region in the neighborhood of position 90 where three consecutive lysines occur in the sequences of the venom PLAs which present a myotoxic activity. Both sites coincide with the location of some of the fifteen residues that could have a role in the toxic properties of snake venom PLAs, mentioned in (27). The "neurotoxic site" proposed here would match part of the interface recognition site mentioned in (27).

(4) The "toxic sites" proposed here, are consistent with a several step toxic mechanism, where the attachment of the toxin to the membrane via a recognition site would be the first step. The phospholipasic activity could be involved in the toxic action as a subsequent step. The myotoxic activity, if present, could also contribute to enhance neurotoxicity.

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