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Elapid venom toxins: multiple recruitments of ancient scaffolds

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Nigroxins A and B, two myotoxic phospholipases A_2 (PLA_2s) from the venom of the American elapid *Micrurus nigrocinctus,* belong to a new PLA_2 subclass. Their primary structures were established and compared with those of PLA_2s that have already been studied with respect to myotoxic activity. The combination of amino acid residues Arg15, Ala100, Asn108 and a hydrophobic residue at position 109 is present exclusively in class I PLA_2s that display myotoxic activity. These residues cluster within a surface region rich in positive charges and are suggested to play a role in the interaction with the target membrane of the muscle fibers. It is concluded that the myotoxic PLA_2s resulted from recruitment of an ancient scaffold. Dendrotoxins and α-neurotoxins are similarly derived from other old structures, which are, however, now also present in nontoxic proteins that are widely distributed throughout the animal kingdom. The evolutionary pathways by which elapid PLA_2s acquired myotoxicity and dendrotoxins acquired K^+-channel blocker activity are traced. They demonstrate how existing scaffolds were adapted stepwise to serve toxic functions by exchange of a few surface-exposed residues.

Keywords: phospholipase A_2; protein evolution; protein family; structural comparisons; toxic site.

Snakes arose from lizards more than 100 million years ago, and some families of modern snakes, Elapidae, Viperidae and Colubridae, independently evolved venom systems [1]. Snake venoms are produced by specialized glands which have a common evolutionary origin with that of the salivary glands [2] but secrete toxic proteins which often target the neuromuscular system. Among these proteins are phospholipases A_2 (PLA_2s), K^+-channel blockers and α-neurotoxins of the three-finger-shape family. Questions arise about how the venom proteins acquired their toxic activities.

The PLA_2s catalyze Ca^{2+}-dependent hydrolysis of the fatty acyl ester bond at the *sn*-2 position of glycerophospholipids and are grouped into different classes depending on disulfide bridge positions and lengths of C-termini [3]. PLA_2s from elapid venoms are grouped into class I with those from pancreatic secretions, whereas PLA_2s from viperid venoms belong to class II with those from inflammatory exudates. The three-dimensional structures share a core of three α helices, a Ca^{2+}-binding site and a hydrophobic channel which binds the substrate fatty-acyl chains [4–6], but the snake venom PLA_2s display one or more toxic activities, such as cytotoxicity, myotoxicity, neurotoxicity, anticoagulant activity and hypotensive effects [7,8].

Dendrotoxins are polypeptides that are highly similar to Kunitz-type serine protease inhibitors which block the K^+ channels at nerve terminals, producing uncontrolled neurotransmitter release [9–11]. The site of interaction between dendrotoxin K and neuronal K^+ channels is formed by at least four residues, which cluster at the surface of the toxin molecule [12].

The three-finger-shape elapid toxins have a folding motif, with four invariant disulfides in a central globular core from which three β-sheet-rich loops emerge [13]. The CD59 membrane receptors, which protect mammalian cells from complement attack, have a similar three-dimensional structure [14]. Two classes of α-neurotoxins are distinguished, the 60/62-residue 'short-chain toxins', with four disulfide bonds, and the 70/74-residue 'long-chain toxins', with five disulfides [13]. The two α-neurotoxin classes bind to the nicotinic acetylcholine receptor (AchR) causing flaccid paralysis of striated muscles, and have a set of strictly conserved residues which are important for the interaction with the receptor [15].

We have now characterized the myotoxic activities and determined the primary structures of nigroxins A and B, two PLA_2s from the venom of the medically most important coral snake of Central America [16]. We identified a group of residues that are conserved among elapid venom PLA_2s associated with myotoxicity. We also traced the evolutionary processes in which myotoxic activity was acquired by these toxic enzymes. They resulted from the recruitment of a scaffold which already existed more than 700 million years ago, when nematodes diverged. Similarly, we found that dendrotoxins and α-neurotoxins originated from other old structures.

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Abbreviations: PLA_2, phospholipase A_2; AchR, acetylcholine receptor.

Enzymes: creatine kinase (EC 2.7.3.2); acetylcholinesterase (EC 3.1.1.7); endoprotease Glu-C (EC 3.4.21.19); endoprotease Lys-C (EC 3.4.21.50); endoprotease Asp-N (EC 3.4.24.33).

Note: The amino acid sequences of nigroxins A and B have been submitted to the SWISS-PROT database with accession numbers P81166 and P81167.

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MATERIALS AND METHODS

Venom and antibodies

Venoms from Central American coral snakes were from the serpentarium of the Instituto Clodomiro Picado. Those from South American coral snakes were supplied by Dr N. J. Da Silva (Universidade Catolica de Groias, Groiana, GO, Brazil),

textilotoxin was provided by Dr P. Mirschin (Venom Supplies, Tanunda, Australia) and notexin by Dr B. Persson (Uppsala University, Uppsala, Sweden). Other venoms and toxins were from Sigma.

Hybridomas were prepared using *Micrurus nigrocinctus nigrocinctus* venom as antigen [17]. MAbs produced in ascitic fluid in mice were purified by ion-exchange chromatography on DEAE-Sepharose. Polyclonal antibodies to nigroxin A were prepared in rabbits by subcutaneous injection of native toxin (12.5 mg) emulsified in Freund's complete adjuvant. Subsequent boosters with increasing amounts of native protein (25–50 mg) were given weekly, three in Freund's complete and eight in incomplete adjuvant. ELISAs were performed as described [16].

Protein purification and biological properties

M. n. nigrocinctus venom proteins (125 mg) in 1.5 mL 0.05 M Hepes buffer, pH 8, were fractionated on Mono S HR 16/10 to yield 64 fractions, screened for PLA_2 activity with a hemolytic assay [17] and for ELISA immunoreactivity with a mAb (9A). Immunoreactive fractions were separated at 1 $mL \cdot min^{-1}$ by reverse-phase HPLC on a Vydac C4 column (4.6 × 250 mm, 5 mm) with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. Material from different FPLC fractions was pooled and homogeneity was verified by capillary electrophoresis using a Beckman P/ACE 2100 instrument and by N-terminal amino acid sequence analysis. Protein concentrations were determined by amino acid analysis or the Coomassie blue method [18].

Enzymatic activity of the purified PLA_2s toward 1,2-diacyl-*sn*-glycero-3-phosphorylcholine was determined using the pH-stat method, and the released fatty acids were titrated using a Radiometer PHM 82 [19]. Myotoxicity was evaluated by measurement of creatine kinase activity in plasma at 1, 2, 3, 6 and 24 h after intramuscular injection of the purified PLA_2s (12 mg) in the gastrocnemius muscle of white Swiss–Webster mice. Creatine kinase activity was determined using a colorimetric assay (Sigma; kit 520). Mice were killed 24 h after injection and the muscle fixed and processed for histological examination of myonecrosis.

Structural analysis

Nigroxins A and B were reduced by incubation with 300 mM dithiothreitol for 2 h at 37 °C in 0.4 M Tris/HCl, pH 8.1, containing 6 M guanidine hydrochloride and 2 mM EDTA. Carboxymethylation was performed with iodo[^{14}C]acetate for 2 h at 37 °C. Amino acid compositions were determined using a Pharmacia Alpha Plus 4151 ninhydrin-based analyzer after hydrolysis at 110 °C for 24 h in evacuated tubes with 6 M HCl containing 0.5% (w/v) phenol. Molecular masses of the carboxymethylated nigroxins were determined by electrospray mass spectrometry using a VG AutoSpec instrument.

Samples of the carboxymethylated proteins were digested with CNBr, *Staphylococcus* Glu-C protease, *Achromobacter* Lys-C protease and *Pseudomonas* Asp-N protease. Enzymatic digestions were performed in 0.1 M ammonium bicarbonate for 4–20 h at 37 °C with protease to substrate ratios of 1 : 20–1 : 100. The resulting peptide mixtures were fractionated by reverse-phase HPLC on a Vydac C4 column, and peptide masses were determined by matrix-assisted laser desorption on a Finnigan MAT Lasermat 2000 instrument. Amino acid sequences were determined with an Applied Biosystems 477A or a MilliGen/Biosearch 6600 instrument, both with on-line phenylthiohydantoin analysis.

Sequence alignments, phylogenetic trees and molecular modelling

Amino acid sequences were aligned using the program CLUSTAL W [20] with a few gap adjustments. Phylogenetic trees were calculated with the same program and were drawn with PHYLO-WIN. Alignment figures were made with the program PRALIN. Three-dimensional models of nigroxin A and *Ophiophagus hannah* PLA_2 were built, using, respectively, the structures of *Naja naja* PLA_2 and the bovine pancreatic PLA_2 as templates, with the ICM program (version 2.7, 1997, Molsoft, Metuchen, NJ, USA). Surface potentials were calculated with the same program.

RESULTS AND DISCUSSION

Nigroxins A and B, a new subclass of myotoxic PLA_2

Nigroxins A and B were purified from crude *M. n. nigrocinctus* venom by a two-step procedure, using Mono S FPLC and reverse-phase HPLC. Several preparations were pooled, and homogeneity of the pooled material was verified by capillary electrophoresis and N-terminal sequence analysis [16]. The enzymatic activities of nigroxins A and B were 1367 ± 109 and 1590 ± 142 $mmol \cdot mg^{-1} \cdot min^{-1}$, respectively, as evaluated by fatty acid release from 1,2-diacyl-*sn*-glycero-3-phosphorylcholine. Both nigroxins A and B are myotoxic on intramuscular injection, as judged by increased creatine kinase activity in the plasma (Fig. 1) and histology of muscle samples after injection.

Polyclonal antibodies against nigroxin A also recognized nigroxins B, C1, C2 and C3, notexin and textilotoxin (Fig. 2A). The antibodies showed a weaker reaction with β-bungarotoxin and *Naja mocambique* PLA_2s, and did not recognize the porcine pancreatic PLA_2. This cross-reactivity pattern differs from those described for other elapid PLA_2s [21] and shows that nigroxin A belongs to a new antigenic PLA_2 subclass. The polyclonal antibodies to nigroxin A recognized venoms from several *Micrurus* species (Fig. 2B). Three mAbs (9A, 28 and 55) against *M. n. nigrocinctus* PLA_2s [17] reacted identically against nigroxins A and B, while three other mAbs (denoted as 4, 27 and 51) reacted more strongly with nigroxin A and two (7H and 21) with nigroxin B, indicating slight differences in the largely similar antigenic properties of these two PLA_2s.

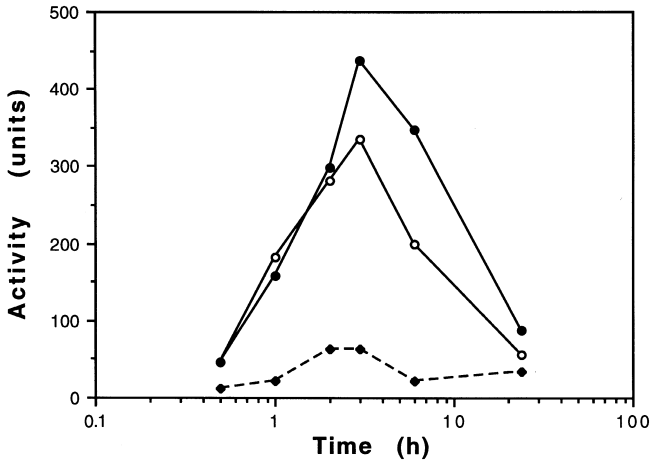

Fig. 1. Myotoxic activity of nigroxins A and B in mice. Time course of the increase in plasma creatine kinase activity after intramuscular injection of 12 μg of nigroxin A (–○–) or B (–●–) in 50 mL of phosphate-buffered saline. Control mice (--◆--) received buffer alone. Each point represents the mean of three determinations.

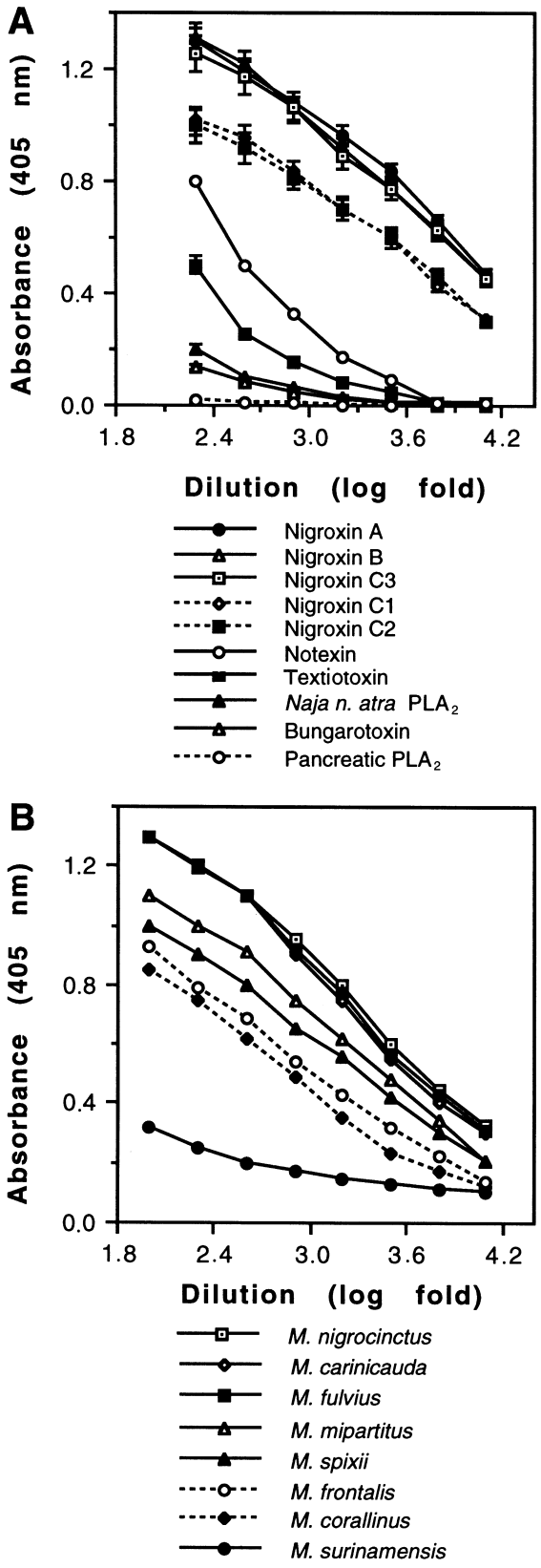

Fig. 2. ELISA cross-reactivities of nigroxin A-specific polyclonal antibodies with purified class I PLA_2s (A) or venoms from different *Micrurus* species (B). Microtiter wells were coated with 0.5 μg of the purified PLA_2 or 1 μg of venom, and incubated with different dilutions of the antibodies. Conjugate and substrate were added and A_{405} recorded 1 h later. Each point represents the mean of three determinations.

Direct sequencer degradation of carboxymethylated nigroxins A and B resulted in interpretable amino acid sequences covering close to half of the molecules. The remaining parts, to give the entire amino acid sequences (Fig. 3), were determined by peptide analysis after four types of cleavage of the carboxymethylated proteins and fragment isolation by reverse-phase HPLC. Electrospray MS of the carboxymethylated nigroxins showed ions corresponding to molecular masses of 14181.5 Da for nigroxin A and 14210.0 Da for nigroxin B, in agreement with the values calculated from the amino acid sequences, 14182.8 for nigroxin A and 14211.8 for nigroxin B.

Nigroxins A and B differ in primary structure at positions 3–5 only (Fig. 3) but have several unique features when compared with all known class I PLA_2s and constitute a new subclass, as shown by their separate grouping in phylogenetic trees (below). However, both are clearly class I PLA_2s, with seven disulfide bridges, an 'elapid loop' at positions 52–54, but no 'pancreatic loop' between positions 57 and 58, and with conserved residues in the catalytic network (H46, Y50 and D92), the Ca^{2+}-binding site (Y26, G28, G30 and D47) and the hydrophobic channel (L2, I9, W17 and Y66) [4]. Previously, class I PLA_2s have been identified only in snakes and mammals, but searching the Genpept database for nigroxin homologs, we now find that there is a protein from *Caenorhabditis elegans* (CEC07E3–3) [22] that has the same pattern of half-cystines and contains the key residues involved in the catalytic network, the Ca^{2+}-binding site and the hydrophobic channel. The existence of a PLA_2 in *Caenorhabditis* indicates that the PLA_2 scaffold already existed $\geq$ 700 million years ago, before the divergence of nematodes [23].

A cluster of surface-exposed residues associated with myotoxicity of class I PLA_2s

To trace the amino acid residues associated with myotoxicity, we aligned the sequences of nigroxins A and B with those of the class I PLA_2s that are known from histological studies to be myotoxic on intramuscular injection [24–27], and with those of the taipoxin γ chain (PA23_OXYSC), the β-bungarotoxin A chain (PA21_BUNMU) and two pancreatic PLA_2s (PA2_BOVIN; PA21_PIG), which are nonmyotoxic [25,28,29] (Fig. 4). A specific pattern of residue conservation was observed among myotoxic class I PLA_2s. It includes Arg15, Val88, Ala100 and Asn108. In addition, positions 83 and 109 are conserved in property, with a positively charged residue and a hydrophobic residue, respectively. Although none of these six residues plays a role in catalysis [4], five (Arg15, Val88, Ala100, Asn108 and hydrophobic residue 109) are remarkably conserved among PLA_2s from elapids which diverged $\geq$ 40 million years ago [1]. Their conservation suggests that they play a role in the myotoxic activity. Accordingly, the weak myotoxicity (as judged by the high dose required to produce myonecrosis) of the *O. hannah* PLA_2 [30] may be related to an Arg15Leu substitution, whereas the lack of myotoxicity of the taipoxin β chain [25] could be related, at least in part, to the presence of His108 and Ser109.

Nigroxin A was modeled into the PLA_2 fold to locate the spatial positions of the conserved residues associated with myotoxic activity. We found that Val88, located at the third α helix, makes contact with Ala53 and Pro61. These contacts probably have a structural importance in PLA_2s lacking the pancreatic loop, because Val88 is strictly conserved in these variants. The other four conserved positions (Arg15, Ala100, Asn108 and the hydrophobic residue at 109) cluster on the protein surface. Electrostatic potential calculations were performed for two structures and two models of class I PLA_2s: one nonmyotoxic (bovine pancreatic), a weakly myotoxic (*O. hannah* PLA_2) and

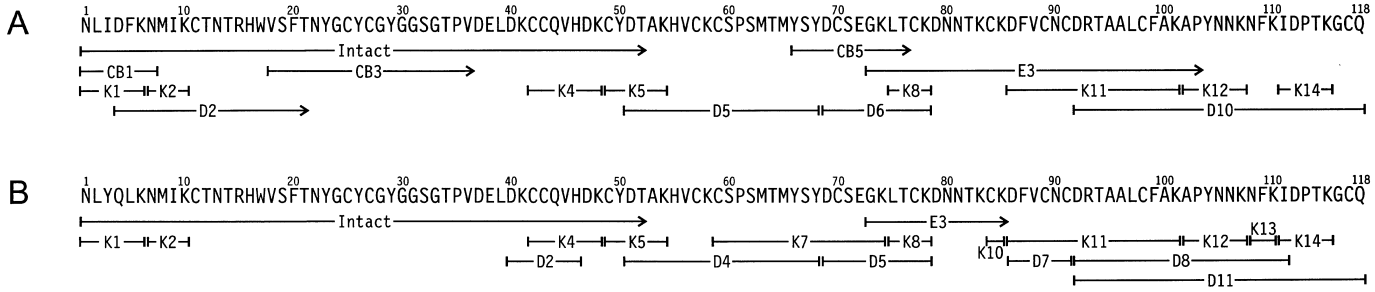

Fig. 3. Primary structures of nigroxins A (A) and B (B). Numbers above the sequences give residue positions in the enzyme. Peptides submitted to structural analysis are denoted by letter and number combinations, according to the type of cleavage: CB, CNBr; K, Lys-C protease; D, Asn-N protease; and E, Glu-C protease.

two potent myotoxins (nigroxin A and notexin). The potent myotoxic PLA_2s have an increased concentration of positive charges at the surface where the residues associated with myotoxicity are located (Fig. 5). Electrostatic forces between basic surface residues and negatively charged phospholipids are important for the interaction of proteins and lipid bilayers [31,32]. The association of conserved residues and a positively charged surface with myotoxic activity in the class I PLA_2s suggests that this part of the toxin molecule plays a role in the interaction with the target membrane of the muscle fibers. Supporting the importance of this region, chemical modification of Trp109 has been shown to reduce by about sevenfold the lethal effect of notexin in mice [33].

Although the molecular mechanisms involved in myotoxicity of snake venom PLA_2s are not fully understood [8,29], it has been assumed *a priori* that the toxic site is the same in PLA_2s from elapids and viperids [34,35]. The target of myotoxic PLA_2s from classes I and II is the plasma membrane [8,36], but the acceptor structure(s) are unknown. The toxin N- and C-terminal segments contribute to the myotoxic effect of the K49 class II PLA_2s [37,38], and several amino acid residues of these regions are conserved among those myotoxins [28]. However, these residues are not present in myotoxic class I PLA_2s and are not among those now identified as being associated with the myotoxic activity in class I PLA_2s (Fig. 4). Therefore, we conclude that (a) the structural determinants of myotoxicity are unique in each PLA_2 class and (b) the myotoxicity of classes I and II evolved independently and may involve binding to different acceptor structure(s) in the muscle cell membrane, although resulting in a similar series of pathological consequences. In agreement with this view, PLA_2s from classes I and II have been reported to interact differently with cultured muscle cells [7]. Hence, a convergent evolution of myotoxicity in class I and II venom PLA_2s appears likely, and this would explain why the prediction of a myotoxic site common to both classes [34] is not valid [8,27].

Myotoxic elapid venom PLA_2s evolved by recruitment of a pancreatic PLA_2-like ancestor

The existing evolutionary model for the secreted PLA_2s suggests that the class I PLA_2s appeared after the divergence of classes III and II [39]. This implies that the features typical of class I PLA_2s (the half-cystines at positions 11 and 70, and the pancreatic and elapid loops) are recent acquisitions [40]. However, the sequence of the *C. elegans* PLA_2 implies a different scenario. The *Caenorhabditis* PLA_2 has 14 Cys and 1 Gly at positions identical with those in the PLA_2s of classes I, II and III, which indicates that it has a three-dimensional structure similar to those of the secreted PLA_2s from higher organisms. This *Caenorhabditis* enzyme has Cys11 and Cys70 typical of class I PLA_2s and a C-terminal extension distinctive of the class II enzymes. Remarkably, it also has the pancreatic loop, characteristic of the mammalian class I PLA_2s, which indicates that all existing class I PLA_2s evolved from a pancreatic PLA_2-like ancestor.

The *Caenorhabditis* PLA_2 was used as an outgroup to derive an evolutionary tree (Fig. 6) from an alignment of 74 class I PLA_2s (available via anonymous ftp from ftp.mbb.ki.se). The phylogenetic relationships among other mammalian PLA_2 classes

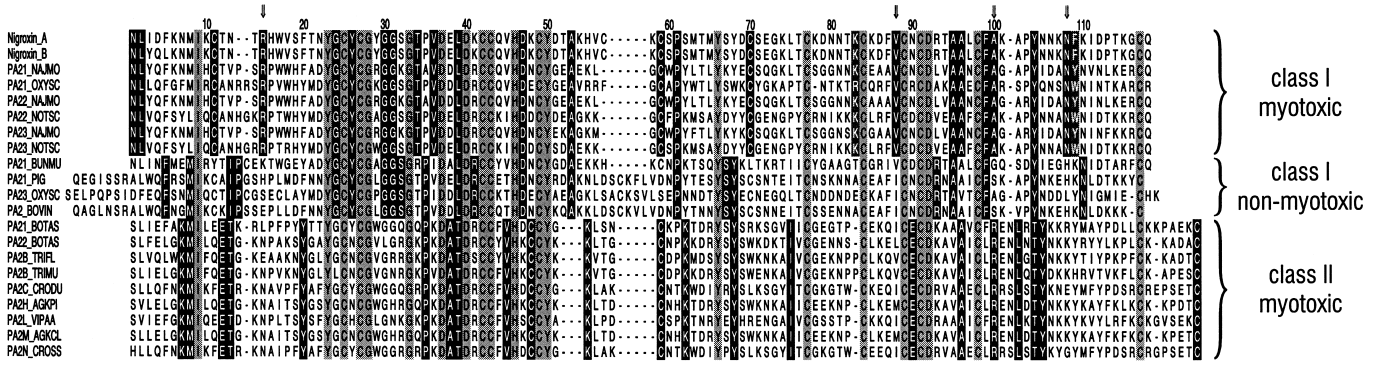

Fig. 4. Sequence alignment of nigroxins A and B with other PLA_2s that have previously had their myotoxic activity evaluated histologically. Accession codes in the Swissprot database are given on the left. The top eight sequences correspond to venom class I PLA_2s which are myotoxic, the following four sequences correspond to nonmyotoxic class I enzymes and the bottom nine sequences correspond to myotoxic class II PLA_2s. Residues conserved in all sequences are given against a light gray background and residues strictly conserved within each group are given against a black background. Positions with conserved properties in the myotoxic class I PLA_2s are given against a dark gray background. Numbers at the top refer to the nigroxin A sequence.

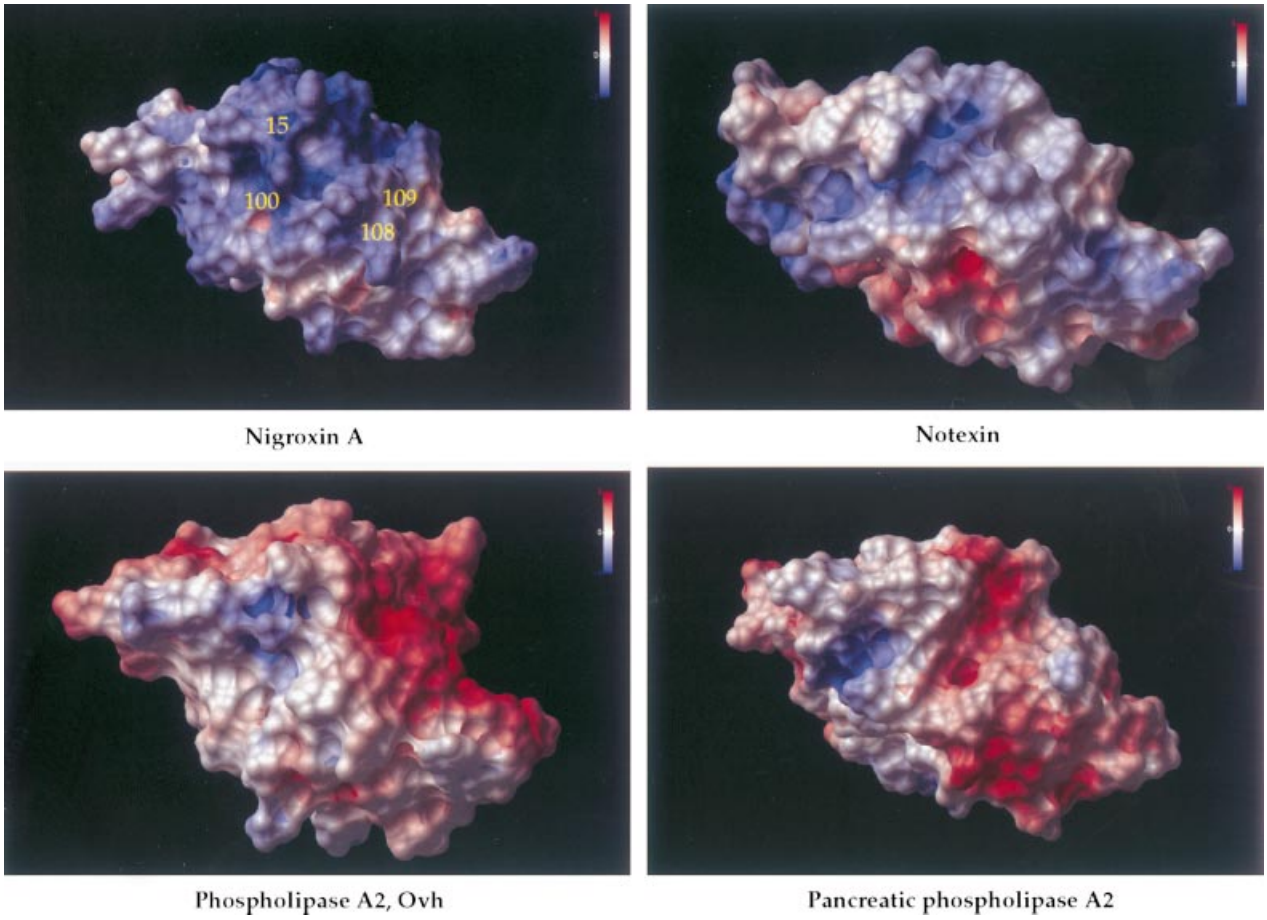

Fig. 5. Electrostatic potential map of the surface proximal to the residues associated with myotoxicity in class I PLA_2s. Surface potentials were calculated for the structures of bovine pancreatic phospholipase and notexin, and for the models of *O. hannah* PLA_2 and nigroxin A. Red, blue and white correspond to negatively charged, positively charged and uncharged regions, respectively. The maps were generated using the ICM program (version 2.7, Molsoft).

and the snake venom class II PLA_2s have been established [41] and therefore were not included in this study. The results show that the mammalian pancreatic PLA_2s and the elapid venom enzymes constitute two separate lineages from a common ancestor. We distinguish six divergent groups within the elapid venom PLA_2s: group IA-1, which has the N-terminal propeptide and the pancreatic loop; IA-2, which lacks the propeptide but retains the pancreatic loop; and IA-3 (from cobras), IA-4 (kraits), IA-5 (marine/Australian elapids) and IA-6 (American coral snakes), which all lack both the propeptide and the pancreatic loop. These phylogenetic relationships from the venom PLA_2s correlate with those from morphological criteria, which place cobras, kraits, marine/Australian elapids and American coral snakes in different taxonomic groups [1]. We conclude that the elapid PLA_2s of the subclasses IA-1 to IA-6 evolved from a pancreatic PLA_2-like precursor, and that the elapid enzymes in subclasses IA-1 and IA-2 are the vestiges of that evolutionary process.

Myotoxicity, previously detected in PLA_2s from Asian/African and Marine/Australian elapids (IA-3 and IA-5), is also present in PLA_2s from American coral snakes (IA-6). The myotoxic activity of these elapid venom PLA_2 lineages could have been acquired through convergent evolution or through a common precursor (during the Cretaceous, before the entire separation of the continents). The finding of a specific residue conservation pattern, associated with myotoxicity in divergent elapid PLA_2 lineages, indicates that the myotoxic activity was acquired by the PLA_2 of an ancestral elapid. The following events most likely occurred during the acquisition of myotoxicity by elapid venom PLA_2s (Fig. 7): (A) a nonmyotoxic pancreatic PLA_2-like ancestor was recruited in the evolving venom gland; (B) the nucleotides corresponding to Ala100 and a hydrophobic residue at position 109, present in all snake venom enzymes, were acquired; (C) a gene duplication occurred, and in one of the duplicates the nucleotides encoding the propeptide were lost while those corresponding to Asn108 were acquired; (D) a second gene duplication occurred, and in one of the duplicates, nucleotides corresponding to Arg15 and Val88 were acquired, while those encoding the pancreatic loop were lost. The elapid enzymes in subclasses IA-1 and IA-2 provide evidence for the gene duplications that occurred in steps (C) and (D), respectively. The importance of these events in the acquisition of myotoxicity is supported by several lines of evidence: the PLA_2s from pancreatic secretions are not myotoxic [28,29]; the taipoxin γ chain, which still has the propeptide and the pancreatic loop but lacks three of the residues associated with myotoxicity (Arg15, Val88, Asn108), is not myotoxic [25]; the *O. hannah* PLA_2, which retains the pancreatic loop but lacks two of the residues associated with myotoxicity (Arg15, Val88), has only weak myotoxic activity [30]; the rest of the fully myotoxic class I PLA_2s lack both the propeptide and the pancreatic loop.

As enzymatic activity is important for myotoxic activity of class I PLA_2s [24], the deletion of the propeptide, which led to a constitutively active PLA_2, was probably crucial in the evolution of myotoxicity. On the other hand, the loss of the pancreatic

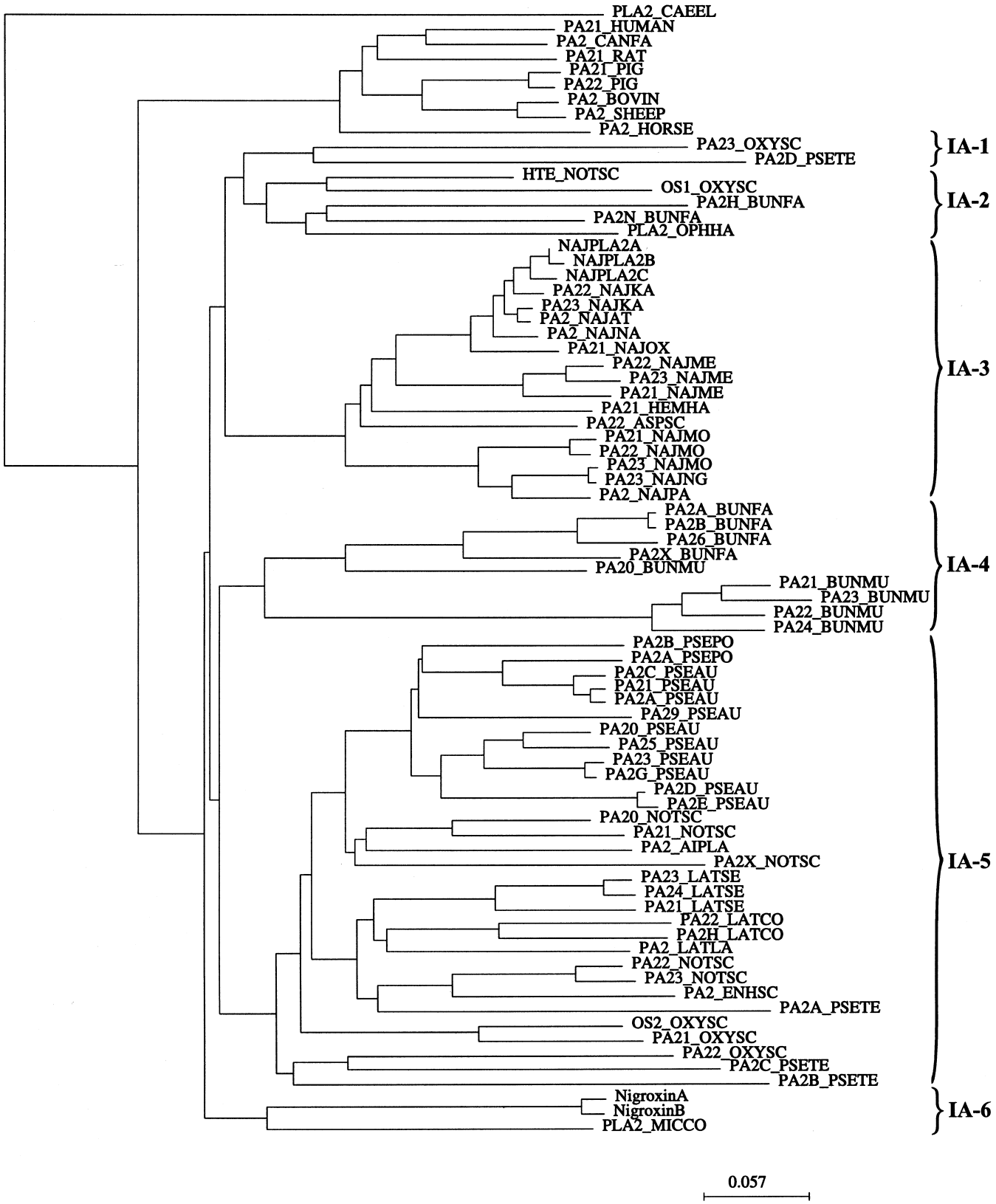

Fig. 6. Phylogenetic tree relating nigroxins to all known class I PLA_2s. The primary structures were aligned using the program CLUSTAL W and the tree was built with the same program using the sequence of the *Caenorhabditis* PLA_2 as outgroup. Subclasses now established are denoted by roman numeral and letter combinations. The length of the branches is proportional to mutational distance.

loop is concluded also to have been advantageous for the development of full myotoxicity because it increases the k_{cat} of these enzymes [42]. The A chain of β-bungarotoxin most likely arose after a new gene duplication of the PLA_2 gene in the line of *Bungarus multicinctus* and may have had a fast mutation rate related to its unique quaternary structure [43]. Thus, the

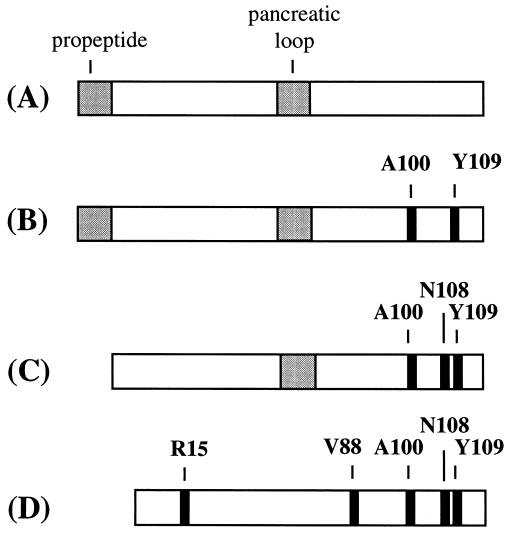

Fig. 7. Schematic representation of the evolutionary pathway in which the residues associated with myotoxicity were acquired by elapid venom PLA_2s. A, B, C and D represent the traced evolutionary steps: (A) a nonmyotoxic pancreatic PLA_2-like ancestor was recruited in the evolving venom gland; (B) the nucleotides corresponding to Ala100 and a hydrophobic residue at position 109, present in all snake venom enzymes, were acquired; (C) a gene duplication occurred, and in one of the duplicates the nucleotides encoding the propeptide were lost while those corresponding to Asn108 were acquired; (D) a second gene duplication occurred, and in one of the duplicates, nucleotides corresponding to Arg15 and Val88 were acquired, while those encoding the pancreatic loop were lost. The residues associated with myotoxicity are indicated in black while the deletions in the structure of the ancestor are colored in gray.

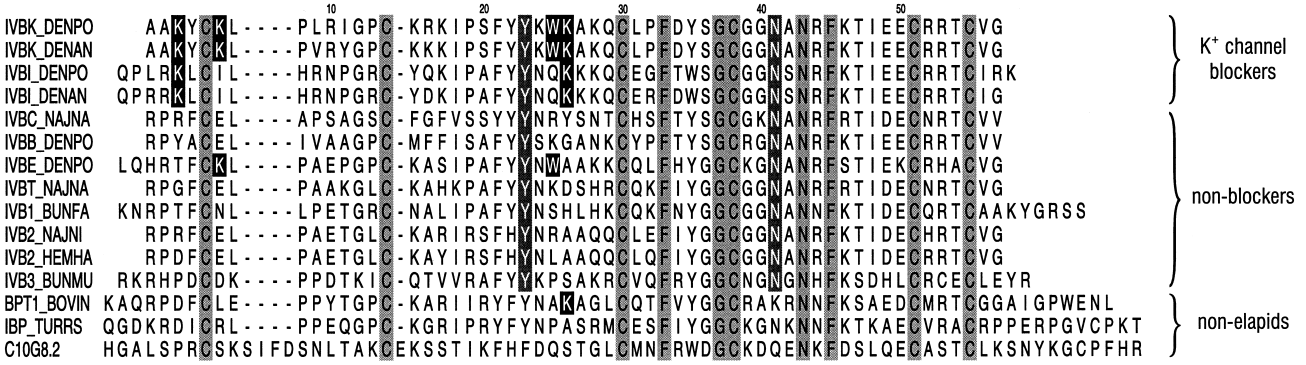

Fig. 8. Sequence alignment of Kunitz-type protease inhibitor homologs from elapid venoms and antiproteases from bovine, turtle and *Caenorhabditis*. Left column contains accession codes for the Swissprot or Wormpep databases. Residues important for the K^+-channel blocker activity of dendrotoxin K are given against a black background. Residues strictly conserved among the whole family are given against a light gray background. Residues conserved exclusively among elapid venom polypeptides are given against a dark gray background. The numbers at the top give positions of dendrotoxin K.

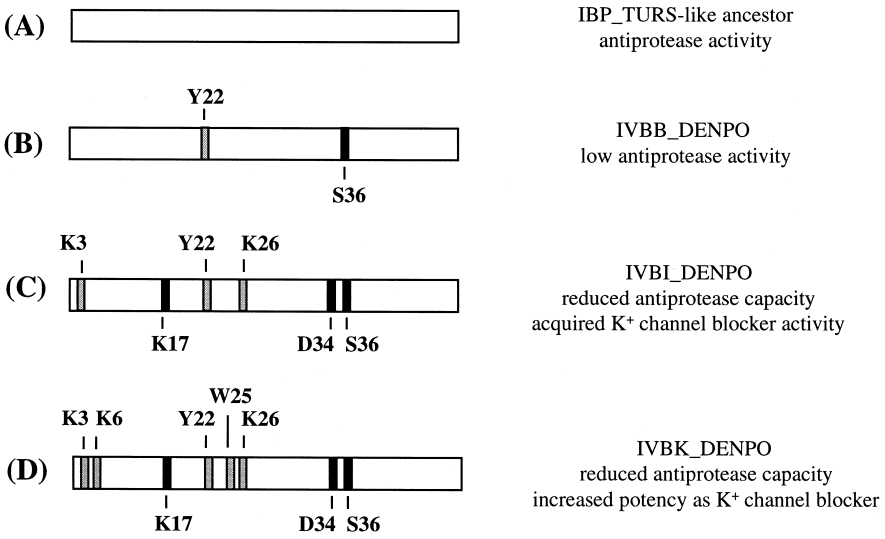

Fig. 9. Schematic representation of the evolutionary pathway in which the residues critical for K^+-channel blocker activity were acquired by dendrotoxins. A, B, C and D represent the traced evolutionary steps: (A) an ancestral IBP_TURS-like antiprotease was recruited by the venom gland; (B) nucleotides corresponding to Tyr22 and Ser36, common to all *Dendroaspis* protease inhibitor homologs, were acquired, leading to a polypeptide with a lowered antiprotease activity; (C) a gene duplication occurred, and in one of the duplicates nucleotides corresponding to Lys3, Lys26, Lys17 and Asp34 were acquired, leading to a polypeptide with K^+-channel blocker activity and a further reduced antiprotease capacity; (D) a second gene duplication occurred, and in one of the duplicates nucleotides corresponding to Lys6 and Trp25 were acquired, which further increased the potency of the K^+-channel blockers. The residues associated with K^+-channel blocker activity are indicated in gray while those which led to a reduced antiprotease activity are indicated in black.

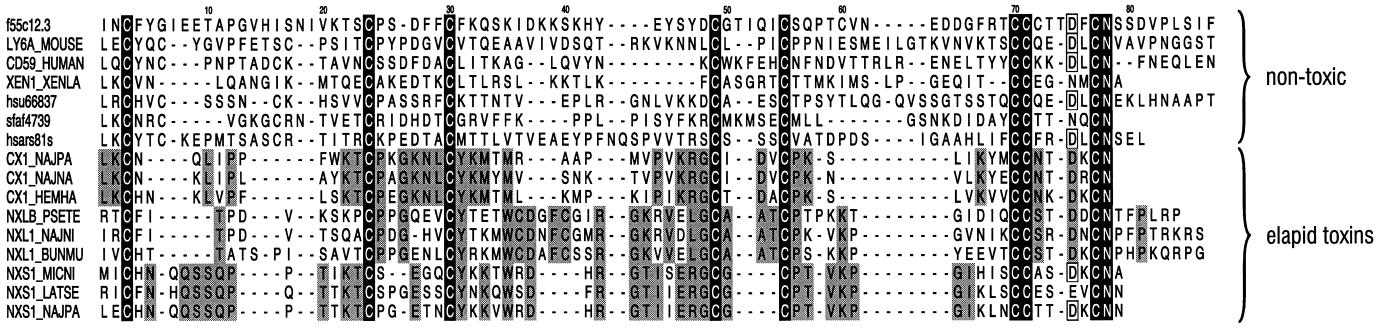

Fig. 10. Sequence alignment of three-finger-shape elapid toxins and their nontoxic counterparts. Abbreviated form of a complete alignment of 169 proteins with the three-finger-shape fold, according to their pattern of residue conservation. Left column contains accession codes for the Swissprot, Wormpep and GenBank databases. The sequences of elapid toxins correspond to cardiotoxins (CX), long-chain and short-chain α-neurotoxins (NXL and NXS). Residues strictly conserved within the whole family are given against a black background, residues conserved more than 85% are boxed and residues conserved within each toxin class are given against a gray background. The numbers at the top give positions in the *Caenorhabditis* protein F55C12.3.

evolutionary history of β-bungarotoxin would explain why it lacks both the pancreatic loop and the residues associated with myotoxic activity.

In conclusion, we found that the elapid venom PLA_2s evolved by recruitment of an ancient scaffold and that myotoxic activity was acquired by changes in the surface-exposed residues. These results prompted us to evaluate the possibility (below) that other elapid toxin families could also have evolved by recruitment in a similar manner.

Dendrotoxins and α-neurotoxins: toxic activities acquired while preserving ancestral structures

Polypeptides homologous to Kunitz-type protease inhibitors have been isolated from several elapid venoms. However, only dendrotoxins have shown the capacity to bind K^+ channels [9] because of the presence of Lys3, Lys6, Trp25 and Lys26 [12]. Nevertheless, all these residues are not present in all dendrotoxins, and accordingly they differ in potency as K^+-channel blockers [44]. Furthermore, dendrotoxins have reduced antiprotease activity because Lys17, Asp34 and Ser36 affect the interaction with target proteases [45]. We now identified in the Wormpept database a protein from *C. elegans* (C10G8.2) which has ≈ 30% residue identity with the Kunitz-type protease inhibitors from higher organisms and also has the same pattern of half-cystines and residues which is critical for antiprotease activity. This protein was used as an outgroup to derive an evolutionary tree from an alignment of the antiproteases from higher organisms (Fig. 8).

The results show that the venom polypeptides share a common ancestor with the nontoxic protease inhibitor from turtles (IBP_TURRS) and indicate that they evolved from an IBP_TURRS-like ancestor. All dendrotoxins share an ancestor with the *Dendroaspis* polypeptide IVBB_DENPO, which lacks the K^+-channel blocker activity [9], but the most potent blockers (IVBK_DENPO and IVBK_DENAN) cluster in a separate group which diverged after the separation of the less potent ones (IVBI_DENPO and IVBI_DENAN). These phylogenetic relationships trace the following steps in which the potent K^+-channel blockers acquired their blocker activity and reduced their antiprotease capacity (Fig. 9): (A) an ancestral IBP_TURS-like antiprotease was recruited by the venom gland; (B) nucleotides corresponding to Tyr22 and Ser36, common to all *Dendroaspis* protease inhibitor homologs, were acquired, leading to a polypeptide with a lowered antiprotease activity; (C) a gene duplication occurred, and in one of the duplicates nucleotides corresponding to Lys3, Lys26, Lys17 and Asp34 were acquired, leading to a polypeptide with K^+-channel blocker activity and a further reduced antiprotease capacity; (D) a second gene duplication occurred, and in one of the duplicates nucleotides corresponding to Lys6 and Trp25 were acquired, which further increased the potency of the K^+-channel blockers. The *Dendroaspis* venom polypeptides IVBB_DENPO and IVBI_DENPO provide evidence for the gene duplications that occurred in steps (C) and (D).

We conclude that the dendrotoxins also evolved by recruitment of an ancient structure, the Kunitz-type protease inhibitor module, in which exchanges of surface residues led to a change from an antiprotease polypeptide to a K^+-channel blocker. However, this recruitment occurred more recently than the PLA_2 recruitment, which took place early in the evolution of Elapidae.

We also found in Wormpept a protein from *C. elegans* (F55C12.3), which has ≈ 30% residue identity with the three-finger-shape toxins from Elapidae, the same half-cystine pattern (Fig. 10), and also conserves Asp75 and Asn78, which are known to play a role in stabilization of the neurotoxin globular core. Further evidence that these proteins belong to the same family (including also *Xenopus laevis* xenoxins [46], CD59 and Ly-6 receptors from mammals [14,47], the fish protein SFAF4739 and the human proteins HSU66837 and HSARS81S) comes from similarities in their gene organization. Their coding regions consist of three exons: the first coding for most of the leader peptide, the second for the first third of the mature protein, and the third for the rest of the polypeptide [48,49]. We therefore conclude that the elapid α-neurotoxins also evolved by recruitment of an ancient structure, which is present in nontoxic proteins widely distributed throughout the animal kingdom. The residues responsible for the binding of α-neurotoxins to AchR [15], which are conserved among Asian/African and Marine/Australian elapids, are also present in the only α-neurotoxin thus far characterized from an American coral snake [50]. This implies that, as for myotoxicity acquisition by elapid PLA_2s, acquisition of AchR-binding activity by α-neurotoxins occurred early during the evolution of Elapidae. The appearance of short-chain and long-chain α-neurotoxins appears to have been advantageous because these toxins differ in toxicity toward different animals: short-chain toxins are most toxic to rodents and birds, long-chain toxins to reptiles and amphibians [13,51].

Elapid toxins: multiple recruitments of versatile scaffolds for new biological functions

The present results suggest that the elapid toxins have evolved through a number of recruitments from different families: myotoxins from PLA_2s, dendrotoxins from Kunitz-type protease

inhibitors and α-neurotoxins from three-finger-shape proteins. Both PLA_2s and the three-finger-shape proteins have diverse functions in several systems. The class I PLA_2s were originally thought to have only a digestive function because they are abundant in pancreatic secretions, but are now known to be expressed also in nonpancreatic tissues and to stimulate cell proliferation [52], extracellular matrix invasion [53], prostaglandin biosynthesis [54] and progesterone release [55]. Subclass IIA PLA_2s have bactericidal [56], proinflammatory [57] and anti-(tumor progression) activities [58], whereas subclass IIC PLA_2s participate in prostaglandin production in testis [59]. In *Xenopus laevis*, the PLA_2 structure has also been identified in the major protein of the aragonitic otoconia [60], suggesting another recruitment during the evolution of the amphibian vestibular system. Similarly, the three-finger-shape fold has also been adapted to fulfill diverse functions in binding other proteins. In mammals this fold is found in at least two groups of monomeric glycosylphosphatidylinositol-anchored proteins: CD59 and Ly-6. CD59 proteins bind the complement proteins C8 and C9, preventing assembly of the lytic complex [14], whereas Ly-6 plays a receptor role in cellular activation [47]. In frogs, the three-finger-shape fold is present in the skin xenoxins [46]. In elapids this structure has been recruited to build toxins that interact with the AchR [13], acetylcholinesterases [61] and L-type Ca^{2+} channels [62].

The wide distributions of the PLA_2s and the three-finger-shape folds and their multiplicities of biological activity provide evidence of their versatility. Recruitments have previously been shown for the crystallin proteins of the eye, which are derived from metabolic enzymes and stress proteins [63,64]. We now show that during the evolution of the elapid venom glands, several versatile scaffolds were similarly adapted to serve toxic functions by changes in a few surface-exposed residues. Thus, recruitment of existing structures during the evolution of new biological functions may be of common occurrence.

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