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Rattlesnake Presynaptic Neurotoxins: Primary Structure and Evolutionary Origin of the Acidic Subunit†

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ABSTRACT: Crotoxin and homologous crotalid presynaptic neurotoxins consist of a toxic, basic subunit and a slightly smaller, nontoxic, acidic subunit. The latter, in turn, consists of three chains, interconnected by disulfide bonds. The complete sequences of two of the three acidic subunit chains of crotoxin, from the venom of the South American rattlesnake *Crotalus durissus terrificus*, have been determined. In addition, all but the ten amino-terminal residues of the third chain have been sequenced. Sequence comparison data suggest that the acidic subunit has been derived from a nontoxic, homodimeric, crotalid phospholipase A_2. When compared with sequences of phospholipases A_2, the acidic subunit lacks a 22-residue amino-terminal segment and two additional segments that are implicated in phospholipid substrate binding. However, it apparently retains an intact active site, the calcium binding loop, and segments involved in subunit binding in homodimeric phospholipases A_2. The C chain of the acidic subunit shows strong homology with mammalian neurophysins, lending possible support to the hypothesis that the acidic subunit functions as a chaperone to prevent nonspecific binding of the toxic basic subunit. Crystals suitable for X-ray diffraction studies have recently been produced [Achari, A., Radvanyi, F. R., Scott, D., Bon, C., & Sigler, P. B. (1985) *J. Biol. Chem. 260*, 9385–9387]; thus with these data it should now be possible to determine the three-dimensional structure of the intact neurotoxin and dissociated subunits.

In 1938, Slotta & Fraenkel-Conrat (1938) isolated a toxic protein from the venom of the South American rattlesnake (*Crotalus durissus terrificus*) that represented some 65–70% of the total venom protein. Named crotoxin, it proved to be a potent presynaptic neurotoxin. This discovery conflicted with prevailing herpetological dogma, which held that neurotoxins were the exclusive province of cobras and their relatives (elapids) and sea snakes (hydrophiids). Accordingly, the South American rattlesnake was viewed for many years as something of an enigma. Although it was suspected that crotoxin could be a mixture of two different proteins, its heterodimeric nature was not determined for 18 years. Fraenkel-Conrat & Singer (1956) eventually not only verified its dimeric structure but also concluded that the larger, basic subunit was rich in lysine

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and arginine residues, while the smaller subunit was quite acidic. The basic subunit was partially sequenced more than 40 years after crotoxin's discovery (Fraenkel-Conrat et al., 1980).

Breithaupt et al. (1974) demonstrated that the acidic subunit of crotoxin consists of three dissimilar polypeptide chains interconnected by seven disulfide bonds. They reported serine as the amino-terminal residue of the A chain, confirming an earlier report (Fraenkel-Conrat & Singer, 1956). However, they were unable to detect any additional amino-terminal residues and concluded that the B and C chains were blocked at the amino terminus.

Proteins present in the venoms of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) (Hendon, 1975; Bieber et al., 1975; Cate & Bieber, 1978; Hendon & Bieber, 1982) and the midget faded rattlesnake (*Crotalus viridis concolor*) (Pool & Bieber, 1981) are structurally and functionally very similar to crotoxin (Aird & Kaiser, 1985). Recent evidence suggests that numerous other rattlesnakes also possess this toxin (Henderson & Bieber, 1984; Glenn & Straight, 1984), although its distribution presently appears almost phylogenetically random. It is unclear whether species that do not produce the neurotoxin actually lack the necessary structural genes or whether neurotoxin production in these taxa is prevented by the action of some regulatory mechanism.

In order to facilitate studies of crotoxin, aimed at elucidating its biosynthesis, three-dimensional structure, mode and site of action, and phylogenetic distribution, we have sequenced the acidic subunit chains. No attempt has been made at present to position the disulfide bonds, although as shown, these may be predicted from disulfide bond positions in probable ancestral sequences.

Experimental Procedures

Venom of the South American rattlesnake was obtained from the Miami Serpentarium (lot CJ13VZ). Crude, lyophilized venom was dissolved in 0.1 M sodium acetate, pH 4.0, and acidified back to pH 4.0 with 1 N HCl. The initial separation was made on a 2.5 × 94 cm Sephacryl S-200 column equilibrated in 0.1 M sodium acetate, pH 4.0. After dialysis and lyophilization, further purification was achieved by passing the third S-200 fraction over a 1.5 × 35 cm DEAE-Sephacel column equilibrated in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.2. Crotoxin subunits were isolated by dissolving the DEAE fraction in 50 mM Tris-HCl, pH 7.2, containing 6 M urea, and fractionating it on a 1.5 × 10 cm DEAE-Sephacel column equilibrated in the urea buffer (Aird & Kaiser, 1985). After gel filtration and DEAE chromatography, the acidic subunit was further purified by reverse-phase high-performance liquid chromatography (HPLC). The reverse-phase A buffer was 0.5 M pyridine–acetate, pH 4.0. The B buffer was similar, except that it contained 40% 1-propanol. The step gradient increased the propanol concentration from 0% to 40% in 4% increments every half hour. The HPLC system employed a postcolumn fluorescamine detection system that detects free primary amines (Lewis, 1984).

The C chain was deblocked by subjecting the intact acidic subunit to pyroglutamate aminopeptidase (Boehringer-Mannheim), as described by Podell & Abraham (1978). Following deblocking the reaction mixture was reduced and carboxamidomethylated. Carboxamidomethylation was carried out in a 1.0-mL volume containing 6 M guanidine hydrochloride (Heico), 0.1 M Tris-HCl, pH 8.2, 1 μM disodium ethylenediaminetetraacetate (Na_2EDTA), and a 5-fold excess of dithiothreitol (DTT) over protein. After reaction for 2 h under N_2, a 2-fold excess of iodoacetamide over DTT was added. After an additional 35 min under N_2 in the dark, the reaction was stopped with glacial acetic acid. Following the pyroglutamate aminopeptidase and carboxamidomethylation reactions, the three chains were separated by reverse-phase HPLC. Buffers used in this separation were as described above. The step gradient, however, increased propanol concentrations by only 1.3% to 2.6% every half hour.

The A and C chains were then sequenced directly on an Applied Biosystems 470A protein sequencer. Because deblocking of the B chain with pyroglutamate aminopeptidase proved unsuccessful, it was incubated in 100% trifluoroacetic acid (TFA) for 2 h at 60 °C prior to sequencing to remove a possible *N*-formyl blocking group. Deblocking was also unsuccessful by this means; however, at this elevated temperature the TFA cleaved an Asp–Pro bond between residues 11 and 12. The carboxy-terminal 23 residues were then sequenced directly. Sequencing employed the NVAC program. PTH-amino acids were identified by HPLC on a Supelco C_{18} column with a Bakerbond C_4 guard column. Buffer A was 66 mM trifluoroacetic acid/4 mM acetic acid adjusted to pH 5.8 with NaOH. Buffer B was 33 mM TFA, 10% acetonitrile, and 65% methanol, adjusted to pH 3.6 with NaOH. The gradient was 28% B at 0 min with linear steps to 30% B at 13 min, 50% B at 17 min, 70% B at 37 min, and 100% B at 38 min. The HPLC system consisted of a Spectra Physics 8700 HPLC equipped with the dynamic mixer, a Waters 710B WISP, and a Kratos 757 UV detector (at 269 nm). Complete separation of all PTH-amino acids was achieved with this system.

To identify protein sequences similar to the acidic subunit sequences, a computer search was conducted by using the protein sequence database of the Protein Identification Resource (National Biomedical Research Foundation, Georgetown University Medical Center) (Lipman & Pearson, 1985).

Results

HPLC purification of the acidic subunit from the DEAE-urea column indicated a minor contaminant representing approximately 10% of the observed fluorescence. The three acidic subunit chains were readily separable with reverse-phase HPLC. The C chain eluted first in 4% propanol while the A and B chains eluted later in 13.3% and 16% propanol, respectively (Figure 1). The C chain, which lacks lysine, was undetectable if the deblocking reaction was omitted. It contains 14 residues (Figure 2) as anticipated from composition data (Breithaupt et al., 1974). The A chain, however, consists of only 38 residues, with one less each of Asx and Ser than indicated by the previous work (Figure 2). Since both the A and B chains contained a single lysine and both were present in equimolar concentrations, both chains should have shown equal fluorescence if the B chain were deblocked after treatment with pyroglutamate aminopeptidase. The B chain exhibited less than half the fluorescence of the A chain, indicating that it was still blocked at the amino terminus (Figure 1). While the B chain has not been completely sequenced, the 23 residues at the carboxy terminus are totally consistent with composition data (Breithaupt et al., 1974).

Comparisons with 3251 sequences showed strong homologies to portions of snake venom phospholipases A_2 (Figure 3). Sequence homologies between the acidic subunit and nontoxic crotalid phospholipases yield *Z* scores of 16.4, 12.0, and 7.5 for the A, B, and C chains, respectively (Lipman & Pearson, 1985).

The A chain has retained both the Tyr-Gly-Cys-Tyr-Cys-Gly-(Trp)-Gly-Gly segment (positions 24–32), common to all

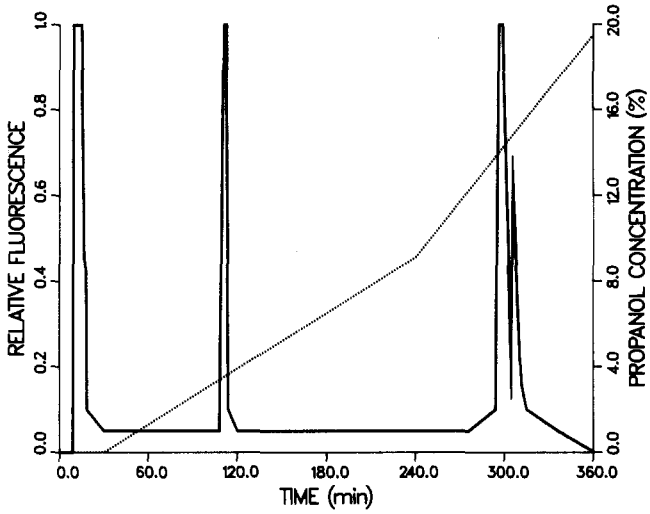

FIGURE 1: Elution of the three acidic subunit chains from a C_{18} reverse-phase column. The equilibration buffer was 0.5 M pyridine–acetate, pH 4.0. The elution buffer differed in that it contained 40% 1-propanol. HPLC was carried out at 22 °C with a flow rate of 0.75 mL/min.

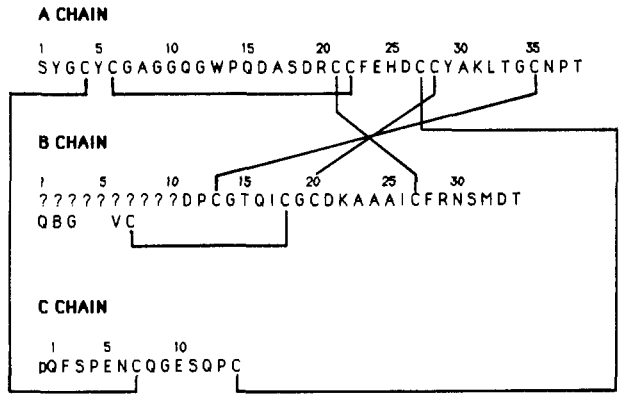

FIGURE 2: Primary structures of the acidic subunit chains of the presynaptic neurotoxin from the South American rattlesnake (*C. durissus terrificus*). The B chain is blocked at the amino terminus but probably not with a formyl group or with pyroglutamate. Probable residues in the unsequenced region are shown below the B chain. Cleavage of the B chain was effected with 100% TFA at 60 °C for 2 h. The C chain was blocked with pyroglutamate. No attempt has been made to position intra- and interchain disulfide bonds; however, probable assignments, based on the work of Heinrikson (1982), are shown.

known snake venom phospholipases, and the Asp-Arg-Cys-Cys-Phe-(Val)-His-Asp-Cys-Cys-Tyr-(Gly) segment (positions 41–52), which is invariant among crotalid and viperid phospholipases. B-chain homologies with phospholipase segments are also very high (positions 71–104, Figure 3). Locations of four to five cysteine residues have been maintained, two of which occur in the highly conserved segment Cys-Asp-Lys-Ala-Ala-Ala-Ile-Cys-Phe-Arg (positions 90–99). The 14-residue C chain is homologous with the carboxy terminus of viperid and crotalid phospholipases, retaining both cysteines at positions 118 and 125 (Figure 3). It also shows high homology with a segment (residues 48–61) of mammalian neurophysins and their precursors.

Discussion

Attempts to complete the B-chain sequence are presently under way; however, this cannot be accomplished by common methods, such as a combination of aminopeptidase and carboxypeptidase. This is because the unsequenced portion is ten residues long, is blocked at the amino terminus by an unknown

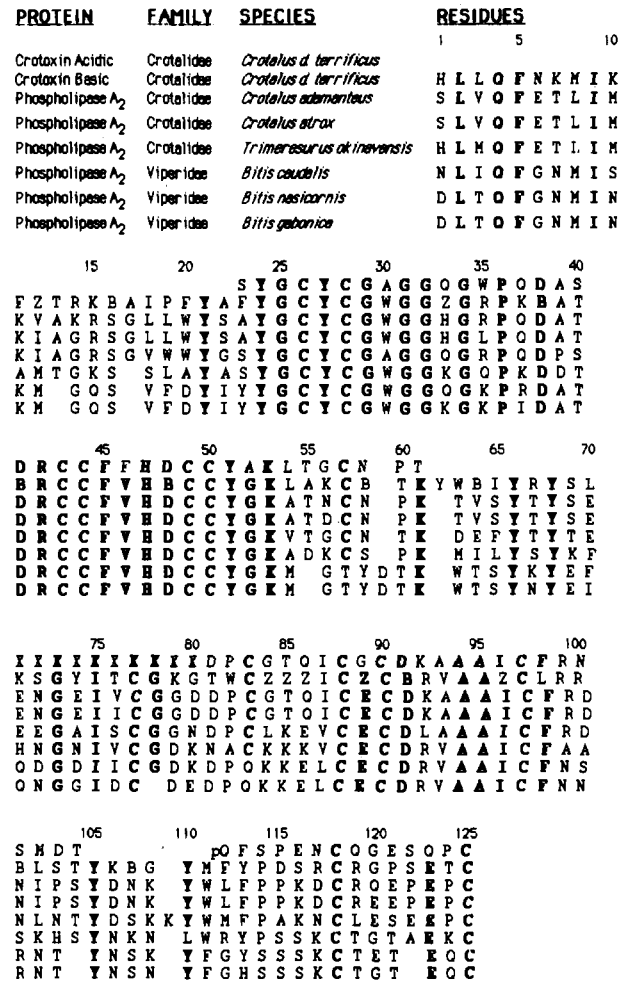

FIGURE 3: Sequences of the acidic and basic subunits of crotoxin relative to other crotalid and viperid phospholipase A_2 sequences. The three phospholipase segments consisting of residues 23–61, 71–104, and 112–125 correspond to the acidic subunit A, B, and C chains, respectively. Original sequence references include Fraenkel-Conrat et al. (1980), Randolph & Heinrikson (1982), Joubert & Haylett (1981), Viljoen et al. (1982), Joubert et al. (1983), and Botes & Viljoen (1974).

agent, and contains five Glx and three Asx. Of the ten residues not sequenced, five can be positioned with considerable certainty on the basis of probable ancestral sequences. These include Gln-71, Asx-72, Gly-73, Val-76, and Cys-77. However, this missing sequence information is trivial in light of sequence homologies discussed herein.

The Tyr-Gly-Cys-Tyr-Cys-Gly-(Trp)-Gly-Gly sequence (positions 24–32, Figure 3) near the amino terminus of the A chain is at the interface between monomers in homodimeric phospholipases A_2 and may constitute part of the subunit binding site (Keith et al., 1981). The Asp-Arg-Cys-Cys-Phe-(Val)-His-Asp-Cys-Cys-Tyr-(Gly) sequence (positions 41–52, Figure 3) contains the phospholipase A_2 active site (Yang & King, 1980) and Asp-48, which is specifically implicated in Ca^{2+} binding (Drenth et al., 1976).

Homology of the C chain is maximal with phospholipase A_2 from the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) (Heinrikson et al., 1977) ($Z = 7.5$). Interestingly, the next 11 most similar sequences are neurophysins and their precursors (Acher, 1976) (Figure 4). These molecules bind noncovalently to neurohypophyseal hormones. While their biological function is unknown, they are believed to function as carrier molecules (Gorbman et al., 1983). Using ^{125}I-labeled toxins, Jeng et al. (1978) demonstrated that the

PROTEIN	TAXON	POSITIONS	RESIDUES
C Chain	*C. d. terrificus*	1-14	pQ F S P E N C Q G E S Q P C
PLA_2	*C. adamanteus*	109-122	L F P P K D C R Q E P E P C
Neurophysin I	*Bos*	48-61	N Y L P S P C Q S G Q K P C

FIGURE 4: Sequence of the C chain of the crotoxin acidic subunit compared with the carboxy-terminal end of the homodimeric, nontoxic phospholipase A_2 from *C. adamanteus* venom and with a partial sequence from mammalian neurophysins and their precursors. This sequence is the same for all mammals listed in the National Biomedical Research Foundation database.

acidic subunit does not bind to erythrocytes or their ghosts, while the basic subunit does. When the intact neurotoxin was injected, the acidic subunit substantially reduced binding of the basic subunit. While residues 48–61 of mammalian neurophysins have not been proven to be involved in the actual binding of hormones, the sequence homology between the C chain and mammalian neurophysins does lend additional credence to the suggestion that the acidic subunit acts as a chaperone to prevent nonspecific binding of the basic subunit (Jeng et al., 1978; Achari et al., 1985).

All cysteine residues common to both toxic and nontoxic phospholipases appear to have been retained in the acidic subunit. It seems highly probable, therefore, that the disulfide bonds correspond with assignments made by Heinrikson for group II phospholipases (Heinrikson, 1982). Numbering the positions as in Figure 2, the cysteine residues would be paired as follows: A4–C7, A6–A22, A21–B27, A27–C14, A28–B20, A35–B13, and B7–B18. Cys-125 is probably covalently linked to Cys-50 in the active site (Randolph et al., 1980; Keith et al., 1981).

The low p*I* of the acidic subunit (3.2, as opposed to 9.7 for the basic subunit; Breithaupt et al., 1974) has been produced partly by the excision of three segments (positions 1–22, 62–70, and 105–111; Figure 3) containing a total of seven basic residues and only one to two acidic residues. These segments are believed to interact with phospholipid substrates in intact, nontoxic, crotalid phospholipases (J. Maraganore, personal communication). From the sequence comparison data it is not possible to determine the amino acid residues involved in the cleavages to generate the three chains. Clearly, it will be important to determine the sequence of the whole precursor in order to assign the sites of cleavage. It will be of interest to know how these cleavages relate to cleavages of precursors of other bioactive peptides.

Homology of both the acidic and basic subunits is greatest with nontoxic, crotalid phospholipases, which exist in solution as stable dimers with dissociation constants of 10^{-9}–10^{-11} M (Shen et al., 1975) (e.g., phospholipases from the venoms of *C. adamanteus* and *Crotalus atrox*). Crotoxin exhibits maximal toxicity only as a dimer, despite the fact that it dissociates upon reaching the target tissue. That is, neurotoxicity is reduced about 10-fold if the dissociated basic subunit is injected alone. While retention of the subunit binding site by the acidic subunit further suggests its origin from a homodimeric phospholipase and suggests that the two active sites of the subunits face one another as proposed by Keith et al. (1981), the loss of enzymatic activity in the acidic subunit is intriguing. Randolph et al. (1980) report that the dimeric PLA_2 from *C. atrox* is completely destabilized when the amino-terminal 10 residues of the protein are removed with cyanogen bromide. In the acidic subunit, 22 residues are missing from the amino terminus. Keith et al. (1981) report that two salt bridges between Asp-48 of one monomer and Lys-61 of its mate (Figure 3) may serve in governing the Ca^{2+}-mediated entry of substrate molecules into the active site. One of these must be missing in crotoxin, where Lys-61 in the acidic subunit has been replaced by a threonine. The role of the acidic subunit may be to recognize specific binding sites and to escort the basic subunit to these receptors, where its phospholipase activity may be selectively directed. Achari et al. (1985) have reported the production of crotoxin crystals suitable for high-resolution X-ray diffraction studies. Given the sequence data reported herein, it should now be feasible to undertake three-dimensional structural analyses of the intact toxin and of the free acidic and substrate-bound basic subunits.

Because protein similarity scores do not follow a normal distribution, the significance of similarity is expressed as a *Z* score rather than as a *P* value (Lipman & Pearson, 1985) and is calculated as Z = (similarity score minus mean of random scores)/(standard deviation of random scores). Lipman & Pearson (1985) consider Z scores >3 as possibly significant, Z >6 as probably significant, and Z >10 as significant. Thus Z scores of 16.4, 12.0, and 7.5 for the A, B, and C chains permit us to discuss acidic subunit homologies with considerable certainty. We have nearly completed the sequence of the crotoxin basic subunit, published in incomplete form by Fraenkel-Conrat et al. (1980). When similarity scores for all three chains are combined, greater similarity is still apparent between the acidic subunit and the homodimeric, nontoxic crotalid phospholipases than between the acidic and basic subunits of crotoxin themselves. This suggests that the two subunits have probably evolved independently from a common, nontoxic, ancestral sequence rather than from each other. Phospholipases from true vipers (Old World) are less closely related to the acidic subunit than are those from pit vipers (New World and Asia). Phospholipases from the venoms of the cobra and sea snake families exhibit still less homology, as one would anticipate from current snake phylogeny (Figure 3). The toxic monomeric phospholipase from the horned puff adder (*Bitis caudalis*) is peculiar in that it shows greater similarity to the basic subunit (our revised, unpublished sequence data) than do the phospholipases from the rhinoceros viper (*Bitis nasicornis*) and the gaboon viper (*Bitis gabonica*). In particular, the *caudalis* enzyme displays four insertions that are characteristic of crotalid phospholipases but not the other viperid phospholipases (Thr-13, Thr-55, Ser-104, and Ala-122; Figure 3). All of these insertions are lacking in the other two viperid sequences. Furthermore, the *caudalis* phospholipase possesses the "crotalid" deletion at position 59, where the gaboon and rhinoceros viper enzymes have an aspartate. Whether these changes arose prior to the divergence of these two snake families or whether such similarities are the product of convergent evolution subsequent to their divergence is difficult to say.

Previously it was thought that crotoxin might represent the product of from one to four loci. The present study suggests that the two subunits of crotoxin are products of separate genes. We are now in a position to make DNA probes to isolate the precursors of both subunits. These data will determine what posttranslational modifications occur in processing these subunits. We will also be able to determine whether production of the two subunits is coordinated or whether their synthesis is independent.

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