Isolation and Amino Acid Sequence of a Neurotoxic Phospholipase A from the Venom of the Australian Tiger Snake Notechis scutatus scutatus*

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The complete amino acid sequence of notechis 5, a neurotoxic phospholipase A from the venom of Notechis scutatus scutatus (Australian tiger snake), has been elucidated. The main fragmentation of the 119-residue peptide chain was accomplished by digesting the reduced and S-carboxymethylated derivative of the protein with a staphylococcal protease specific for glutamyl bonds. Tryptic peptides were used to align and complete the sequence of the four staphylococcal protease peptides. The sequence was determined by Edman degradation by means of the direct phenylthiohydantoin method. Notechis 5 differs in seven positions from the recently elucidated sequence of the presynaptic neurotoxin notexin from the same venom. Notechis 5 has a 50% higher specific phospholipase A activity than notexin when assayed against egg yolk but is only one-third as toxic.

Snake venom neurotoxins act on the peripheral nervous system, blocking transmission across the cholinergic neuromuscular junction by either a pre- or postsynaptic mode of action. Presynaptic neurotoxins interfere with the release of acetylcholine from the motor nerve terminals, whereas postsynaptic (curarimimetic) neurotoxins block the nicotinic acetylcholine receptors of the muscle motor end plate.

The two most thoroughly characterized presynaptic venom neurotoxins, notexin from the venom of Notechis scutatus scutatus (1-8) and B-hungarotoxin from the venom of Bungarus multicinctus (9-13), exhibit phospholipase A activity, which might be involved in their neuromuscular blocking action (3-6, 12-13). No information is as yet available, however, as to which structural features endow these phospholipases with a high degree of neurotoxicity, whereas most phospholipases A are non- or poorly neurotoxic.

In the communication on the isolation of notexin (1) the presence in tiger snake venom of another neurotoxin, toxin 5, with the same number of amino acid residues was reported. Toxin 5 was reported to be 20-fold less toxic than notexin, and was thought to have a postsynaptic mode of action based on the observation of the symptoms elicited in mice. In conjunction with the sequence work on notexin (3), toxin 5 (in the future referred to as notechis 5) was submitted to preliminary characterization. Comparison of the amino acid composition of notechis 5 with that of notexin revealed only four differences between the two proteins, making it unlikely that the two neurotoxins could differ as greatly as originally reported. On the contrary, it seemed that notechis 5 should also be a potent presynaptic neurotoxin with phospholipase A activity. The biological activity and amino acid sequence of notechis 5 have been investigated in order to better understand structure-function relationships in presynaptic neurotoxins with phospholipase A activity.1

MATERIALS AND METHODS

The crude Notechis scutatus scutatus venom was purchased from Eric Worrell's Australian Reptile Park, P.O. Box 192, Gosford, N.S.W. 2250, Australia.

Gel filtration of the crude venom, ion exchange chromatography on Bio-Rex-70, reduction and S-carboxymethylation, digestion with proteolytic enzymes, purification of peptides, manual Edman degradation, identification of phenylthiohydantoin derivatives, and amino acid analysis were performed as described earlier in the elucidation of the notexin sequence (3).

Toxicity assays were performed by intravenous injection in isotonic saline into the tail vein of white mice weighing about 20 g. Four to eight mice were used at each dose level. The toxin concentration was determined spectrophotometrically with the use of an extinction coefficient determined by amino acid analysis of a sample of known absorbance. The LD50 was approximated as the dose killing four of eight mice. Phospholipase A assays were performed by a modification of the method of de Haas et al. (14) as described previously (6).

RESULTS

Isolation of Notechis 5—Notechis 5 was isolated from the crude venom in the same way as notexin, gel filtration on

1 Most of the data are presented in a miniprint format immediately following this paper. (Figs. 1 through 6 and Tables I through V are found on pp. 7346-7347. For the convenience of those who prefer to obtain that data in the form of full size photocopies, they are available as JBC Document No. 76M-236. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $2.10.

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Amino Acid Composition and NH2-terminal Sequence—Notechis 5 differs in amino acid composition from notexin only by 2 more residues each of serine and arginine and by 1 less residue each of glycine, alanine, phenylalanine, and lysine. Two cycles of Edman degradation on the intact reduced and S-carboxymethylated derivative established the sequence Asn-Leu. The remainder of the NH2-terminal sequence of the protein was established by degradation of the 40-residue NH2-terminal staphylococcal protease peptide in order to minimize the “background” encountered in extended degradation of the intact protein.

Complete Amino Acid Sequence of Notechis 5—Gel filtration of the 18-h staphylococcal protease digest of reduced and S-carboxymethylated notexin 5 on Sephadex G-50 yielded four peaks, each of which was due to only one major peptide. Fraction A (Peptide SP-1)4 required no further purification. The major components of Fractions B, C, and D were freed from trace amounts of contaminants by column electrophoresis at pH 5.0 or 7.5, yielding the three pure peptides SP-2, SP-3, and SP-4, respectively. Manual Edman degradation of the four staphylococcal protease peptides established the sequence of 55 of the 110 residues in the protein. The COOH terminal sequence was established by eight cycles of degradation on a chymotryptic subfragment of peptide SP-4 which represented residues 111-119. Tryptic peptides accounting for 115 residues were isolated, and these provided sufficient information to align and complete the sequences of the staphylococcal protease peptides. The experimental data which led to the elucidation of the complete sequence are detailed in the supplement to this communication. The complete amino acid sequence of notexin 5 is shown in Fig. 1.5

DISCUSSION

Examination of a recent homology alignment of pancreatic and snake venom phospholipases A (5) shows that none of the substitutions between notexin 5 and notexin involves hitherto invariant residues. Five of the substitutions observed could arise from single base changes in the codons for the amino acids.

Fig. 1. Complete amino acid sequence of notexis 5. The sequence differs from that of notexin at seven positions as indicated on the line above.
involved, whereas the Trp/Ala substitution at position 31 and the Ser/Asp substitution at position 58 each require two base changes.

All amides are conserved between the two proteins. In the elucidation of the notexin sequence slight doubt arose about the identification of residue 24 as aspartic acid and not asparagine. Residue 24 is unmistakably aspartic acid in notechis 5, and there is no reason to suspect deamination during the peptide purification.

Perhaps the most interesting result from the structure-function standpoint is the nearly absolute identity between notexin and notechis 5 in the region between Lys-57 and Cys-90, which is the stretch in which notexin differs most greatly from all other phospholipases. As pointed out previously, however, this is also the region where there is least homology among the non-nicotinamide phospholipases.

Methodological Aspects of Sequence Determination—The results of the staphylococcal protease digestion of notechis 5 surpassed those obtained in the elucidation of the notexin sequence, where non-glutamyl cleavage was observed and was thought to be due to active autolytic fragments having a different specificity than the native enzyme. The staphylococcal protease preparation used in the present investigation was gel-filtered on Sephadex G-150 prior to use, and no parasitic cleavage of notechis 5 was observed. Autolytic fragments of the enzyme were thus probably the cause of the parasitic cleavage of notexin, but these fragments were undoubtedly present in the enzyme preparation prior to use and were not generated during the digestion of notexin as thought originally (3).

No evidence was found for cleavage at Glu-56, and the yield of the staphylococcal protease peptide SP-2 containing this internal glutamic acid residue was not significantly lower than that of the peptide obtained in highest yield. Apparently the two basic lysine residues 57 and 58 immediately following the glutamic acid residue 56 inhibit cleavage by the staphylococcal protease.

The substitutions at positions 53, 54, and 56 are interesting from a methodological point of view as they illustrate the danger of inferring sequences by homology. The tryptic peptide (47–57) from notechis 5 differs in composition from the corresponding peptide from notexin by a Ser/Gly replacement, suggesting a Ser/Gly substitution at position 56. In reality, however, three substitutions are involved: Ser/Asp at position 53, Asp/Glu at position 54, and Glu/Gly at position 56. Two homologous peptides differing by only 1 residue in amino acid composition thus differ in sequence at three positions.

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REFERENCES
### Table I

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### Figure 1

- **Figure 1A**: Comparison of the enzyme activity of the two enzymes (Enzyme A and Enzyme B) across different pH values. The chart shows a significant increase in activity at pH 7.5 compared to other pH levels.

- **Figure 1B**: Comparative analysis of the enzyme activity at various temperatures. The enzyme activity remains stable up to 40°C before showing a slight decrease at higher temperatures.
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