TAIPOXIN, AN EXTREMELY POTENT PRESYNAPTIC SNAKE VENOM NEUROTOXIN

Elucidation of the primary structure of the acidic carbohydrate-containing taipoxin-subunit, a prophospholipase homolog

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1. Introduction

Taipoxin, the most potent animal toxin known, is a ternary complex of three subunits designated α, β, and γ which are linked together by non-covalent interactions [1]. Taipoxin causes neuromuscular blockade by interfering with transmitter release at motor nerve terminals [2]. Ultrastructurally the poisoned nerve terminals show the following lesions: reduction of the content of transmitter-storing vesicles, profuse Ω-shaped indentations in the neurolemma, degenerative changes in the mitochondria and generally increased electron density with deposits of granular material. The data have been interpreted to indicate that the principal lesion caused by presynaptic snake venom neurotoxins is the inhibition of reformation of functional vesicles [3]. This hypothesis is supported by the observation that these toxins inhibit the high-affinity choline uptake of purified nerve-terminal elements; namely, T-sacs (synaptosomes) from *Torpedo marmorata* [4].

The α- and β-subunits appear to be strict homologs of venom and pancreatic phospholipases A2. This paper concerns the primary structure of the glycoprotein γ-subunit, which is strictly homologous in sequence to the proenzyme form of pancreatic phospholipase A2.

2. Materials and methods

Taipoxin was isolated as described earlier [1] by gel filtration on Sephadex G-75 of taipan (*Oxyuranus s. scutellatus*) venom obtained from C. Tanner, Cooktown, Australia. Pure γ-subunit was obtained by gel filtration in 6 M guanidine hydrochloride as described earlier, except that the new gel Sephacryl S-200 [5,6] was used instead of Sepharose 6B.

The reduced and *S*-carboxymethylated derivative was labelled with iodo-[^{14}C]acetate to facilitate eventual identification of the phenyl thiohydantoins of aspartic acid and carboxymethylcysteine, which are encountered very often in the sequencing of this molecule and have similar chromatographic properties in the thin-layer systems used [1].

Cyanogen bromide cleavage was done with a 30-fold molar excess of reagent in 70% formic acid [7].

Digestions with staphylococcal protease, trypsin and chymotrypsin were done in 0.1 M ammonium bicarbonate at room temperature or 37°C. Peptide purification was done by initial group separation on Sephadex G-50 followed by electrophoresis in columns of cellulose powder (see [14]). The purity of peptides was assessed by paper electrophoresis and amino acid analysis. Edman degradation and the identification of the phenyl thiohydantoins were done as described [1].

3. Results and discussion

The integral amino acid composition derived from the sequence (table 1) differs from that reported [1] by one less residue each of Glx and Gly. The peptides and degradation steps upon which the sequence determination is based are indicated in fig.1. The

Table 1
Amino acid compositions of three cyanogen-bromide fragments and the intact RCM γ-subunit

	CNBr-1,2	CNBr-3	CNBr-4	RCM-γ
CM-Cysteine	2.86 (3)	11.87 (12)	0.73 (1)	16.22 (16)
Aspartic acid	2.36 (2)	19.82 (20)		22.45 (22)
Threonine	1.02 (1)	5.80 (6)		7.05 (7)
Serine	3.61 (4)	5.77 (5)		8.87 (9)
Glutamic acid	5.83 (6)	8.67 (7)	1.10 (1)	14.73 (14)
Proline	2.89 (3)	4.28 (4)		6.76 (7)
Glycine	1.15 (1)	9.06 (9)		10.63 (10)
Alanine	1.10 (1)	7.00 (7)		8.35 (8)
Valine		1.76 (2)		2.02 (2)
Methionine	0.17 (2)	0.22 (1)		2.68 (3)
Isoleucine	2.83 (3)	3.71 (3)	1.01 (1)	6.83 (7)
Leucine	1.96 (2)	4.96 (5)		7.05 (7)
Tyrosine	0.99 (1)	6.75 (7)		7.90 (8)
Phenylalanine	1.95 (2)	2.03 (2)		4.02 (4)
Glucosamine		4.93 (5)		
Histidine		1.33 (1)	0.99 (1)	1.84 (2)
Lysine		4.08 (4)	0.98 (1)	4.86 (5)
Tryptophan				0
Arginine		1.96 (2)		2.10 (2)
Homoserine	1.01	0.91		
Total	31	97	5	133

The integral values in parentheses are based on the sequence

numbering is the same as in the homology fig.2. The alignment posed no problems, since the cyanogen bromide fragments placed themselves and the subsequent glutamoyl-specific cleavage fragments of CNBr-3 were easily aligned by means of tryptic and chymotryptic peptides. Although chymotryptic peptides accounting for the complete sequence were isolated, only those used for sequencing are indicated in fig.1. Problems were encountered with the stretch from residues 23–27, but the sequence as written is justified by a chymotryptic split at Leu-28, a staphylococcal protease split at Glu-26 and the failure of the post-proline cleaving enzyme [8] to cleave at Pro-22 suggests that it is followed by an acidic residue (carboxymethyl-cysteine).

The carbohydrate-containing peptides were easy to isolate by gel filtration since they were always larger than all other peptides present. The carbohydrate moiety is attached to Asn-78, which is followed by Asp-79 and Thr-80 according to the general scheme Asn–X–Ser/Thr. Glu-75 was not cleaved by the glutamoyl-enzyme, but it has been reported before that a Glu–Pro bond is resistant to attack [9]. No cleavage occurred at Glu-84 and cleavage at Glu-100 was incomplete. Apparently hydrolysis is inhibited when Glu is followed by another acidic amino acid–carboxymethyl cysteine in both cases here.

The sequence 1–8 corresponding to the activation peptide of the pork pancreatic proenzyme cannot easily be cleaved off from the γ-chain by trypsin. Ten other elapid phospholipases A for which sequence data are available lack residues 1–8 and 70–74 [10–15]. Apart from the carbohydrate moiety and the extra disulfide, the γ-subunit of taipoxin is thus a strict homolog of the pig pancreatic proenzyme. It would be very interesting indeed to study the pancreatic phospholipase of the taipan.

The highly conserved stretch 33–60 contains the invariant histidine-56 and tyrosine-36 which are thought to be part of the active site [16,17].

If the 14 invariant half-cystines are paired as

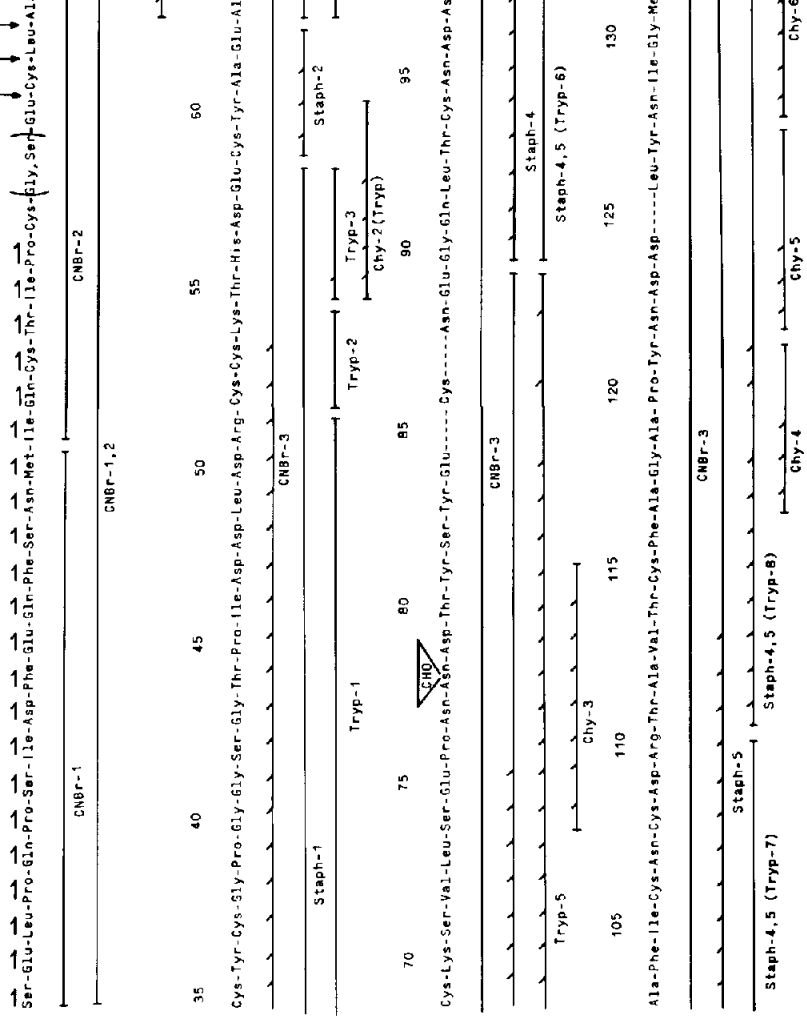

Fig.1. Alignment of the γ-subunit indicating the peptides used to establish the sequence. With the exception of the tryptic peptides 6–8, which were derived from a staphylococcal overlap peptide Staph 4,5, the tryptic peptides indicated were obtained by cleavage of the staphylococcal peptide indicated on the line above. The chymotryptic peptides were derived by cleavage of the intact reduced and S-carboxymethylated γ-chain. Vertical arrows indicate the points at which CNBr-2 was cleaved by thermolysin (Thn), the staphylococcal protease, and by chymotrypsin. Gaps (– – –) are placed at positions 85, 87, 125, 134 and 135 so as to have the same numbering as in the homology fig.2. The peptide chain is only 133 residues long.

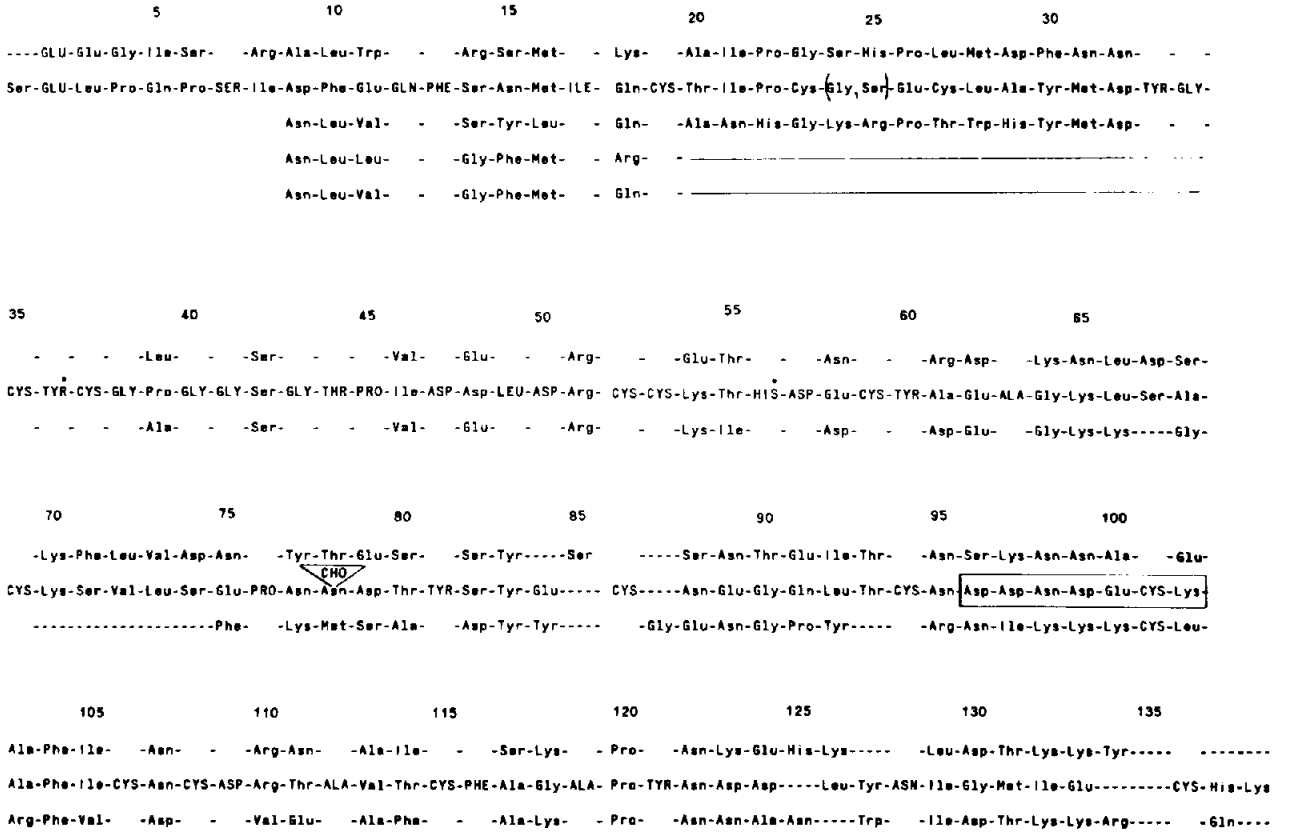

Fig.2. Alignment illustrating the homology between the γ-subunit of taipoxin, the pork pancreatic prophospholipase A2, and notexin. The amino terminal sequences of the α- and β-subunits of taipoxin are also indicated [1,19,20]. The amino acid sequences above are presented in the order: Pork pancreatic prophospholipase; Taipoxin γ-subunit; Notexin; Taipoxin α-subunit; Taipoxin β-subunit.

reported for the pancreatic proenzyme the γ-chain should contain an extra short disulfide loop connecting Cys-23 and Cys-27 [17].

The inaccessibility of His-56 to modification by *p*-bromophenacyl bromide [18] is puzzling. Perhaps the carbohydrate moiety sterically hinders the reaction.

The negatively charged stretch from residues 96–102 seems the likely candidate for the binding of the very basic α-subunit. This interaction apparently depends mainly on electrostatic bonds [1], and notexin has a very basic stretch in exactly the same region. By the same reasoning the C-terminus Glu–Cys–His–Lys might interact specifically with the β-subunit.

The γ-subunit of the taipoxin complex has been suggested to serve mainly as a chaperone to protect the essential α-subunit from fast renal elimination and proteolytic degradation and to sharpen the specificity at the target, perhaps helping to orient the complex to ensure proper binding and/or action of the α-subunit [18]. The elucidation of the primary structure has not shed light on this, but it is a prerequisite for future modification studies and for the crystallographic analysis now in progress. In the case of taipoxin, one ought to be able to map the target interaction surface directly since many surfaces on the α-subunit must be covered in the complex by interaction with the γ- and β-subunits.

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References

[1] Fohlman, J., Eaker, D., Karlsson, E. and Thesleff, S. (1976) Eur. J. Biochem. 68, 457–469.
[2] Kamenskaya, M. and Thesleff, S. (1974) Acta Physiol. Scand. 90, 716–724.
[3] Cull-Candy, S. G., Fohlman, J., Gustavsson, D., Lüllmann-Rauch, R. and Thesleff, S. (1976) Neuroscience 1, 175–180.
[4] Dowdall, M. J., Fohlman, J. and Eaker, D. (1977) Nature, in press.
[5] Belew, M., Fohlman, J. and Janson, J.-C. (1977) Separation News, Pharmacia, in press.
[6] Belew, M., Porath, J., Fohlman, J. and Janson, J.-C. (1977) J. Chromatogr., in press.
[7] Gross, E. and Witkop, B. (1962) J. Biol. Chem. 237, 1856–1862.
[8] Koida, M. and Walter, R. (1976) J. Biol. Chem. 251, 7593–7599.
[9] Austen, B. M. and Smith, E. L. (1976) Biochem. Biophys. Res. Commun. 72, 411–417.
[10] Joubert, F. (1975) Biochim. Biophys. Acta 379, 345–359.
[11] Joubert, F. (1975) Eur. J. Biochem. 52, 539–544.
[12] Joubert, F. (1977) Biochim. Biophys. Acta 493, 216–227.
[13] Obidairo, T. K., Tampitag, S. and Eaker, D. (1977) to be published.
[14] Halpert, J. and Eaker, D. (1975) J. Biol. Chem. 250, 6990–6997.
[15] Halpert, J. and Eaker, D. (1976) J. Biol. Chem. 251, 7343–7347.
[16] Volwerk, J. J., Pieterson, W. A. and De Haas, G. H. (1974) Biochemistry 13, 1446–1454.
[17] Drenth, J., Enzing, C. M., Kalk, K. H. and Vessies, J. C. A. (1976) Nature 264, 373–377.
[18] Fohlman, J., Eaker, D. and Dowdall, M. J. (1977) Eur. J. Biochem. submitted.
[19] Evenberg, A., Meyer, H., Gaastra, W., Verheij, H. M. and De Haas, G. H. (1977) J. Biol. Chem. 252, 1189–1196.
[20] Halpert, J. and Eaker, D. (1975) J. Biol. Chem. 250, 6990–6997.