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Identification of a Locality in Snake Venom α-Neurotoxins with a Significant Compositional Similarity to Marine Snail α-Conotoxins: Implications for Evolution and Structure/Activity

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Summary. α-neurotoxins from elapid snake venoms and α-conotoxins from marine snails bind specifically and with high affinity to nicotinic cholinoceptors. Although both types of toxin are polypeptides, there is more than a fourfold difference in size between the two and no clear sequence homology is evident. A systematic computer search of the three-dimensional structure of erabutoxin b (an α-neurotoxin from the false sea snake *Laticauda semifasciata*) was performed to identify the locality that most closely matched the amino acid compositions of the smaller α-conotoxins (from the marine snails *Conus magus* and *Conus geographus*). The area of greatest similarity centered on residue position 25 of erabutoxin b, a locale that is conserved throughout the snake α-neurotoxins and their homologues. Six proteins unrelated to erabutoxin b were compared to the α-conotoxins to show that the extent of the erabutoxin b/α-conotoxin match was too high to be coincidental. Homologues of erabutoxin b, namely α-cobratoxin from *Naja naja siamensis* and cytotoxin $V^{II}4$ from *Naja mossambica mossambica*, were also analyzed. The extent of the matching with the α-conotoxins decreased in the series erabutoxin b > α-cobratoxin > cytotoxin $V^{II}4$, and this also relates the order of similarity to the pharmacological properties of the α-conotoxins.

The α-conotoxin-like area of the snake α-neurotoxins is peripheral to the site previously considered important for binding to the cholinoceptor, even though it seems to represent the focus of evolutionary convergence between the two types of neurotoxin. The area of resemblance does, however, have strong associations with the conformational behavior of the snake toxins. Hence, the outcome of this study has important consequences for the current ideas on snake α-neurotoxin structure/activity relationships and the evolutionary origins of neurotoxicity.

Key words: α-Conotoxin — α-Neurotoxin — Erabutoxin b — Evolution — Venom

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Introduction

Elapid snakes (i.e., the cobras, kraits, mambas, tiger snakes, and sea snakes) and marine predatory snails (i.e., the Conidae) both use potent neurotoxins to capture their prey. Of particular interest are the postsynaptically active α-neurotoxins contained in the snake venoms (Dufton and Hider 1983; Endo and Tamiya 1987) and the postsynaptically active α-conotoxins from the Conidae (Olivera et al. 1985).

The α-neurotoxins from snake venom (e.g., α-bungarotoxin) are highly specific for the nicotinic acetylcholine receptors of skeletal muscle neuromuscular junctions (Chang 1979). In vivo, this action leads to a neuromuscular blockade and paralysis of the animal. The α-conotoxins also produce a selective block of skeletal muscle cholinoceptors (McManus et al. 1981; Hashimoto et al. 1985;

McManus and Musick 1985). It has been demonstrated that an α-conotoxin can compete with ^{125}I-labeled α-bungarotoxin for binding sites on mouse muscle preparations (McManus et al. 1981) and also that α-conotoxin can prevent the binding of monoclonal antibodies raised against the α-bungarotoxin binding site of *Torpedo* cholinoceptors (McManus and Musick 1985). The major differences between the α-conotoxins and the snake α-neurotoxins are quantitative rather than qualitative: the α-neurotoxins have higher affinities and are less readily reversible than the α-conotoxins. Because the two types of molecule bind to the same molecular target, despite their very different origins, their structural resemblances are of great interest to those wishing to understand how the specificity is achieved.

The snake α-neurotoxins have been studied in great detail (Dufton and Hider 1983; Endo and Tamiya 1987). They are proteins of 60–74 amino acids in length and include such examples as α-bungarotoxin, α-cobratoxin, and erabutoxin b. The three-dimensional structures of these three toxins have been solved by X-ray crystallography, and complementary spectroscopic studies have revealed their solution properties and their responses to chemical and environmental modification. Their target, the nicotinic cholinoceptor, is also very well characterized (Endo and Tamiya 1987), but as yet there is no clear picture of how the two molecules actually interact. However, very confident proposals have been made as to the key interactive areas of the α-neurotoxins (Dufton and Hider 1983; Endo and Tamiya 1987).

In contrast, the α-conotoxins are peptides of 13–15 residues and are therefore a quarter of the size of the snake neurotoxins. Although conformational data are lacking and there is no crystal structure available, three structural models have been proposed by Gray et al. (1985), Hider (1985), and Kobayashi et al. (1986). The first is based on circular dichroism spectra and secondary structure predictions, whereas the second is closely modelled on the conformation of those parts of the snake α-neurotoxins considered to be crucial for activity. The third model was obtained by NMR and a distance geometry algorithm. Gray et al. (1985) and Hider (1985) postulate that certain residues in the α-conotoxins imitate the juxtaposition of the major chemical moieties in the curare-like alkaloids. As this type of mimicry also has been proposed for several demonstrably important residues in the snake α-neurotoxins, the similar specificity of the snail and snake toxins is explainable with this hypothesis.

The evolutionary rationale that underlies the above reasoning is that the snake α-neurotoxins and the α-conotoxins have acquired specific chemical resemblances independently, because there are a very limited number of ways in which to achieve high activity and specificity toward nicotinic cholinoceptors. That is, they have converged during evolution toward the most optimal three-dimensional arrangement of the necessary chemical moieties. Certainly, the amino acid sequences of the α-conotoxins do not betray any direct evolutionary relationship with those of the snake α-neurotoxins, so convergent evolution seems the most likely explanation.

In the analyses of α-conotoxin structure/activity described above, it is clear that much has depended on the existing concept of how the snake α-neurotoxins achieve their toxicity. What have not been considered in detail, however, are the implications that the α-conotoxins hold for the snake α-neurotoxins. Because the α-conotoxins achieve a similar specificity with less than 25% of the bulk of the snake toxins, there is the potential to define even more closely the determinants of specificity in the latter. If the postulates concerning mimicry of curare-like compounds are correct, one might expect that the point of greatest chemical similarity between the snake and snail toxins would involve the curare-mimic region of the snake toxins. Although this is implied by previous studies on the α-conotoxins, no systematic search for the closest possible match between the α-conotoxins and a similar-sized locality in the snake α-neurotoxin molecules has ever been undertaken. This study was performed to remedy this omission and thereby to reexamine the current ideas as to how neurotoxicity is achieved.

Methods

Preliminary Considerations. The sequences of the four known naturally occurring α-conotoxins (from the species *Conus magus* and *Conus geographus*) (Olivera et al. 1985) are shown below using the standard one-letter code.

GI			E	C	C	N	P	A	C	G	R	H	Y	S	C	
GIA			E	C	C	N	P	A	C	G	R	H	Y	S	C	G	K
GII			E	C	C	H	P	A	C	G	K	H	F	S	C	
MI		G	R	C	C	H	P	A	C	G	K	N	Y	S	C	

(The sequences of two snake α-neurotoxins are shown in Tables 1 and 2.)

Although no direct sequence homology between the α-conotoxins and the snake toxins has been remarked upon by other investigators, nevertheless, a systematic search was performed for this study using the RELATE routine of the Protein Identification Resource (National Biomedical Research Foundation) as mounted in the SERC Daresbury laboratory. According to the criteria of this search routine, there was no significant similarity between these two kinds of toxin. This lack of sequence homology lessens the likelihood that the snake and snail toxins have evolved from the same ancestral peptide/protein and instead centers attention on the possibility that they have evolved convergently.

The identification of a locality in a protein that has evolved convergently with a smaller peptide is problematical for a number of reasons. Foremost is the fact that the course of the polypeptide backbone could be different and even discontinuous in such a

locality of the protein, so only the positions of the amino acid side chains may constitute the resemblance to the peptide structure. Secondly, some of the larger amino acid side chains possess considerable degrees of freedom, especially if they are located at the molecular surface. This means that there can be considerable difficulty in deciding if a given type of side chain occupies an equivalent position when two molecules are compared. At present, most of the information about the possible juxtapositions of the α-conotoxin side chains is contained in the largely theoretical models cited previously. NMR has provided more detailed evidence for some side chain proximities in the model by Kobayashi et al. (1986), but these experiments were performed in dimethylsulfoxide rather than in physiological media, and the results have not been published in a form for computer manipulation.

Overall, this lack of specific data meant that any search for an α-conotoxin-like environment within the superstructure of the snake toxins would have to be based solely on the composition of amino acid side chains within a specified area. In other words, the search would be for the closest local match to the amino acid composition of the α-conotoxins.

Analytical Procedure. The search procedure to be described has been incorporated into the molecular graphics package INTERCHEM (available from Interprobe Chemical Services Ltd., University of Strathclyde), which is mounted on the VAX cluster of computers at the University of Strathclyde.

The initial step was to select one of the three snake α-neurotoxins of known tertiary structure. Erabutoxin b from the false sea snake *Laticauda semifasciata* was chosen because, like the α-conotoxins, it would be optimized against marine prey (i.e., both the sea snake and snails are piscivorous). Thereby, the chemical differences between the two types of neurotoxin due to differences between the nicotinic cholinoceptors of the prey species should be at a minimum. Additionally, in terms of reversibility of the neurotoxic effect, the α-conotoxins resemble erabutoxin b more closely than they resemble α-bungarotoxin or α-cobratoxin (McManus et al. 1981; Dufton and Hider 1983; Hashimoto et al. 1985; McManus and Musick 1985; Endo and Tamiya 1987).

Next, an approximation was required of the molecular dimensions and shape of a typical α-conotoxin. According to the NMR studies of conotoxin GI, it is a compact molecule; this is also implied by the theoretical models proposed (Gray et al. 1985; Hider 1985; Kobayashi et al. 1986). Moreover, there are two disulfide bridges in the conotoxins that are linked so as to preclude a very extended configuration. Therefore, the conotoxins were viewed as roughly spherical molecules that might tend toward maximum compactness. Using other proteins as a guide, it was found that a sphere of radius 7 Å was around the minimum volume that could encompass successfully from 13 to 15 main-chain α-carbon atoms (64 instances of 13–15 α-carbons within a 7-Å-radius sphere were found, in contrast to only two instances of 16 or more α-carbons). Hence a 7-Å-radius sphere was taken to approximate the lower limit of the volume that the 13–15-residue conotoxin α-carbon skeleton might occupy.

The basic plan of the computer program was to proceed along the polypeptide chain of erabutoxin b, pausing at each of its 62 α-carbons to record all the other α-carbons of amino acid residues within 7 Å of that point. Each cluster of amino acids defined in this way (i.e., 62 clusters ranging from 3 to 15 amino acids) was then compared in terms of composition to the α-conotoxin amino acid compositions. Because there are four known conotoxin variants (GI, GIA, GII, and MI), the exercise was repeated for each variant. The degree of correspondence between the compositions of the clusters derived from erabutoxin b and the α-conotoxins was recorded simply as the number of matches.

e.g., Composition of 7-Å-radius cluster based on erabutoxin b residue 25 compared to composition of conotoxin MI

Residue	Erabutoxin b	Conotoxin MI	Matches
Alanine	0	1	0
Serine	2	1	1
Half-cystine	5	4	4
Tyrosine	1	1	1
Histidine	1	1	1
Lysine	1	1	1
Arginine	1	1	1
Glycine	2	2	2
Asparagine	1	1	1
Proline	0	1	0
Total match score			12

The amino acids were all ranked equally in importance and no attempt was made to weight some residue matches as being more significant than others. For example, because cystine residues are commonly regarded as purely structural, it might be supposed that they are not directly relevant to receptor recognition. Consequently, it may be argued that notable matches will be restricted to the cystine-rich regions of the proteins being analyzed. Although this is obviously true to some extent, we did not consider it warranted to reduce the significance of a half-cystine-to-half-cystine match (or for that matter, a proline-to-proline or glycine-to-glycine match) while we are in ignorance of the actual interaction between the toxins and their receptor. Indeed, the conformational properties bestowed on the molecules by the cystine bridges are thought to be important (see later). We did, however, ensure that relatively disulfide-rich proteins (i.e., phospholipase A_2 and bovine pancreatic trypsin inhibitor) were among those we anlayzed as controls (see below).

Other methods of expressing the compositional similarity were considered in an effort to take into account the variation in the number of residues specified within any given 7-Å radius (i.e., clusters could contain between 3 and 15 residues). For instance, the number of matches obtained for any cluster could be recorded as a percentage based on the total number of residues within the cluster. However, this system has the disadvantage of not distinguishing between, say, 3 matches in a cluster of 3 or 10 matches in a cluster of 10. Clearly, in terms of probability, the latter is far more noteworthy. Instead, we decided to retain the match score data in their simple form and to establish from other proteins which matches were within the bounds of coincidence and which were significantly unusual. This meant that comparisons did not have to be made between clusters that contained different numbers of residues. Hence, 1068 other 7-Å-radius clusters from cytochrome C, trypsinogen, carboxypeptidase A, phospholipase A_2, myoglobin, and bovine pancreatic trypsin inhibitor (which have no apparent relation to the mode of action of the α-conotoxins) were also matched to the four conotoxins.

Analysis of Other Snake Toxins. For completeness, the tertiary structures of two homologues of erabutoxin b, namely α-cobratoxin (from *Naja naja siamensis*) and cytotoxin $V^{II}4$ (from *Naja mossambica mossambica*), were searched for α-conotoxin matches in the same way. These toxins have basically the same folding pattern as erabutoxin b, but they differ to varying degrees in their structure and pharmacology. Whereas α-cobratoxin retains specificity for cholinoceptors despite some contrasts to erabutoxin b, cytotoxin $V^{II}4$ has no affinity for cholinoceptors, but does appear to interact with other types of membrane component (Dufton and Hider 1983, 1988; Endo and Tamiya 1987). Hence it is of interest to see if the extent of the α-conotoxin match in these examples reflects the increasing differences compared to erabutoxin b. For the cytotoxin, a predicted model of its tertiary structure was used, as described in Breckenridge and Dufton (1987).

The actual X-ray structure has been deduced (Rees et al. 1987), but at the time of writing, the coordinate data have not been placed on record. The predicted model is a very close approximation of the actual structure, however.

It is also necessary to point out here that the conformation of α-cobratoxin in solution differs from that in the crystal: the vicinity of position 19 is sensitive to the low pH of the crystallizing media (Walkinshaw et al. 1980; Hider et al. 1982). However, the conformational disturbance does not seem dramatic in terms of the α-carbon backbone, so the use of this crystal structure is still appropriate.

Snake Toxin Sequence Conservation. The significance of any given cluster highlighted in erabutoxin b also depends on how that cluster has been maintained during the evolution of this neurotoxin type as a whole. To this end, 27 sequential homologues of erabutoxin b (i.e., the "short" neurotoxins that contain 60–62 residues and four disulfide bridges) were compared, and the number of alternative side chains possible at each residue position was noted (Dufton and Hider 1983; Endo and Tamiya 1987). Then, using the 7-Å-radius cluster information, the average number of accepted amino acid changes per position was obtained for each cluster. Thus, every 7-Å-radius cluster in the erabutoxin b could be assessed in terms of evolutionary conservancy. This analysis was also performed for the homologues of α-cobratoxin (i.e., the "long" neurotoxins) and the homologues of cytotoxin $V^{II}4$.

Results

Comparison of α-Conotoxin Composition with Local Compositions in Erabutoxin b

In Table 1 is a detailed listing of the match scores obtained by comparing erabutoxin b with the four α-conotoxins. To place these results in a three-dimensional context, Fig. 1 represents the α-carbon backbone of erabutoxin b as revealed by X-ray crystallography (Low et al. 1976; Tsernoglou and Petsko 1976). At the site of each α-carbon, the number of residue composition matches found within the 7-Å radius is depicted by circles of varying diameter. As there are four α-conotoxin variants, the number of matches represented at each position is the average of the four results pertaining to that position. It is clearly shown by Fig. 1 that the highest match scores originate from near the disulfide-bridged area in the molecule. This is, of course, where the 7-Å-radius clusters will include the highest totals of α-carbons. Although the relationship between cluster size and match score will be considered further in the discussion, the highest match score concerns the 7-Å vicinity of position 25. Out of the 14 members of this cluster, up to 12 matches are obtained with α-conotoxins GIA and MI.

Comparison of α-Conotoxin Composition with Local Compositions in α-Cobratoxin and Cytotoxin $V^{II}4$

In Tables 2 and 3 are the match scores for α-cobratoxin and cytotoxin $V^{II}4$, respectively. There are

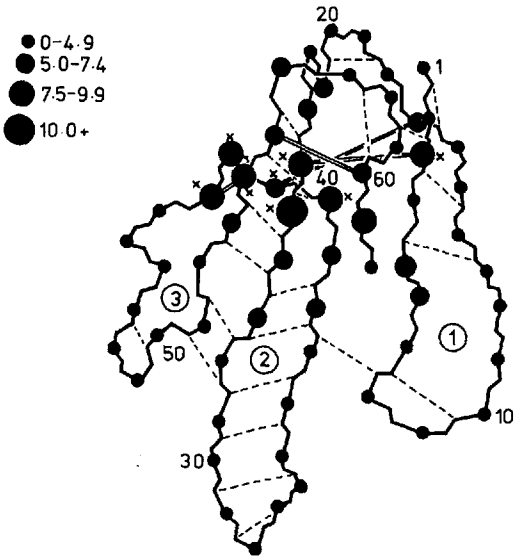

Fig. 1. The polypeptide backbone of erabutoxin b (Low et al. 1976; Tsernoglou and Petsko 1976) showing the disulfide bridges (double lines) and major hydrogen bonds (broken lines). At the site of each α-carbon, the diameter of the circle corresponds to the average match score found between the 7-Å-radius vicinity and the compositions of the four α-conotoxins. The larger the circle, the greater the number of matches (see key). An X denotes unusually high matches (see Fig. 2 and Discussion), whereas the circled numbers identify the major loops in the structure.

fewer high-scoring clusters in these two toxins, the maxima being the 9 matches out of 14 obtained at α-cobratoxin positions 19 and 20 (corresponding to erabutoxin b positions 23 and 24) and the 10 matches out of 15 obtained at cytotoxin position 3 (corresponding to erabutoxin b position 3). As with erabutoxin b, the highest scores relate to the central core areas of the molecules where the 7-Å-radius clusters are the most densely packed (the folding patterns of α-cobratoxin and cytotoxin $V^{II}4$ are very similar to that shown in Fig. 1). The addition of an extra disulfide bridge and a C-terminal extension in α-cobratoxin compared to erabutoxin b/cytotoxin $V^{II}4$ creates a relatively high-scoring cluster based on residue 30 (scores of 7 with α-conotoxins GIA, GII, and MI), which has no parallel in the other two toxins. This area approximates to erabutoxin b residues 30–34.

Comparison of α-Conotoxin Composition with Local Compositions in a Selection of Other Proteins

Cytochrome C, myoglobin, trypsinogen, carboxypeptidase A, phospholipase A_2, and bovine pancreatic trypsin inhibitor were analyzed to guide judgement in deciding what might be significantly high match scores for a given cluster size in the snake toxins. In total, these proteins provided 1068 7-Å clusters for comparison with the four α-conotoxin compositions (i.e., a total of 4272 comparisons).

Table 1. Local similarity between erabutoxin b and the α-conotoxins

Position	Sequence	Cluster size	Match GI	Match GIA	Match GII	Match MI
1	R	8	5	5	4	4
	I	9	4	4	4	4
	C	15	[8]	[9]	[8]	[8]
	F	12	4	5	4	5
5	N	10	5	6	5	6
	H	12	6	6	5	5
	Q	10	5	5	3	4
	S	8	3	3	3	2
	S	7	3	3	3	3
10	Q	7	3	3	3	3
	P	6	2	2	2	2
	Q	8	2	2	3	2
	T	8	2	3	3	3
	T	8	2	3	3	3
15	K	9	3	4	4	4
	T	7	4	5	4	5
	C	10	7	8	7	7
	S	6	5	5	5	4
	P	5	5	5	5	4
20	G	5	4	4	4	3
	E	9	6	7	5	6
	S	6	4	4	3	3
	S	12	8	8	7	7
	C	13	9	[10]	7	9
25	Y	14	[10]	[12]	8	[12]
	H	11	7	8	5	7
	K	11	5	6	4	5
	Q	9	2	3	3	2
	W	10	2	3	3	3
30	S	6	2	2	2	2
	D	6	3	3	3	3
	F	4	2	2	2	2
	R	4	2	2	2	2
	G	7	3	3	3	3
35	T	6	2	2	2	2
	I	10	4	4	4	3
	I	10	4	5	5	4
	E	11	5	6	6	5
	R	12	6	7	6	6
40	G	10	8	[9]	6	8
	C	9	7	[8]	6	[8]
	G	10	[9]	[10]	8	[9]
	C	9	[8]	[8]	7	[8]
	P	5	3	3	3	3
45	T	5	2	3	3	3
	V	6	1	2	2	2
	K	6	2	3	3	3
	P	4	2	3	3	3
	G	6	2	3	3	3
50	I	8	2	3	3	3
	K	7	1	2	2	2
	L	9	3	4	4	4
	S	7	5	6	5	6
	C	11	[9]	[9]	8	8
55	C	10	8	8	6	7
	E	8	6	6	6	5
	S	7	4	4	4	3
	E	7	5	5	4	4
	V	9	5	5	5	4
60	C	10	7	7	6	6
	N	11	8	8	6	8
	N	4	2	2	1	2

Significant matches (according to Fig. 2) are boxed

Table 2. Local similarity between α-cobratoxin and the α-conotoxins. Continued on page 360

Position	Sequence	Cluster size	Match GI	Match GIA	Match GII	Match MI
1	I	9	5	5	4	5
	R	10	5	6	5	6
	C	15	7	[8]	7	[8]
	F	13	7	8	7	8
5	I	10	5	5	5	5
	T	9	2	2	3	2
	P	5	1	1	1	1
	D	6	1	1	1	1
	I	6	2	2	2	2
10	T	6	2	2	3	2
	S	5	1	2	3	2
	K	7	4	5	5	5
	D	7	4	5	4	5
	C	9	6	7	5	7
15	P	8	6	6	4	6
	N	5	5	5	4	5
	G	8	6	6	5	6
	H	8	6	6	5	6
	V	14	[9]	[9]	8	[9]
20	C	14	[9]	[9]	7	[9]
	Y	13	7	8	6	8
	T	11	5	6	4	6
	K	10	1	2	1	2
	T	10	1	2	1	2
25	W	9	3	4	3	4
	C	6	3	4	3	4
	D	5	3	3	4	3
	A	4	2	2	3	2
	F	8	5	5	5	5
30	C	10	6	7	7	7
	S	6	4	4	4	4
	I	6	4	4	4	4
	R	6	4	4	4	4
	G	9	4	5	5	5
35	K	9	4	5	4	5
	R	7	3	4	3	4
	V	8	1	2	1	2
	D	8	1	2	2	2
	L	10	3	4	2	4
40	G	12	6	6	6	6
	C	11	7	8	6	8
	A	8	4	5	4	5
	A	8	5	5	5	5
	T	5	3	3	3	3
45	C	7	5	5	4	5
	P	5	2	2	2	2
	T	5	2	3	3	3
	V	7	1	2	2	2
	K	5	0	1	1	1
50	T	5	1	2	2	2
	G	4	1	1	1	1
	V	9	1	2	2	2
	D	7	0	1	1	1
	I	7	0	1	1	1
55	Q	6	2	3	2	3
	C	8	6	6	5	6
	C	8	6	6	5	6
	S	8	5	5	5	5
	T	7	4	4	3	4
60	D	10	6	6	4	6
	N	8	4	4	3	4
	C	9	5	4	5	5

Table 2. Continued from page 359

Position	Sequence	Cluster size	Match GI	Match GIA	Match GII	Match MI
	N	11	6	6	5	6
	P	7	3	3	3	3
65	F	3	1	1	2	1
	P	8	2	3	3	3
	T	6	2	3	2	3
	R	5	2	3	2	3
	K	8	3	4	3	4
70	R	6	2	3	2	3
	P	5	2	3	2	3

Significant matches (according to Fig. 2) are boxed

The resulting 4272 match scores are recorded versus respective cluster size in Fig. 2a. Some combinations (especially dense cluster/high score) are not observed in the data, although they do occur in the analysis of the three toxins. In particular, match scores of 10 or above are not observed, whereas scores of 8 and 9 are relatively rare (1% and 0.02% occurrence, respectively) and depend on cluster size. The data also show that a 7-Å-radius sphere is close to the lower limit for defining a 13–15-member α-carbon skeleton. The most common combinations are cluster sizes 6–11 with match scores 3–5.

Evolutionary Conservation at the 7-Å-Radius Cluster Level in Homologues of the Three Snake Toxins

Figure 3 again represents the α-carbon backbone of the erabutoxin b, but should now be understood as representing "short" neurotoxins in general. In this figure the size of the circle at each α-carbon site reflects the average number of different side chains per position that exists in the short neurotoxin sequence data for the 7-Å-radius cluster centered on that point. Thus, the smallest circles represent the most highly conserved localities.

Figure 3 shows there to be two noteworthy areas of conservation in this type of toxin. The largest (in terms of the 7-Å-radius clusters) is the extremity of the triple-stranded β-sheet, which involves part of the disulfide-bridged core. The second, smaller locality is the tip of the central loop centered on residues 32 and 33.

The homologues of α-cobratoxin (the "long" neurotoxins) reveal a very similar pattern of conservation despite the differences that exist in the polypeptide backbone in both the relevant areas (i.e., an insertion of two residues in the core-associated conserved area and an additional disulfide bridge/C-terminal extension near the central loop extremity). For the homologues of cytotoxin $V^{II}4$, the general level of evolutionary conservation is much

Table 3. Local similarity between cytotoxin $V^{II}4$ and the α-conotoxins

Position	Sequence	Cluster size	Match GI	Match GIA	Match GII	Match MI
1	L	8	3	4	3	4
	K	9	4	5	3	5
	C	15	9	10	6	9
	N	13	7	8	5	8
5	K	13	8	9	6	9
	L	10	5	6	4	6
	I	6	2	3	3	3
	P	7	3	4	3	4
	I	6	3	3	2	3
10	A	9	4	5	3	5
	Y	7	5	6	4	6
	K	10	6	7	5	7
	T	7	3	4	4	4
	C	10	5	6	6	6
15	P	6	4	5	5	4
	E	5	4	5	5	4
	G	5	4	5	4	4
	K	10	5	6	5	5
	N	7	3	4	3	4
20	L	12	7	8	6	8
	C	12	8	8	6	8
	Y	13	8	9	6	9
	K	11	4	5	2	5
	M	11	2	3	1	3
25	M	10	2	3	2	3
	L	10	2	3	3	3
	A	6	3	3	3	3
	S	6	2	3	3	3
	K	4	1	2	2	2
30	K	4	1	2	2	2
	M	7	3	4	4	4
	V	6	3	3	3	3
	P	8	2	3	3	3
	V	8	2	3	2	3
35	K	9	3	4	3	4
	R	11	4	5	2	5
	G	11	7	8	5	8
	C	11	7	8	6	8
	I	8	4	5	4	5
40	N	8	3	4	3	4
	V	6	3	3	2	3
	C	7	5	6	4	6
	P	5	3	4	3	4
	K	5	4	5	4	5
45	N	7	4	5	4	5
	S	5	3	4	3	4
	A	5	3	3	2	3
	L	4	1	1	1	1
	V	9	4	5	3	5
50	K	7	2	3	1	3
	Y	7	1	2	1	2
	V	6	2	3	2	3
	C	9	6	7	6	7
	C	8	6	6	5	6
55	S	8	5	5	4	5
	T	6	4	4	3	4
	D	8	4	5	4	5
	R	8	4	5	3	5
	C	8	5	5	3	5
60	N	10	6	7	4	7

Significant matches (according to Fig. 2) are boxed

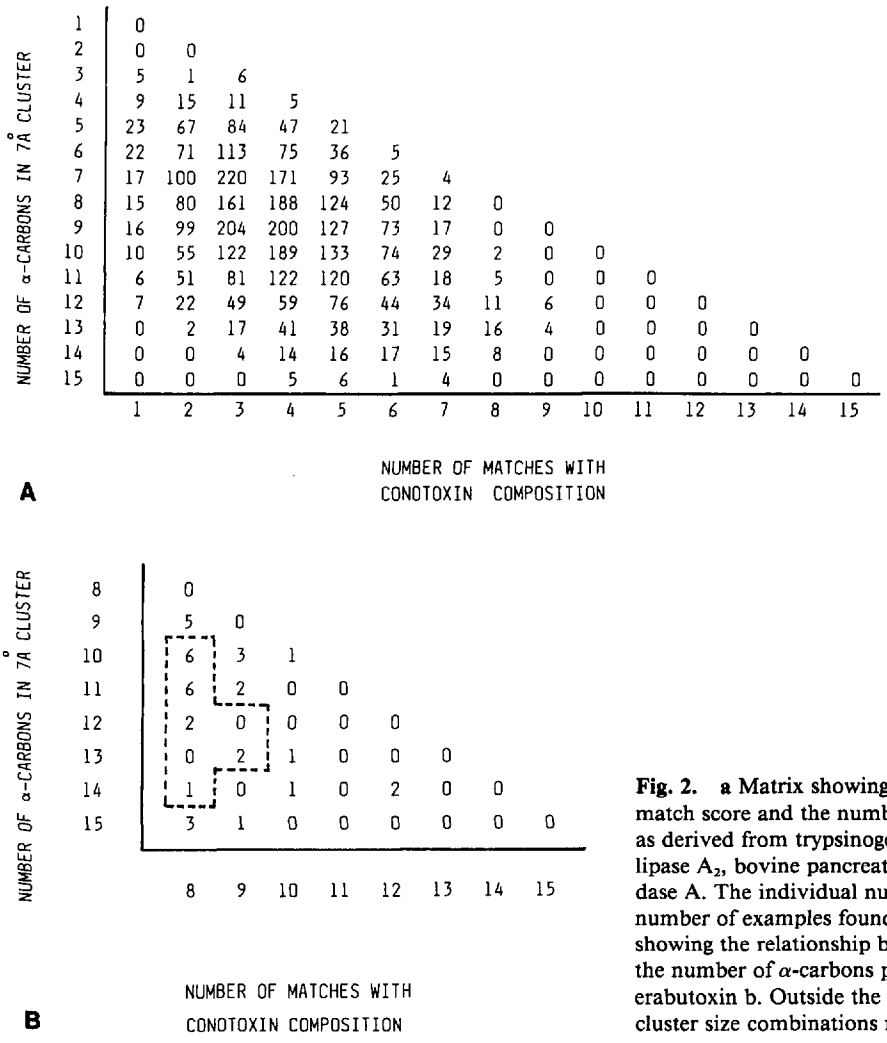

Fig. 2. **a** Matrix showing the relationship between α-conotoxin match score and the number of α-carbons per 7-Å-radius cluster as derived from trypsinogen, cytochrome C, myoglobin, phospholipase A_2, bovine pancreatic trypsin inhibitor, and carboxypeptidase A. The individual numbers within the matrix denote the number of examples found for each combination. **b** Partial matrix showing the relationship between α-conotoxin match score and the number of α-carbons per 7-Å-radius cluster as derived from erabutoxin b. Outside the boxed section are the 19 match score/cluster size combinations not occurring in Fig. 2a.

higher, but the extremity of the β-sheet near the disulfide bridges is again prominent as the most highly conserved 7-Å-radius locality. It centers on residue 21, which is analogous to erabutoxin b residue 24. Unlike the two types of neurotoxin, however, the tip of the central loop is not conserved. Instead, a second highly conserved area is centered on residue 13 (analogous to erabutoxin b residue 16).

Discussion

Significance of Match Scores

The outcome of the comparison of all four α-conotoxin compositions with erabutoxin b is that the greatest compositional similarity exists in the 7-Å neighborhood of tyrosine 25. However, before this result is interpreted, it is necessary to consider how much emphasis can be placed on individual match scores. The most obvious factor to consider is that the clusters vary in size between 3 and 15 members, so the number of matches obtainable for any cluster will be highly dependent on this. The information contained in Fig. 2a provides a means of judging unusual match scores because it presumably reflects the random expectation from proteins that have no known connection with the neurotoxin/cholinoceptor mechanism. This figure shows that as the number of residues in a cluster increases beyond seven, the occurrence of maximum, or very high, match scores decreases until an upper limit of nine matches is obtained for cluster sizes 12 and 13. Even the score of 9 is obtained only in 10 instances. The cluster size/match score data for erabutoxin b show a distribution that is much more biased toward high match scores for cluster sizes 9–15 (Fig. 2b). So much so, that there are 19 instances of match scores 8–12 that do not occur in Fig. 2a. The eight residue positions giving rise to these unusually high matches are highlighted in Fig. 1 and Table 1. A detailed listing of the 7-Å neighborhood of these positions is given in Table 4.

Thus, although it is inevitable that the highest match scores obtained with erabutoxin b will arise from the most densely packed parts of the molecule, the extent of the matching is consistently higher over

Table 4. The 7-Å radius clusters in the snake neurotoxins that show unusually high similarity to α-conotoxin composition

Erabutoxin b/short neurotoxins
1(R), 2(I), 3(C), 4(F), 5(N), 14(T), 15(K), 16(T), 17(C), 24(C), 40(G), 41(C), 58(E), 59(V), 60(C)
3(C), 23(S), 24(C), 25(Y), 40(G), 41(C), 42(G), 43(C), 54(C), 55(C), 56(E), 60(C), 61(N)
23(S), 24(C), 25(Y), 26(H), 27(K), 39(R), 40(G), 41(C), 42(G), 43(C), 53(S), 54(C), 55(C), 61(N)
3(C), 4(F), 5(N), 24(C), 25(Y), 38(E), 39(R), 40(G), 41(C), 42(G)
3(C), 17(C), 23(S), 24(C), 25(Y), 40(G), 41(C), 42(G), 43(C)
21(E), 23(S), 24(C), 25(Y), 40(G), 41(C), 42(G), 43(C), 44(P), 54(C)
23(S), 24(C), 25(Y), 41(C), 42(G), 43(C), 44(P), 45(T), 54(C)
23(S), 24(C), 25(Y), 26(H), 42(G), 43(C), 52(L), 53(S), 54(C), 55(C), 56(E)
α-cobratoxin/long neurotoxins
1(I), 2(R), 3(C), 4(F), 5(I), 12(K), 13(D), 14(C), 19(V), 20(C), 40(G), 41(C), 60(D), 61(N), 63(N)
1(I), 3(C), 17(G), 18(H), 19(V), 20(C), 21(Y), 41(C), 42(A), 43(A), 57(C), 58(S), 59(T), 60(D)
3(C), 19(V), 20(C), 21(Y), 22(T), 40(G), 41(C), 42(A), 56(C), 57(C), 58(S), 60(D), 62(C), 63(N)

The central residue of each cluster is underlined

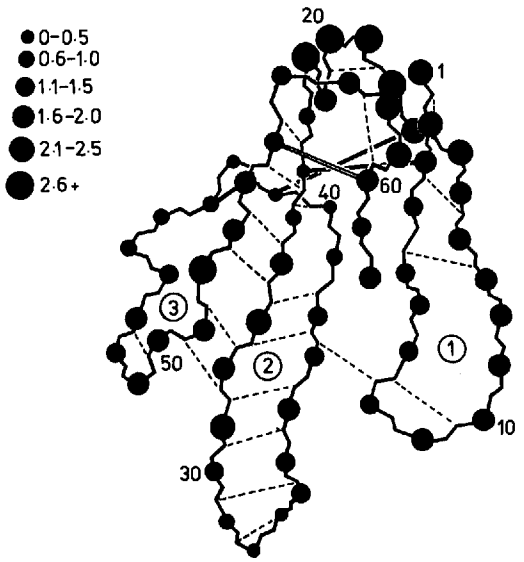

Fig. 3. The polypeptide backbone of erabutoxin b showing the sequence variation among the short neurotoxins. The diameter of the circle at each α-carbon corresponds to the average number of known side chain variants per residue for its 7-Å-radius vicinity (see key). The smaller the diameter of the circle, the less the 7-Å-radius vicinity has undergone change during the course of evolution. The circled numbers identify the major loops in the structure.

a range of cluster sizes compared to the extent seen with any of the control proteins. Although it is true that many of the significant match scores are in fact on the periphery of the distribution in Fig. 2a, the 10 matches out of 10 for the cluster around position 42 (with conotoxin GIA) and the 12 matches out of 14 for the overlapping cluster at position 25 (with conotoxins GIA and MI) are outstanding. With α-cobratoxin and cytotoxin $V^{II}4$, there are fewer match scores that fall outside the distribution established in Fig. 2a: α-cobratoxin exhibits eight instances (Table 2), whereas cytotoxin $V^{II}4$ exhibits only three (Table 3). Hence, although all three types of snake toxin show unusually high local resemblances to α-conotoxin composition, the extent is evidently erabutoxin b > α-cobratoxin > cytotoxin $V^{II}4$. From among the total of 30 snake toxin clusters that show higher than expected match scores in relation to their size, some deserve additional comment. That is because they involve match scores of 8 and 9 for 14- and 15-residue clusters, yet the same scores are observed in Fig. 2a for smaller cluster sizes (i.e., 10–13 members). Despite the progressively lower incidence of clusters with 13, 14, and 15 members, it might have been expected that the average chance of a match score at the level of 8 or 9 would increase, not decrease. One possible explanation is that the specified α-conotoxin compositions are difficult to accommodate in the most closely packed clusters obtainable because of the average side chain bulk. Thus, in these instances of close packing natural selection may conspire to reduce the likelihood of match scores at the higher end of the range. Hence, the unusual match scores obtained with the neurotoxins at cluster sizes 14 and 15 can still be significant. Accordingly, in assessing the worth of the match scores in erabutoxin b, α-cobratoxin, and cytotoxin $V^{II}4$, certain match scores of 8 and 9, and all greater, can be considered as progressively more unlikely to have arisen by coincidence.

One proviso that should be mentioned concerns the proportion of the α-conotoxin molecule that is necessary for neurotoxicity. If less than 50% of the molecule determines its function, then Fig. 2a shows that it is not possible to distinguish a significant match from a coincidental match. In other words, obtaining seven matches or less between the snake toxins and the α-conotoxins is within the bounds of coincidence, so if there was indeed a functionally significant match at these levels, it would not be highlighted. However, even if this were the case, the fact remains that the α-conotoxin molecule as a whole has a uniquely high resemblance to part of the snake neurotoxin structure. In this context, there

are two pieces of circumstantial evidence that tend to support the significance of the match scores. First, the α-conotoxin compositions match better with erabutoxin b than with either α-cobratoxin or cytotoxin $V^{II}4$. As pointed out earlier, α-conotoxins resemble erabutoxin b more in terms of reversibility of the neuromuscular blockade, and erabutoxin b has more relevance to marine prey than α-cobratoxin. Cytotoxin $V^{II}4$, meanwhile, does not bind to cholinoceptors. Second, of all the α-conotoxin variants, GII consistently gives lower match scores than the others (Tables 1–3). In comparisons of potency between conotoxin variants GI, GII, and MI, GII is markedly the least potent (Nishiuchi and Sakakibara 1984; Hashimoto et al. 1985).

Evolutionary Conservation in the Snake Toxins

From Figs. 1 and 3, it will be noted that the area of highest evolutionary conservation in the short neurotoxins coincides with the area that produces the most significant match scores in erabutoxin b. This is the extremity of the triple-stranded β-sheet near the disulfide bridges. Hence, if this region is indeed the focus of evolutionary convergence between the α-conotoxins and erabutoxin b, it is maintained throughout the short neurotoxins. The locality of position 3 in erabutoxin b also produces a significant match with the α-conotoxins, but it only partially overlaps the main area of resemblance, and it has undergone appreciable evolutionary change in related sequences. The second, smaller center of evolutionary conservation, which does not coincide with any resemblance to the α-conotoxins, includes a region that may constitute an acetylcholine mimic (Dufton and Hider 1983; Endo and Tamiya 1987).

For α-cobratoxin and its homologues, much the same description is appropriate. The extremity of the β-sheet near the disulfide bridges is again outstanding, both from the point of view of its resemblance to the α-conotoxins and its evolutionary conservation in the long neurotoxins as a whole. However, there is generally less conservation in this area and less resemblance to the α-conotoxins relative to erabutoxin b (Table 2). A significant match with the α-conotoxins also exists in the vicinity of position 3, but as with erabutoxin b, the area is not strongly conserved in the known sequence homologues.

Cytotoxin $V^{II}4$ shows significant matches with the α-conotoxins only at position 3 (directly equatable with position 3 in both erabutoxin b and α-cobratoxin). This is not the most highly conserved part of the molecule when the other homologues are considered. In fact, the area that does compare on this basis is again the extremity of the β-sheet in the vicinity of residue 21.

The findings for all three types of toxin can be summarized as follows. First, all three exhibit significant matches with the α-conotoxins at position 3, but in no case is the area very prominent in terms of evolutionary conservation within each toxin family. Second, all three toxin types show the strongest evolutionary conservation at the extremity of their β-sheet near the disulfide bridges, but the number of significant matches obtained from this area decreases in the series erabutoxin b (15) > α-cobratoxin (6) > cytotoxin $V^{II}4$ (0). It is particularly interesting to note that if the snake toxin sequence data were treated as a whole instead of being subdivided into short neurotoxins, long neurotoxins, and cytotoxins, it would be the position 3 vicinity that would appear as the most conserved locality.

As will be described in the following section, previous investigations have suggested that the β-sheet/disulfide bridge region given prominence above is involved primarily in determining molecular structure, not target recognition. Although it is always difficult to distinguish structural and functional roles for residues within a protein, this is a clear instance where a predominantly structural role is regarded as most likely. To guide our judgement on this point, we applied the method of determining the average number of alternative side chains per 7-Å-radius cluster to the "Kunitz" type serine proteinase inhibitors. The rationale behind the exercise was to discover if the most evolutionary conserved 7-Å-radius clusters were related to the interactive site [which has been very well characterized experimentally (Chen and Bode 1983)] or to the highly conserved folding motif in this series of molecules. These inhibitors are similar in size to the snake neurotoxins, contain three disulfide bridges, and a wide variety of homologues are known. The outcome of this analysis, based on bovine pancreatic trypsin inhibitor and the sequence variation in 14 homologues with known antiprotease activity (Dufton 1985) was the clear identification of the 7-Å-radius neighborhood of residue 36, which constituted almost the entire interactive site, as the most conserved. Although it is arguable whether it is valid to compare the evolutionary behavior of the snake toxins and the proteinase inhibitors, it has been generalized by others, for example by Bajaj and Blundell (1984), that functionally important sites show the greatest evolutionary conservation because passive structural features can be achieved in a variety of ways.

Significance to Current Concepts of Snake Neurotoxin Structure/Activity

According to the established view of the interactive site of the snake α-neurotoxins, the crucial area involves the emergent parts of loops 2 and 3 (Fig. 1),

which comprise the body of the triple-stranded β-sheet in the molecule (Dufton and Hider 1983; Low and Corfield 1986; Endo and Tamiya 1987). In particular, one face of this β-sheet is thought to provide the main interaction via (in erabutoxin b) residues 25, 27, 29, 31–34, 36, 38, 40, 44, 46, 47, 49, 50, and 52 (Low and Corfield 1986). Functionally prominent among these are lysine 27, tryptophan 29, aspartate 31, phenylalanine 32, arginine 33, and lysine 47 (Endo and Tamiya 1987). There are very good reasons for focusing attention on this area, such as the results obtained from chemical modification, antibody neutralization, and the evolutionary conservation of side chain character. The residues named above, by reason of their character and juxtaposition, have been described as forming a tubocurarine and/or an acetylcholine mimic (Dufton and Hider 1983; Endo and Tamiya 1987).

However, comparing this putative interactive area with the composition of the most α-conotoxin-like environment in erabutoxin b [i.e., that centered on tyrosine 25 (Fig. 1 and Table 4)], it can be seen that the only common components are tyrosine 25, lysine 27, and glycine 40. Instead, many of the residues that form the α-conotoxin-like locality are those that are thought to be important from a structural point of view. Besides the glycine and cystine residues, which are often expected to be limited to the performance of intramolecular structural duties, both asparagine 61 and tyrosine 25 are heavily implicated in structure-oriented roles (Low and Corfield 1986; Endo and Tamiya 1987). Nevertheless, because this tyrosine is an integral part of the proposed interactive site, it has been included among the other functional residues. Most notably, Tamiya et al. (1980) included it in their proposal for a *d*-tubocurarine mimic. Residues 23 (serine), 26 (histidine), 39 (arginine), and 53 (serine) are not regarded as essential for neurotoxicity; this is partly because of their observed evolutionary variance, and partly because of their disposition away from, or on the wrong side of, the β-sheet. Histidine 26, however, has received attention because spin-labeling experiments show that along with residues 1 and 2, it is perturbed by the binding process. This has been difficult to reconcile with the notion that only one face of the β-sheet is directly involved in the toxic interaction (Endo and Tamiya 1987).

As regards structural properties, it should be noted that the snake neurotoxins are not entirely rigid molecules under physiological conditions. There is a large body of evidence to show that there is a conformational equilibrium present that is readily perturbable by heat, specific residue modification, and changes in solvent. This equilibrium has been identified throughout the short and long neurotoxins and it may also be present in the related cytotoxins, so the suggestion has been made that it is fundamental to the achievement of toxicity, whether neurotoxicity, cardiotoxicity, or otherwise (Dufton and Hider 1983). The scope, origin, and maintenance of this conformational balance have proved difficult to define, but there are several experimental indicators to the effect that the tyrosine 25 vicinity is partially or wholly involved (e.g., Inagaki et al. 1978, 1980, 1982; Tsetlin et al. 1979). Overall, it would appear that at least five members of the α-conotoxin mimic in erabutoxin b (i.e., tyrosine 25, histidine 26, lysine 27, serine 53, and half-cystine 54) are involved in a locale that is conformationally flexible or mobile under physiological conditions.

α-cobratoxin also displays evidence of a very similar conformational equilibrium under physiological conditions. In particular the temperature dependence of its NMR has highlighted the involvement of valine 19, half-cystine 20, lysine 23, leucine 39, and half-cystine 41 (these correspond to erabutoxin b residues 23, 24, 27, 39, and 41, respectively) (Hider et al. 1982). Moreover, in addition to there being a conformational change around histidine 18 associated with a lowering of pH (as previously mentioned), there is evidence that an equilibrium between similar before-and-after conformational states exists in related long neurotoxins at physiological pH (Endo and Tamiya 1987). Thus, the area in the long neurotoxins that corresponds most closely to the α-conotoxin composition is involved in an equilibrium between a dominant (90%) native state and a minority pH denatured type state. In fact, Endo and Tamiya (1987) already have postulated that this conformational interconversion might influence the biological function of these molecules. As was also shown by these authors, the temperature-sensitive and pH-sensitive conformational locales of α-cobratoxin overlap in the very region that bears most resemblance to the conotoxin compositions [i.e., *all* the members of the 7-Å-radius cluster at position 20 (Table 4) are implicated in the pH-sensitive and temperature-sensitive domains].

To sum up, those areas of the snake α-neurotoxins that bear most compositional resemblance to the α-conotoxins have more involvement with the fundamental structural and conformational organization of the snake toxin group than with the supposed functional locale. The two issues are not separate, however, because the evidence also suggests that the properties of the structural and functional residues are interdependent. If the vicinity of tyrosine 25 in erabutoxin b and its relatives is indeed the actual focus of evolutionary convergence with the α-conotoxins, it is implied that intramolecular structural criteria may dominate the achievement of neurotoxicity. In other words, it suggests that a particular conformational property, such as a specific shape

and/or a defined allosteric response, might be the major prerequisite. Certainly, as regards the α-conotoxins, up to 50% of the residues are cystine, proline, and glycine, and some dramatic losses of toxicity have followed either rearrangement of the disulfide bridges or replacement of the proline and glycine residues (Nishiuchi and Sakakibara 1984; Hashimoto et al. 1985). Most significantly perhaps, conotoxin MI has shown evidence of two slowly interchanging conformational states in solution (Gray et al. 1988).

Concluding Remarks

The results presented herein do not question the functional importance of those areas of the snake α-neurotoxins that already have been identified as being involved in receptor binding. Instead, they could mean that a change in emphasis is needed. Thus, the evolutionary origins of neurotoxicity in the snake toxins appear to be firmly connected with that part of the structure which is common throughout and provides the fundamental definition of this type of protein. Whereas the major part of the triple-stranded β-sheet in the snake neurotoxins has been looked upon as being central to the neurotoxic capability, it may now have to be seen as an extension of, or a directing influence upon, the more evolutionarily ancient α-conotoxin-like area. This latter area, containing as it does disulfide bridges and other structural residues, may have been dismissed too lightly in its envisaged role of solely determining the correct remote framework on which the major site could be built. The possibility now has to be entertained that it is this region that provides the basic means for targeting certain types of membrane-bound receptor.

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