THE CRYSTAL STRUCTURE OF A POST-SYNAPTIC NEUROTOXIN FROM SEA SNAKE AT 2.2 Å RESOLUTION

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1. Introduction

Among the most lethal proteins in the venoms of sea snakes and elapid land snakes are the post-synaptic neurotoxins. These act by blocking the nicotinic acetylcholine receptor of the muscle motor end plate [1], thus preventing transmission across the cholinergic synapse. Because of their extremely specific and tight binding to the receptor (K_D is on the order of 10^{-11}), these toxins have been the object of intensive chemical study [2], and have also been used to isolate the receptor protein [3,4]. Up to now there has been no information on the three-dimensional structure of the neurotoxins to aid in understanding their mode of action. We have solved the crystal structure of a neurotoxin from the sea snake *Laticauda semifasciata* from the Philippines Sea. This is a 'short' neurotoxin of 62 amino acids cross-linked by four disulfide bridges [5]. The structure has been determined at 2.2 Å resolution. The molecule is a disc with one extended loop containing most of the residues believed essential for toxicity. We conclude that this structure is common to all snake venom neurotoxins, and that the post-synaptic toxins act by inserting the loop into a cleft or channel in the acetylcholine receptor.

2. Experimental

Purified neurotoxins a and b from Philippines sea snake were a gift of Dr A. T. Tu. Crystals were grown from 40% saturated ammonium sulfate adjusted to pH 7.5 with 5 mM phosphate buffer. Their space group is $P2_12_12_1$ with one molecule per asymetric unit. The unit cell dimensions are a = 49.5 Å, b = 46.6 Å, c = 21.1 Å for toxin a . The corresponding values for toxin b are 49.9, 46.6, and 21.3 Å. (For a general review of the terms and method of protein crystallography, Eisenberg's article [6] may be consulted.)

Survey of isomorphous heavy atom derivatives proved extremely difficult, requiring over 300 experiments, probably due to the small number of reactive groups of the toxin and to close packing of the crystals (V_m = 1.77 Å^3/dalton) which makes them inaccessible to bulkier heavy atom compounds. Our task was made even harder by the fact that due to the scarcity of toxin all the experiments were performed on 1.5 mg of material.

Intensities of the native crystals of toxin b were measured to a resolution of 1.38 Å (over 90% are greater than 2 σ). Two heavy atom derivatives, platinum and mercury, were measured to 2.2 Å; anomalous differences were also measured. Phases were calculated in the usual way [6]. The mean figure of merit was 0.76. Full details of the crystallographic procedures will be published elsewhere.

3. Results

An electron density map was constructed, from which it was possible to trace the entire chain of the protein. A portion of the map is shown in fig.1. The map is of excellent quality showing carbonyl oxygen bumps and resolved sulfur atoms in the disulfide bridges.

Since the sequence of our toxin is not known but its composition is reported to be only slightly different

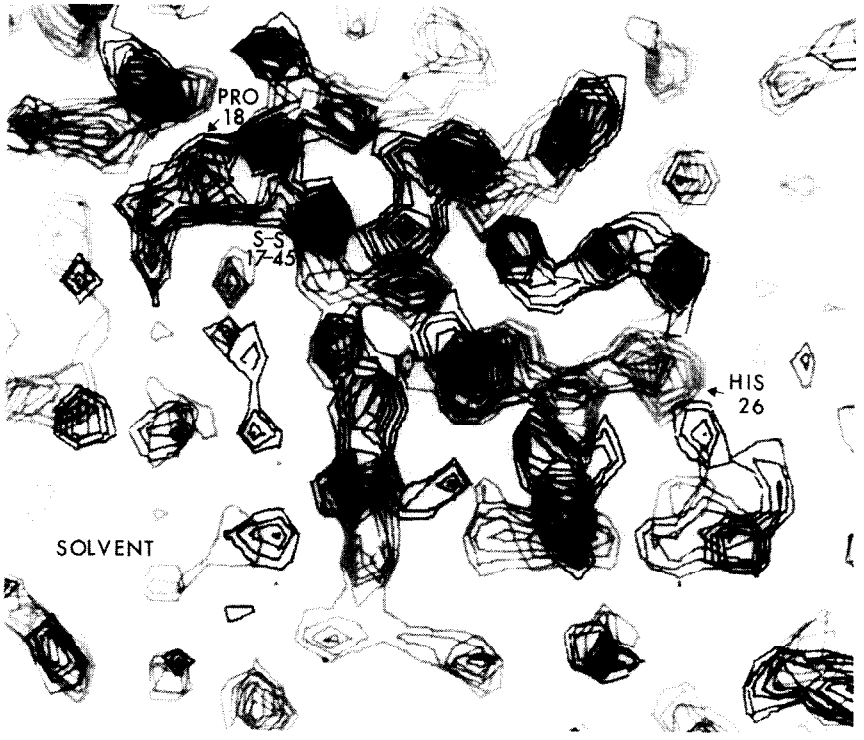

Fig.1. 4 Å slice through the electron density map of the neurotoxin. Contours are drawn at intervals of 0.2 e/Å^3, the first contour level being 0.4 e/Å^3 above the average density in the unit cell. Several residues are labeled, and an intermolecular solvent region is indicated.

from that of erabutoxin b [5], the latter sequence was fitted to the electron density map. This was quite satisfactory. Though it is impossible to obtain unambiguous sequence information from a protein electron density map, it appears that our toxin is very similar, or perhaps identical, to erabutoxin b. We have additional evidence based on our work on toxin a (to be published) that the differences between toxins a and b are smaller than reported earlier [5].

The molecule is extremely flat and notably extended in one direction (figs.2 and 3). It may be described as a disc with one protruding loop of chain. This loop contains residues 25 through 44 (homology alignment sequence numbering, reference 7) which form antiparallel β-pleated sheet. The other principal structural feature is the core of clustered disulfide bridges. All of the disulfides are close together and the molecule is folded around them. All the sequences of the snake venom toxins determined so far, including the 'long' neurotoxins such as α-bungarotoxin, can be accommodated in the structure with only minor adjustments. The deletion from 5 through 7 is on an external loop and the insertions 32 through 36 and 47 to 48 could both form short external loops without disturbing the remainder of the structure. In particular, the 32–36 'extra disulfide' stretch in the long neurotoxins can fit onto the end of the protruding loop without altering the relative positions of the important residues Trp 29 and Arg 37. The extra run of seven amino acids at the end of the long toxins poses no difficulty, as the C-terminus is free and on the surface of the molecule. We therefore predict that all snake venom neurotoxins, short and long, will have the same general structural features as our toxin. Further, we expect the cardiotoxins and direct lytic factors to be similar. Our structure is unlike that predicted from indirect chemical evidence [7].

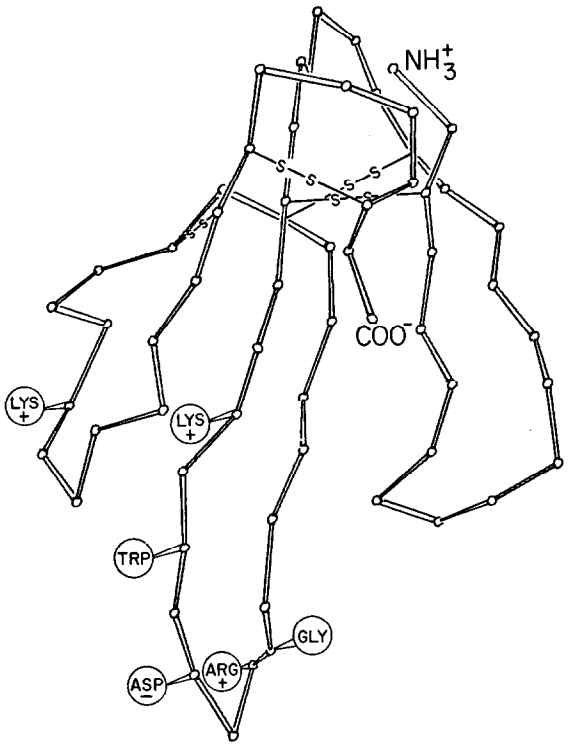

Fig.2. Perspective drawing of the course of the polypeptide chain of the sea snake neurotoxin. The circles indicate approximate α-carbon positions. The six residues believed essential for toxicity are indicated.

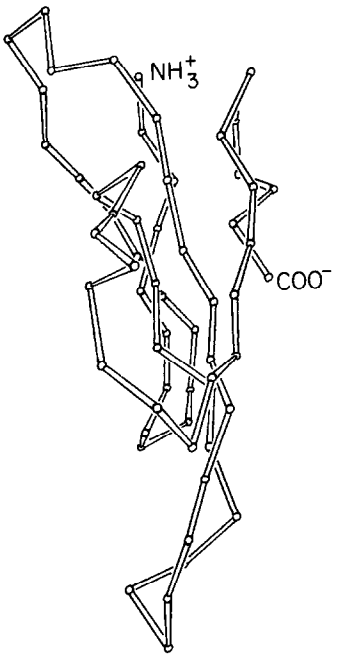

Fig.3. Drawing of the course of the main chain in a view orthogonal to that of fig.2. The molecule has been rotated 90° about a vertical axis lying in the plane of the paper. The disulfide bridges have been omitted to avoid excessive overlap.

4. Discussion

Comparison of the amino acid sequences of snake neurotoxins, cardiotoxins and lytic factors has led to the identification of twelve invariant residues as essential for maintaining tertiary structure. At least six other residues invariant in all neurotoxins but different in non-neurotoxins are thought to be involved in neurotoxic activity. The latter is supported by chemical modification studies. All of these observations (summarized in reference [7]) are explained by our structure:

The internal position of the four disulfides indicates that they are critical for maintaining the core of the molecule. Tyr 25 is completely buried, and is involved in the formation of the hydrophobic interior; in addition, the phenolic –OH seems to be involved in internal hydrogen bonding. Residue 44 must always be glycine since it is in an area where two disulfides come close together, leaving no room for a longer side chain. The proline at position 50 is necessary to force the chain away from the disulfide core for the third external loop, and Asn 67 seems to be involved in a number of hydrogen bonds. These interactions will be described in detail in a future paper.

The positions of the 'toxic' residues are even more striking. Five of them are contained in the extended loop of antiparallel β-pleated sheet pointing straight down in figs.2 and 3.

Five of the six (all but Gly 38) are also pointing in the same direction (into the plane of the paper in fig.2), forming an 'active surface'. The β-pleated sheet would seem to have the dual role of maintaining a rigid conformation for the extended loop and ensuring that most of the 'toxic' side chains lie on the same of the loop. The inescapable conclusion is that this active

surface is the principal site of interaction with the acetylcholine receptor, and a number of inferences can be drawn about the nature of this interaction. There are three residues on this surface which bear a positive charge at physiological pH: Lys 27, Lys 53, and Arg 37. Any one may bind to the receptor site for the positive charge of acetylcholine, but because of its position at the tip of the loop Arg 37 is most attractive for this crucial role. We believe Asp 31 interacts with the receptor either by ionic or hydrogen-bonding, and Trp 29, which in the unbound toxin is exposed to the solvent, contributes to the tightness of binding by hydrophobic interaction with some residue or residues of the receptor. The roles of Lys 27, Lys 53 and Gly 38 are less clear, but it seems likely that they too interact with the receptor to stabilize the complex. Briefly, we believe that this neurotoxin (and therefore by analogy all snake venom post-synaptic neurotoxins) binds to the acetylcholine receptor through a combination of ionic and hydrophobic interactions, the principal driving forces being the binding of Arg 37 to (most likely) the acetylcholine cation binding site, and the burying of the exposed hydrophobic side-chain of Trp 29. We also believe the structure of the neurotoxin provides insight into the structure and function of the acetylcholine receptor itself. The toxin structure is striking for its remarkable shape and the concentration of key side-chains on one extended loop. This suggests that the toxin binds by inserting the loop into a cleft or channel in the membrane-bound receptor. Indeed, the size of the extended loop provides some information about the size of the cleft or channel. It must be at least 19 Å deep and 7 Å wide. (This is on the assumption that the cleft is defined by a single receptor promoter. If the receptor is a complex of four to six subunits, however, and the cleft is located at the center of the complex and defined by all the subunits, then it can be 30 to 40 Å wide if each subunit binds one toxin.) The acetylcholine binding site(s) is about 20 Å from the surface according to this model.

If, as many workers believe, the receptor and ion channel are one and the same, the positive charges on the active surface may be attracted into the channel, aiding the binding of the toxin. This would also explain the large size of the cleft. Further studies on the structure of the receptor itself (e.g., [8]), and of homologous neurotoxins and cardiotoxins (underway in our laboratory) will shed light on these ideas.

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