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Structure and Function of Snake Venom Curarimimetic Neurotoxins

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SUMMARY

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A hydrogen-bonded ion pair between the guanidinium group of the arginine at position 37 and the side-chain carboxylate of the aspartic acid residue at position 31 of sea snake venom neurotoxin has stereochemical features resembling those of acetylcholine. The results of crystallographic determination of the structure of the neurotoxin suggest that such an ion pair can form at the tip of the "toxic loop" and may be involved in inhibition of the cholinergic receptor.

INTRODUCTION

Curarimimetic neurotoxins are small proteins found in the venom of elapid land snakes and sea snakes. There are two classes: short neurotoxins of 60–62 amino acids with four disulfide bridges, and long toxins with 71–74 residues and five disulfide bridges. These proteins act by blocking the nicotinic acetylcholine receptor of the muscle motor end plate, preventing transmission across the cholinergic synapse (1). Binding of toxin to receptor is tight ($K_d \sim 10^{-11}$ M) and specific (there is no detectable binding to acetylcholinesterase), which makes these neurotoxins valuable tools for isolation and assay of the membrane-bound receptor (2). Currently available data indicate that curarimimetic neurotoxins and cholinergic ligands bind in a mutually competitive manner (3). The physiological effects of administration of the purified neurotoxins are very similar to those of the alkaloid *d*-tubocurarine, which is believed to bind to the same receptor site as acetylcholine without causing a change in membrane permeability (4).

The amino acid sequences of over 50 neurotoxins have been determined, and summaries have been provided by Ryden *et al.* (5) and Karlsson (6), among others. There is a homology that covers not only the short neurotoxins (such as sea snake neurotoxins) and long neurotoxins (such as α-bungarotoxin) but also the cardiotoxins, a related group of proteins that change the permeability of membranes in a great variety of cells and tissues but do not specifically bind to the nicotinic receptor. Ryden *et al.* included an extensive tabulation of the variability of the amino acid residues. From their work it is apparent that the areas around the four disulfide bonds common to all three toxin classes are invariant, presumably because of their role in preserving the structural integrity of the molecules. In addition, certain other amino acids—Lys-27, Lys-53, Trp-29, Asp-31, Arg-37, and Gly-38—appear to be conserved in α-neurotoxins but not in cardiotoxins.[1] These residues are presumed to be involved

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[1] The numbering used in this paper is the general homology sequence numbering (5, 6) (Fig. 1).

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in the physiological function of the neurotoxins and are termed "functionally invariant" (in fact, only Arg-37, Trp-29, and Gly-38 are completely conserved; this will be discussed in more detail below). All these residues appear to be located in the same area of the molecule according to the recently determined three-dimensional structures of two sea snake neurotoxins (9–11). Figure 2 shows the folding of the polypeptide chain in the short toxin from Philippine sea snake, which was determined in our laboratory from a 2.2-A-resolution electron density map. We had noted earlier that five of the "toxic" residues are contained in the extended loop of the antiparallel β-pleated sheet, suggesting that the toxin binds by inserting this loop into a cleft or channel in the membrane-bound receptor. In this report we attempt to examine, in the light of the three-dimensional structure, the possible roles of the toxic residues and, in particular, to identify that part of the structure which may bind to the receptor site for acetylcholine.

Functionally Invariant Residues

Because we draw on chemical modification studies of both short and long neurotoxins in our discussion, we have examined the effect of the insertions in the 70-type toxins on the short toxin structure. We had predicted earlier that both classes would have the same general structural features (9, 11), and we have been able to build a model of the long neurotoxins in accord with this hypothesis. In particular, the "extra disulfide" insertion of residues 32–36 in the long toxins can be fitted onto the end of the "toxic loop" without altering the

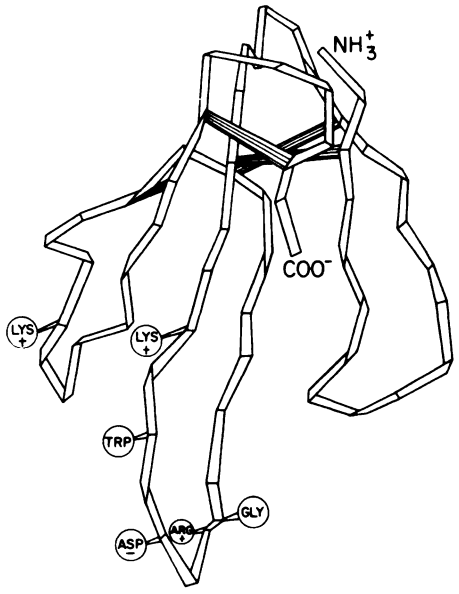

FIG. 2. *Perspective ribbon drawing of folding of polypeptide chain of short α-neurotoxins (9, 11)*

The α-carbon positions are shown by folds; the disulfide bridges are striped. The functionally invariant residues are labeled; they all point in the same direction (into the plane of the paper), forming a "toxic surface."

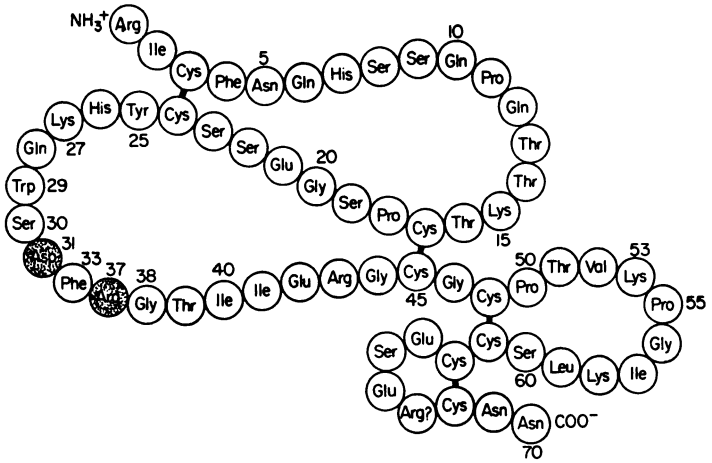

FIG. 1. *Amino acid sequence of Philippine sea snake α-neurotoxin b (7, 8)*

The identity of Arg-67 is still uncertain. The general homology sequence numbering (5, 6) is used, and the residues forming the proposed ion pair are shaded.

relative positions of Arg-37, Trp-29, and Asp-31. In our model for the 70-type toxins the extra residues continue the β-pleated sheet, which folds up out of the plane of the paper in Fig. 2. (The direction of the fold is dictated by the disulfide bond between residues 30 and 34, since the β-pleated sheet forces the side chain of residue 30 to project that way. A β bend is formed at residues 34 and 35.)

Chemical modification studies on both short and long neurotoxins, reviewed extensively by Karlsson (6), suggest that none of the functionally invariant residues is absolutely essential for toxin binding, but confirm their involvement in toxicity. Since α-neurotoxins are highly basic proteins, whereas cholinergic agonists and antagonists are cations, much attention has focused on the lysine residues. Neither is completely conserved in all known sequences, but modification of either does reduce toxicity to some extent. However, modifications that retain the positive charge on the lysines have either little or no effect on toxicity (6). We therefore conclude that these residues serve as ionic binding points but may not have any specific geometrical requirements.

The role of the invariant Gly-38 has up to now been uncertain. According to our three-dimensional model this residue may be conserved because a longer side chain would interfere with the interaction of Arg-37 and the receptor.

A striking feature of the tertiary structure of the Philippine sea snake neurotoxin is the positioning of functionally invariant Arg-37 and Asp-31 opposite each other at the tip of the toxic loop. The β sheet brings these residues close enough so that their side chains could interact without any change in backbone conformation (Figs. 3 and 4). Ion pairing between a protonated guanidinium group and a deprotonated carboxylate has been observed in many protein crystal structures (12–15), and also in crystal structures of peptides (16) and free amino acids (17, 18). We were struck by the stereochemical resemblance between such an ion pair and acetylcholine (Fig. 5). When the centers of positive charge are brought into coincidence, the oxygen atoms may be superimposed, implying that the two structures might have similar hydrogen bond acceptor characteristics. The length of the hydrogen bond allows it to substitute for the ethanoxy bridge in acetylcholine, and the O—C—O bond angle of 123° in the carboxylate ion is the same as the O_1—C_6—O_2 angle observed in various crystal forms of acetylcholine.

Although the C—O bond lengths in acetylcholine are not precisely equal to the 1.25 A for the carboxylate ion, the differences are very small. Of course, acetylcholine is a much more hydrophobic molecule than a guanidinium-carboxylate ion pair, but the hydrophobic environment could be provided by other parts of the neurotoxin molecule. It seems to us that the role of Arg-37 may be to bind to the choline cation binding site in the receptor and that the role of Asp-31 may be to provide the hydrogen bond acceptors normally provided by the ester and carbonyl oxygens of acetylcholine. These residues could perform this role whether or not they formed a salt link

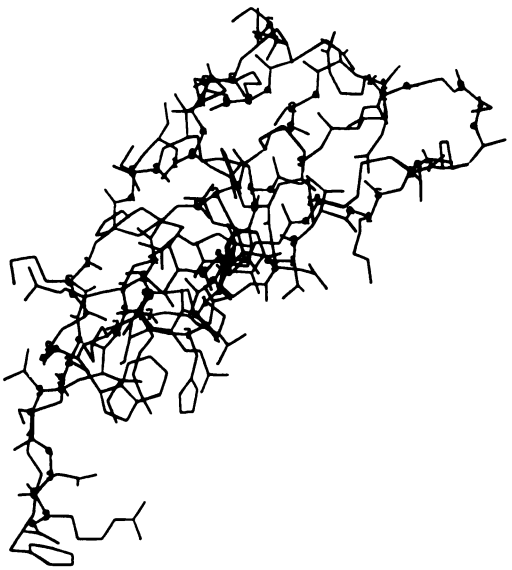

FIG. 3. *Complete three-dimensional structure of Philippine sea snake α-neurotoxin b*

The view of the molecule is orthogonal to that of Fig. 2 (90° rotation about a vertical axis in the plane of the paper). Arg-37 and Asp-31 are the residues pointing out to the right at the bottom of the molecule; Phe-32 is below them, and Trp-29 is above.

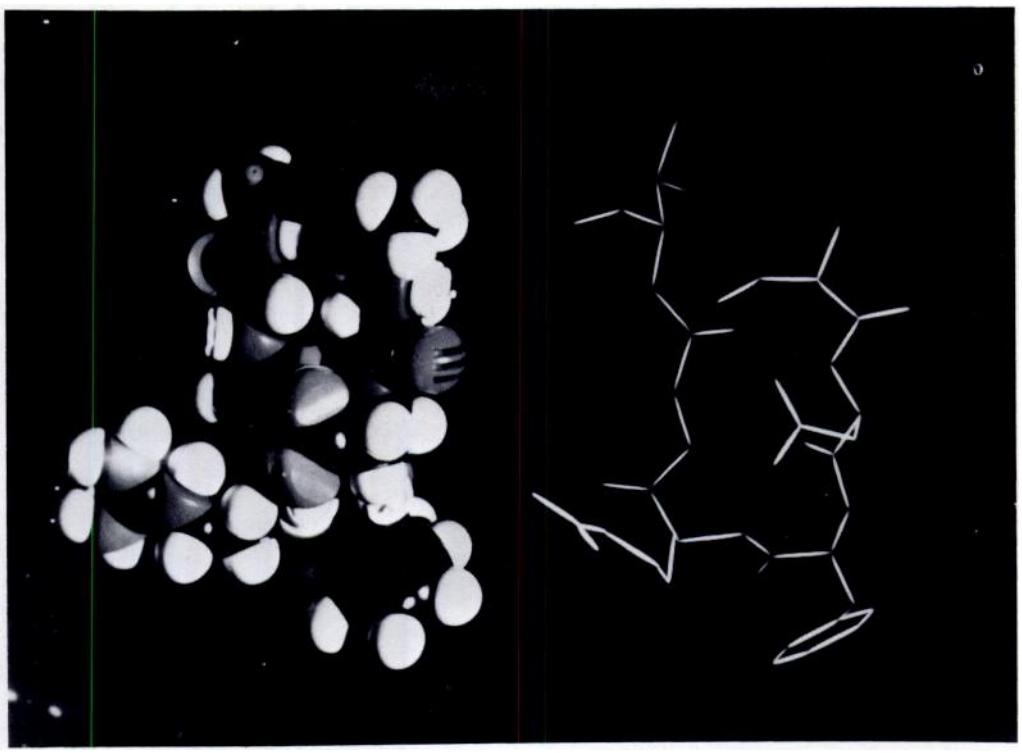

FIG. 4. *Space-filling model of tip of toxic loop and computer graphics drawing of the same residues*
This side-by-side illustration emphasizes the juxtaposition of Arg-37 and Asp-31 and the rigidity of the structure.

to each other, but in the discussion that follows we assume this is the case.

Structure and Activity of Acetylcholine

Various models for the interaction of acetylcholine with nicotinic receptors have stressed the role of the quaternary positively charged nitrogen and the ester and carbonyl oxygens. Unfortunately, acetylcholine is a flexible molecule; rotation around the C_4—C_5 and C_5—C_1 bonds allows it to assume many different conformations of similar energy. The exact conformation of acetylcholine when bound to the nicotinic receptor is still under debate, but this really does not affect our hypothesis regarding the acetylcholine-like character of an arginine-aspartic acid pair. Rotation about the hydrogen bond, plus the possible nonlinearity of the bond itself, gives a guanidinium-carboxylate ion pair conformational flexibility of its own, and any of the proposed acetylcholine structures can be mimicked.

To simplify our discussion of the structural-functional properties of the proposed ion pair, we have chosen a representative structure of such a pair and of acetylcholine for display (Fig. 6). The acetylcholine conformation is that proposed by Beers and Reich (19), based on comparison of the structures of known nicotinic agonists and antagonists. We have compared this with a guanidinium-carboxylate ion pair constructed with standard covalent bond lengths and angles. A distance of 2.80 A was chosen for the N—H··O hydrogen bond (17, 18), and a linear hydrogen bond was assumed. The ζ carbon of guanidinium and the nitrogen of acetylcholine were superimposed, and the acylate function was rotated about the linear hydrogen bond into coincidence with the acyloxy function of acetylcholine. The computed distance between the ζ carbon and the center of the van der Waals surface of the hydrogen bond acceptor site provided by carboxylate oxygen (r_1) was found to be 5.9 A. Beers and

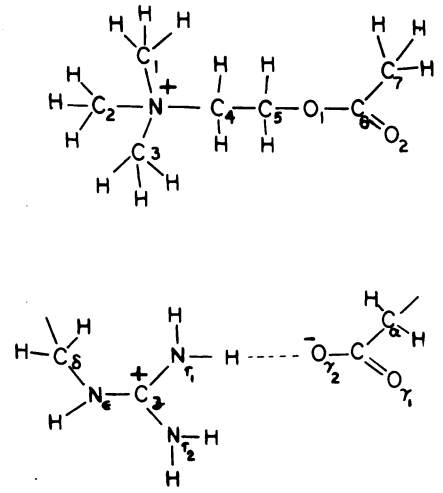

FIG. 5. *Schematic diagram of acetylcholine molecule and guanidinium-carboxylate ion pair*

This diagram shows the numbering scheme used and the general structural similarity between the two entities.

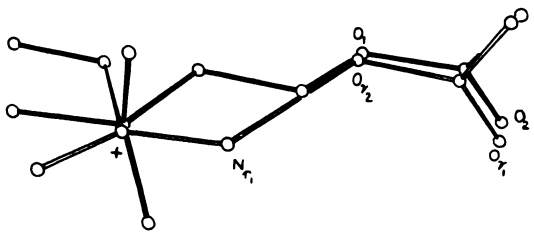

FIG. 6. *Computer-produced perspective drawing of direct comparison of Beers and Reich conformation of acetylcholine (19) and model guanidinium-carboxylate ion pair*

Acetylcholine is drawn with black bonds, and the ion pair hydrogen bond is striped.

Reich (19) have proposed that the specific binding of nicotinic agents to the receptor is mediated by electrostatic interaction involving the cation and a hydrogen bond that depends on an acceptor group and is formed 5.9 A from the positive charge (Fig. 5).

An alternative conformation for acetylcholine has been proposed by Pauling and his co-workers (20, 21). This is the conformation found in crystals of acetylcholine chloride (22) and is believed from nuclear magnetic resonance spectroscopy to predominate in solution (23). There is some evidence that this conformation may be relevant to interaction at least with the muscarinic receptor (21). This structure can be imitated by a guanidinium-carboxylate ion pair in a different conformation, such as that found in the crystal structure of ribonuclease S between Arg-10 and Glu-2 (12, 24).

Toxin-Receptor Association

We hypothesize that the structural resemblance between the guanidinium-carboxylate hydrogen-bonded ion pair and acetylcholine contributes to the specific binding of snake venom neurotoxins to the nicotinic acetylcholine receptor. Although ion pair formation is not essential for interaction between Arg-37, Asp-31, and the corresponding receptor sites for acetylcholine, such interactions bring these two amino acids close enough to hydrogen bond to one another, and so this feature is included in our model. The model requires an initial association between toxin and receptor involving other points of contact, including the functionally important lysines. The resulting desolvation of the tip of the toxic loop promotes the formation of the Arg-37–Asp-31 ion pair, which binds to the acetylcholine binding site.

It must be emphasized that Arg-37 and Asp-31 are not within hydrogen bonding distance in the unbound toxin (at least in our crystal form), but this is not surprising. These residues are exposed to solvent, and each can form many hydrogen bonds to water molecules (in fact, the ordered water is clearly visible in our electron density map). However, the moderately hydrophobic nature of acetylcholine and other cholinergic agonists and antagonists suggests that solvent exclusion probably takes place in binding to the receptor. Removal of solvent from the environment of Arg-37 and Asp-31 would promote ion pairing. The proper conformation can be achieved by only single-bond rotations about side-chain bonds and does not require any backbone rearrangement. Similarly, the Arg-10–Glu-2 ion pair in ribonuclease S is not formed until S-peptide complexes with S-protein (24). Ion pairing in the toxin would also be aided by the presence (Fig. 3) of the invariant Trp-29. This large hydrophobic group participates in the initial hydrophobic as-

sociation between toxin and receptor in the model we propose and serves some of the role normally provided by the hydrophobic part of acetylcholine, but it may also have another interesting role. Our refinement of the neurotoxin structure at 1.38 A resolution indicates that Trp-29 is within hydrogen bonding distance to one of the carboxylate oxygens of Asp-31. This fixes the orientation of Asp-31, possibly making hydrogen bonding to the receptor and/or Arg-37 entropically more favorable when the complex is formed.

Support for the Model

Our model explains a considerable amount of existing data on neurotoxin sequence and chemistry. Arg-37 is the only completely invariant cationic side chain in all long and short neurotoxins (6), and phenylglyoxalation of this residue causes a substantial reduction in neurotoxicity (6). Asp-31 is invariant in all neurotoxins except Ls III, a minor component of *Laticauda semifasciata* venom (where it is replaced by asparagine), and the minor components CM-10 and CM-12 of *Naja haje annulifera* venom (where it is replaced by glycine). All three toxins have extremely low toxicity (10%, 2%, and 0.2% of normal, respectively). Amidation of accessible carboxylate functions in cobrotoxin by reaction with glycine methyl ester reduced toxicity by 25%, but because the sites and extent of modification were not directly determined, this study gives no quantitative data for the importance of Asp-31 (6). It is particularly interesting that none of the cardiotoxins sequenced thus far has either an arginine in position 37 or an aspartic or glutamic acid in position 31. Presumably this is because such a molecule would have some neurotoxic character.

Although chemical modification data do not indicate that either Arg-37 or Asp-31 (or, for that matter, any of the functionally invariant residues in the α-neurotoxins) is absolutely necessary for toxicity, this is not surprising. Karlsson has shown that the interaction between toxin and receptor is not primarily electrostatic, since binding of the toxin to the solubilized receptor is not prevented by 1.5 M NaCl (6). The binding is very strong ($K_d \sim 10^{-11}$ M) and is therefore probably the result of a large number of contacts between the two molecules. Our model proposes that binding involves hydrophobic interactions from Trp-29, ion pairing with Lys-53 and possibly Lys-27, and additional hydrophobic and hydrogen bonds with other residues in the toxin. The association is that of a toxic surface with a corresponding region of the membrane-bound receptor. Since there are many contact points, chemical modification of one should weaken binding and reduce but not eliminate toxicity. In this model the role of Arg-37 and Asp-31 is to block the acetylcholine binding site on the receptor. The positive charge of Arg-37 binds to some anionic region in the receptor that normally binds the quaternary nitrogen of acetylcholine. The carboxyl group accepts hydrogen bonds from donors on the receptor that normally bind to the ester and carbonyl oxygens of acetylcholine. This provides increased specificity and tighter binding for the neurotoxin. Formation of an ion pair between the two residues is not essential for our hypothesis but seems to be a reasonable feature.

Many of the reported nicotinic agonists and antagonists, such as decamethonium, have functional groups that may provide multiple contact points. Particularly interesting is the approximately 11-A distance between positive charges found in tubocurarine and its derivatives (25). A number of such distances are found in the neurotoxin structure between functionally important residues (Arg-37–Lys-27, 11.9 A; Lys-27–Lys-53, 11.7 A; Lys-15–Lys-27, 10.8 A, to list a few). This is not surprising, since the toxins mimic the action of curare. However, the toxins exhibit substantially greater specificity and strength of binding. We propose that this may be due to the added presence and acetylcholine-like character of Arg-37 and Asp-31 in these neurotoxins.

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