J. Pharm. Pharmacol. 1991, 43: 785–789
Received January 29, 1991

Importance of Two Arginine Residues in *Lapemis* Postsynaptic Neurotoxins: Re-examination using Acetylcholine Receptor-neurotoxin Complex Instead of Free Toxin

NOBUHIRO MORI AND ANTHONY T. TU

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523, USA

Abstract—The role of the three arginine residues in *Lapemis* neurotoxin has been re-examined using an acetylcholine receptor-toxin complex. The receptor-bound neurotoxin was treated with phenylglyoxal to modify available arginine residues. The positions of the modified arginine residues were then identified from the amino acid sequences of proteolytically digested fragments of the detached neurotoxin. The result was compared with the result from modification of the free *Lapemis* toxin (unbound). Arg-31 and Arg-34 were not modified when *Lapemis* toxin was bound to receptor although they were modified when the toxin was not bound to receptor. It was concluded that Arg-31 and Arg-34 residues are involved in the toxin-receptor interaction.

Neurotoxicity of sea-snake venom is due to the binding of neurotoxin to acetylcholine receptor causing the paralysis of the muscle, but the detailed mechanism needs to be further clarified. Hydrophiidae (sea snake) neurotoxins have not been studied as much as neurotoxins of Elapidae; however, a number of neurotoxins have been isolated from sea snake venoms, and the amino acid sequences of some toxins have been determined. *Lapemis* toxin is a postsynaptic neurotoxin isolated from the venom of one species of Hydrophiidae, *Lapemis hardwickii* (Hardwick's sea snake), and binds to the acetylcholine receptor (Ishizaki et al 1984; Allen & Tu 1985).

Chemical modification has been carried out on free toxins, to determine the mechanism of this binding; however, the conclusions from such an approach are less reliable than methods using receptor-bound toxin (Tu & Toom 1971; Tu et al 1971; Karlsson 1979). Comparing the results of chemical modification of receptor-bound neurotoxin with those from modification of free neurotoxin will yield more precise information as to the role of a particular amino acid on toxin binding to the receptor. Reactivity of the lysine residue in *Naja naja siamensis* postsynaptic neurotoxin decreased when the toxin became attached to the acetylcholine receptor (Balasubramaniam et al 1983). This suggests that some lysine residues are involved in the neurotoxin-acetylcholine receptor binding. Recently Garcia-Borron et al (1987) chemically modified lysine residues of the receptor-bound α-bungarotoxin. Reactivity of Lys-26, Lys-38 and other lysine residues to methylation is different when the toxin is bound to the receptor, although the extent of this protection is much more pronounced for Lys-26.

Using the same approach, we have studied the binding of arginine residues in *Lapemis* toxin to the nicotinic acetylcholine receptor.

Correspondence: A. T. Tu, Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523, USA.

Materials and Methods

Materials

Sephadex G-50 and G-10, CM-cellulose, CNBr-activated Sepharose 4B, and molecular weight standards were purchased from Sigma Chemical Co. *Torpedo californica* electroplax, excised and then quick frozen in liquid nitrogen, was obtained from Pacific Bio-Marine Laboratories (Venice, CA) and stored at −70°C until needed. [^{125}I]-α-Bungarotoxin and Econofluor were purchased from New England Nuclear (Boston, MA). Phenylglyoxal monohydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Phenyl[2-^{14}C]glyoxal was obtained from Research Products International Corp. (Mount Prospect, IL). Endoproteinase Glu-C (protease V8) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Extracti-Gel D and PITC (phenylisothiocyanate) were purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals were of analytical or reagent grade.

Venom

Sea snakes, *L. hardwickii*, were captured in the Gulf of Thailand in 1986, and the venom was extracted as previously described (Tu & Hong 1971).

Neurotoxin

The major neurotoxin was isolated by a modification of the method of Tu & Hong (1971) using a two-step gel chromatography procedure at 4°C.

Toxicity was checked after each step of the isolation procedure. After G-50 chromatography, three to five Swiss mice were injected with protein from peak III dissolved in 0·9% NaCl at a concentration that was twice the LD50 of crude venom. The number of mice that died in 24 h was recorded.

The homogeneity of the toxin was checked using polyacrylamide gel electrophoresis with the β-alanine acetate system previously described (Tu et al 1975).

Aliquots of *Lapemis* toxin were injected onto a Beckman ultrasphere-ODS column (4·6 mm × 25 cm) operated at a flow rate of 1·0 mL min^{-1}. Gradient conditions were as follows: from solvent A (0·1% trifluoroacetic acid) to 50% solvent B (0·1% trifluoroacetic acid in acetonitrile) in 30 min.

Receptor isolation

Acetylcholine receptor was isolated from *T. californica* electroplax tissue using the method of Froehner & Rafto (1979). Cobrotoxin affinity resin was prepared as previously described (Brockes & Hall 1975) using CNBr-activated Sepharose 4B.

Receptor assay

Toxin binding to receptor was determined using the method of Schmidt & Raftery (1973). All assays were done in *Torpedo* Ringer's buffer (containing (mM): 250 NaCl, 5 KCl, 4 $CaCl_2$, 5 sodium phosphate (pH 7·0)), and 0·1% bovine serum albumin at room temperature (21°C) in a total volume of 125 μL, for 1 h. Two Whatman DE-81 filter disks were placed on a Millipore filter holder connected to a vacuum flask and were rinsed with buffer containing 100 mM NaCl, 0·1% Triton X-100, and 10 mM sodium phosphate (pH 7·4). Fifty μL of the incubation mixture was applied to the DE-81 filter disks and allowed to soak for 10 min. The disks were then rinsed five times with 5 mL of buffer. The filters were finally dried under a heat lamp, placed in 5 mL of Econofluor, and counted in a Beckman LS 7800 liquid scintillation counter.

Amino acid analysis

The analyses were performed on a Beckman Model 344 M HPLC system using a reverse-phase column after precolumn derivation by PITC. The preparation of the PTC amino acids was as described by Heinrikson & Meredith (1984).

Sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis

Electrophoresis was performed on SDS-polyacrylamide gel (10%) using a procedure modified from Laemmli (1970). The sample buffer contained 2% SDS and 5% β-mercaptoethanol. Samples were boiled for 5 min before electrophoresis. Bovine serum albumin (66000), egg albumen (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20000), and α-lactalbumin (14000) were used as standards for molecular weight determinations (Weber & Osborn 1969). Receptor and standard were mixed and applied to the same well for molecular weight determinations. Gels were stained with Coomassie brilliant blue.

Arginine modification

Modification of arginine residues in *Lapemis* toxin with phenylglyoxal was performed by previously published procedures (Takahashi 1968; Yang et al 1974) with minor modification. The commercial [^{14}C]phenylglyoxal employed throughout this study had specific activities of 25-35 mCi $mmol^{-1}$ and was freed of impurities by chromatography on Silica Gel 60.

To a solution of *Lapemis* toxin (0·6 μmol) in 1 mL of 0·2 M *N*-ethylmorpholine acetate buffer (pH 8·0), a 100-fold molar excess of phenylglyoxal in 0·3 mL of the same buffer was added, and the reaction was allowed to proceed at 27°C for 3 h. The mixture was passed through a column of Sephadex G-10 (2·5 × 50 cm) followed by CM-cellulose chromatography with a linear gradient of increasing salt concentration from 0·0 to 0·4 M NaCl in 0·01 M ammonium acetate, pH 6·8. The fractions of the main protein peak were lyophilized and desalted through a column of Sephadex G-10 (2·5 × 50 cm) equilibrated with 1% acetic acid. The protein fractions were then pooled and lyophilized.

Identification of arginine residues modified by phenylglyoxal

The modified derivatives were digested with endoproteinase Glu-C (protease V8) after reduction and *S*-carboxymethylation. Reduction and *S*-carboxymethylation were performed by the method described by Crestfield et al (1963). The *S*-carboxymethylated derivatives were dissolved in 0·02 M $NaHCO_3$ buffer (pH 7·2) to give 1% solutions, and endoproteinase Glu-C (50:1) was added. Digestion was carried out at 27°C for 16 h.

Arginine-containing peptides from endoproteinase Glu-C digests were separated by RP-HPLC (Beckman ultrasphere-ODS, 4·6 mm × 25 cm). Gradient conditions were as follows: from 95% solvent A (0·1% trifluoroacetic acid) and 5% solvent B (0·1% trifluoroacetic acid in acetonitrile) to 40% solvent B in 40 min. Peptides were detected by their absorbance at 214 nm. The eluting peptides were collected manually, dried, and stored at −20°C until needed for amino acid analysis.

Arginine modification of Lapemis *toxin in the presence of receptor*

Acetylcholine receptor (5 mg) from cobrotoxin-affinity chromatography was concentrated by ultrafiltration using a Centriflo membrane cone (mol. wt cut-off 25000 daltons, Amicon Corp.). Ultrafiltration was carried out at 750 *g* at 4°C, and reduced the 10 mL initial volume to 2 mL. In an arginine-modification experiment, the required amount of receptor was mixed with a twofold excess of *Lapemis* toxin. After 1 h incubation at room temperature, the receptor solution was applied onto a Sephadex G-50-80 column (2·5 × 90 cm) previously equilibrated with 0·2 M *N*-ethylmorpholine acetate buffer (pH 8·0) containing 0·1% Triton X-100. The receptor-bound *Lapemis* toxin fractions were pooled and concentrated by ultrafiltration using a Centriflo membrane cone. Chemical modification of bound neurotoxin was carried out using [^{14}C]phenylglyoxal. A 100-fold molar excess of [^{14}C]phenylglyoxal was added, and the reaction was allowed to proceed at 27°C for 3 h. The mixture was dialysed using Spectrapor membrane tubing, mol. wt cut-off 12000–14000, for 12 h against 0·01 M ammonium acetate, pH 6·8.

The labelled *Lapemis* toxin was released from the receptor by adding 6 M NaCl and incubating at 37°C for 6 h. Separations of *Lapemis* toxin from receptor and removal of 0·1% Triton X-100 from the reaction mixture were carried out using an Extracti-Gel D, detergent-removing gel, column (0·8 × 1·5 cm). The homogeneity of the toxin was checked by SDS-polyacrylamide gel electrophoresis with Phast Gel Gradient 8-25 using Pharmacia PhastSystem, and also by RP-HPLC. The concentration of the *Lapemis* toxin was

determined using a MicroBCA protein assay kit (Pierce Chemical Co.) with bovine serum albumin as a standard.

Results

Purity of toxin

The homogeneity of the *Lapemis* toxin was established by two independent methods, polyacrylamide gel electrophoresis and analytical HPLC using a Beckman ultrasphere-ODS column (4·6 mm × 25 cm). A single band was observed on acrylamide gels after applying 10 μg of *Lapemis* toxin. The HPLC chromatography pattern also showed that only one protein was present. There was only one sharp band at the retention time of 18·52 min. Toxity of purified *Lapemis* toxin was tested on 3 white mice; at the dose of 0·15 μg g^{-1} all the mice died. The reported LD50 value was 0·06 μg g^{-1} (Tu & Hong 1971).

Free Lapemis *toxin*

In a preliminary experiment phenyl [2-^{14}C]glyoxal was added to free *Lapemis* toxin; the specific activity reached a maximum in 2-3 h and we used the reaction time of 3 h for all modifications. From amino acid analysis it was evident that 2 out of 3 arginine residues were modified (Table 1).

Table 1. Amino acid composition of arginine-modified *Lapemis* toxin.

	Residues per mole of protein		
Amino acid	Present experiment		Published* composition
Asp	5·6	6	6
Glu	8·4	8	8
Ser	4·6	5	5
Gly	4·3	4	4
Thr	7·3	7	7
His	1·9	2	2
Ala	1·3	1	1
Pro	3·3	3	3
Arg	1·2	1	3
Tyr	1·0	1	1
Met	0·5	1	1
Val	1·3	1	1
Half-Cys	8·5	9	9
Ile	2·3	2	2
Leu	1·2	1	1
Lys	5·2	5	5

* Fox et al (1977).

The proteolytic digests were separated into four fragments by HPLC using a C_{18} reverse-phase column (Fig. 1). The amino acid composition of each fragment is summarized in Table 2.

Since the amino acid sequence of *Lapemis* toxin is known, the cleavage sites can be determined (Fig. 2). Fragment 3 showed 1 mol of arginine, but fragment 4 contained no arginine (Table 2). Thus Arg-31 and Arg-34 were modified, while Arg-37 was not modified. Apparently Arg-37 was unavailable for modification because it was situated in a less accessible part of the toxin.

Table 2. Amino acid composition of fragments obtained from Glu-C-digestion of free *Lapemis* toxin.

	Fragment			
Amino acid	1	2	3	4
Asp	2·8 (3)	2·3 (2)		0·6 (1)
Glu	0·7 (1)	2·6 (3)	2·3 (2)	0·8 (1)
Ser		2·1 (2)		3·1 (3)
Gly			3·0 (3)	1·2 (1)
Thr	1·2 (1)	4·1 (4)		1·8 (2)
His	1·0 (1)			1·1 (1)
Ala		1·3 (1)		
Pro		1·3 (1)	2·3 (2)	
Arg			1·0 (1)	0·3 (0)
Tyr				1·0 (1)
Met		0·6 (1)		
Val			0·75 (1)	
Half-Cys	3·1 (3)	2·8 (3)	2·0 (2)	1·1 (1)
Ile			1·1 (1)	0·8 (1)
Leu			1·2 (1)	
Lys		0·6 (1)	2·4 (2)	1·7 (2)

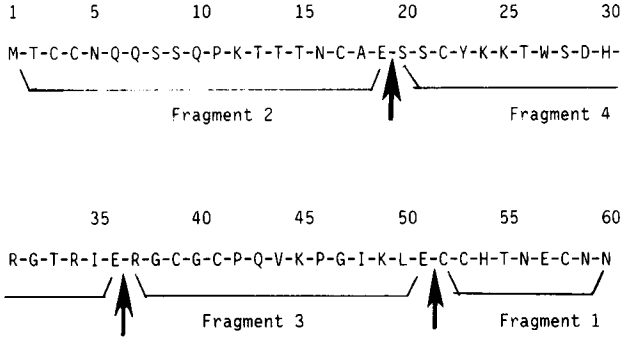

FIG. 2. Sites of cleavage of endoproteinase Glu-C on *S*-carboxymethylated *Lapemis* toxin.

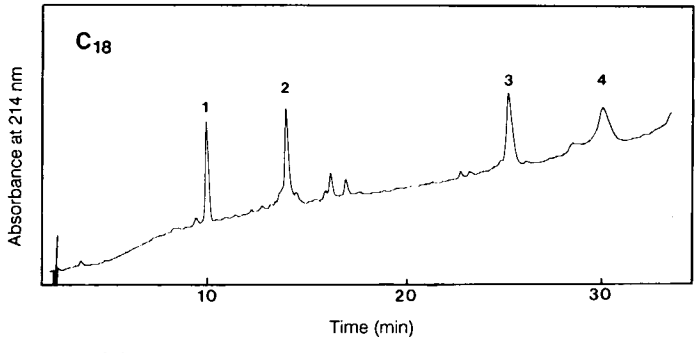

FIG. 1. RP-HPLC separation of the products resulting from endopeptidase Glu-C-digestion of phenylglyoxal-treated *Lapemis* toxin. Samples of endoproteinase Glu-C treated *S*-carboxymethylated *Lapemis* toxin were injected in a Beckman ultrasphere-ODS column (4·6 mm × 25 cm) operated at a flow rate of 1·0 mL $min.^{-1}$.

The modified toxin was non-toxic to mice at 0·5 $\mu g\ g^{-1}$, while the LD50 of unmodified toxin was 0·06 $\mu g\ g^{-1}$.

Receptor Lapemis *toxin*

Lapemis toxin was mixed with receptor at a molar ratio of 2:1. When the complex was formed, the neurotoxin peak that normally would appear at tube 95 on Sephadex G-50 chromotography was not present (Fig. 3). Further evidence of formulation of *Lapemis* toxin and receptor complex is shown in Fig. 4, SDS electrophoresis showed the complex dissociated into α, β, γ, and δ subunits of receptor and *Lapemis* toxin.

The receptor-bound toxin was modified with phenyl[2-^{14}C]glyoxal at 100-fold excess. When phenyl[2-^{14}C]glyoxal was used to modify arginine residues, a very large amount of ^{14}C was incorporated. In two separate experiments, the ^{14}C incorporated was 3756 and 3793 counts min^{-1}, more than double the amount (1688 and 1501 counts min^{-1}) incorporated into free neurotoxin. This suggests that many arginine residues in the receptor of the receptor-toxin complex were modified by this reagent.

The complex was incubated in NaCl at 37°C for 6 h and the receptor removed by precipitation. After removing Triton X-100, the *Lapemis* toxin was purified by HPLC (Fig. 5). Amino acid analysis of the appropriate HPLC fraction confirmed the fraction as *Lapemis* toxin. Background levels of radioactivity only (176–185 counts min^{-1}) were associated with this fraction, indicating the two arginine residues were no longer available to chemical modification in the bound toxin.

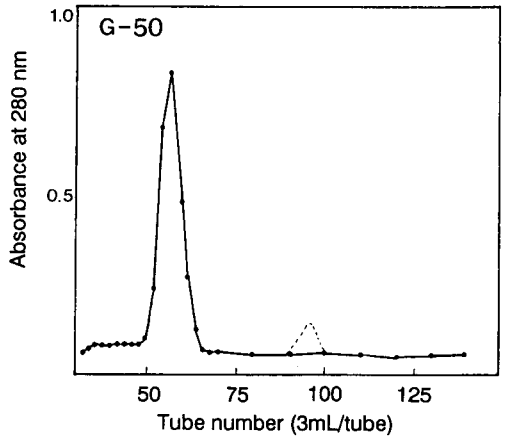

FIG. 3. Sephadex G-50 chromatography of *Lapemis* toxin (0·25 mg) mixed with 5 mg of acetylcholine receptor. The expected location of the eluted *Lapemis* toxin is shown by the dotted curve. Since no absorption is observed, it can be assumed that free toxin did not exist in measurable quantities in the mixture with acetylcholine receptor. The one major peak observed (tube 50-69) is the receptor *Lapemis* toxin complex.

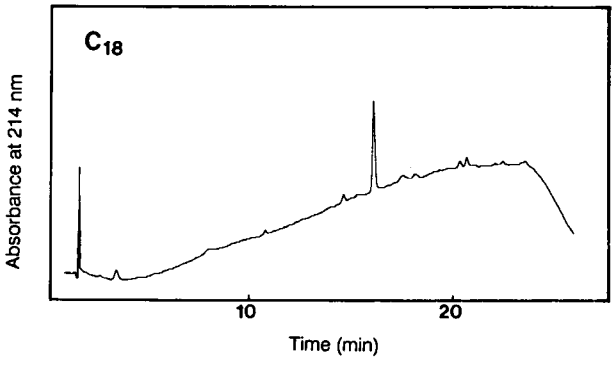

FIG. 5. HPLC of *Lapemis* toxin detached from the receptor on a Beckman ultrasphere ODS column (4·6 mm × 24 cm) operated at a flow rate of 1·0 mL min^{-1}. Gradient condition: from 95% solvent A (0·1% trifluoroacetic acid) and 5% solvent B (0·1% trifluoroacetic acid in acetonitrile) to 50% solvents A and B in 25 min.

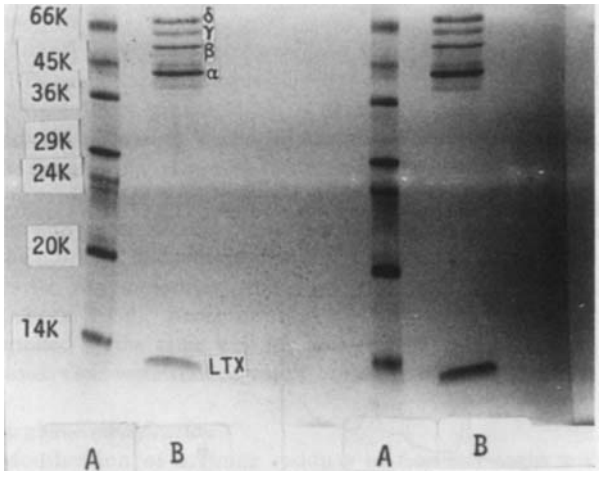

FIG. 4. A. SDS polyacrylamide gel electrophoresis of standard proteins (66 K, bovine albumin; 45 K, egg albumen; 36 K, glyceraldehyde-3-phosphate dehydrogenase; 29 K, carbonic anhydrase; 24 K, trypsinogen; 20 K, trypsin inhibitor; 14 K, α-lactalbumin). B. Acetylcholine receptor showing dissociation to constituent subunits α, β, γ, and δ. Complex and standards were treated with sample buffer containing 4% SDS, 20% glycerol, and 10% β-mercaptoethanol for 5 min at 100°C and then run on 12% polyacrylamide gel. LTX (*Lapemis* toxin) is clearly seen after the dissociation of the complex.

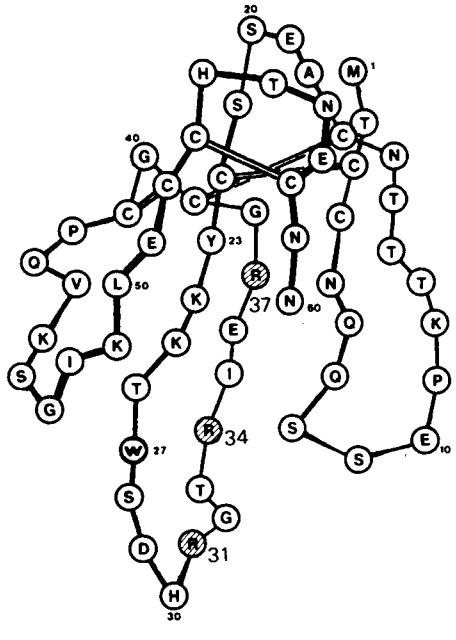

FIG. 6. The amino acid sequence and structure of *Lapemis* toxin. The structure is based on the crystal structure of a similar neurotoxin, toxin b (Tsernoglou & Petsko 1976).

Discussion

Lapemis toxin contains three arginine residues at positions 31, 34, and 37. In free (unbound) toxin Arg-31 and Arg-34 were modified, but Arg-37 was inaccessible to the modifying reagent presumably because it is embedded in the protein coiling. Fig. 6 shows that Arg-31 and Arg-34 are relatively exposed. However, when arginine residues of the neurotoxin were modified, in the receptor complex, neither Arg-31 nor Arg-34 were modified, suggesting that both Arg-31 and Arg-34 are involved in receptor binding.

Although we have shown that two out of three arginine residues are important, binding of neurotoxin to acetylcholine receptor is complex. As Garcia-Borron et al (1987) indicated, lysine residues are also involved and the tryptophan residue in the central loop is also essential (Raymond & Tu 1972). On the other hand, the C-terminal tail is considered to be non-essential for toxin conformation (Endo et al 1987), although the binding activity to the receptor is reduced when the C-terminal loop is eliminated.

Acknowledgement

This work was supported by contract No. DAMD-86-C-6063.

References

Allen, M., Tu, A. T. (1985) The effect of tryptophan modification on the structure and function of a sea snake neurotoxin. Mol. Pharmacol. 27: 79–85

Balasubramaniam, K., Eaker, D., Karlsson, E. (1983) An attempt to identify amino groups of *Naja naja siamensis* neurotoxin that interact with acetylcholine receptor by a comparison of their reactivities in free and receptor-bound neurotoxin. Toxicon 21: 219–229

Brockes, J. P., Hall, Z. W. (1975) Acetylcholine receptors in normal and denervated rat diaphragm muscle. I. Purification and interaction with [^{125}I]-α-bungarotoxin. Biochemistry 145: 2092–2099

Crestfield, A. M., Moore, S., Stein, W. H. (1963) The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. J. Biol. Chem. 238: 622–627

Endo, T., Oya, M., Tamiya, N., Hayashi, K. (1987) Role of C-terminal tail of long neurotoxins from snake venoms in molecular conformation and acetylcholine receptor binding: proton nuclear magnetic resonance and competition binding studies. Biochemistry 26: 4592–4598

Fox, J. W., Elzina, M., Tu, A. T. (1977) Amino acid sequence of a snake neurotoxin from the venom of *Lapemis hardwickii* and the detection of a sulfhydryl group by laser Raman spectroscopy. FEBS Lett. 80: 217

Froehner, S. C., Rafto, S. (1979) Comparison of the subunits of *Torpedo californica* acetylcholine receptor by peptide mapping. Biochemistry 18: 301–307

Garcia-Borron, J.-C., Bieber, A. L., Martinez-Carrion, M. (1987) Reductive methylation as a tool for the identification of the amino groups in α-bungarotoxin interacting with nicotinic acetylcholine receptor. Ibid. 26: 4295–4303

Heinrikson, R. L., Meredith, S. C. (1984) Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. Anal. Biochem. 136: 65–74

Ishizaki, H., Allen, M., Tu, A. T. (1984) Effect of sulfhydryl group modification on the neurotoxic action of a sea snake toxin. J. Pharm. Pharmacol. 36: 36–41

Karlsson, E. (1979) Chemistry of protein toxins in snake toxins. Handb. Exp. Pharmacol. 52: 159–212

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Raymond, M. L., Tu, A. T. (1972) Role of tyrosine in sea snake neurotoxin. Biochim. Biophys. Acta 285: 498–502

Schmidt, J., Raftery, M. A. (1973) A simple assay for the study of solubilized acetylcholine receptors. Anal. Biochem. 52: 349–354

Takahashi, K. (1968) The reaction of phenylglyoxal with arginine residues in proteins. J. Biol. Chem. 243: 6171–6179

Tsernoglou, O., Petsko, G. A. (1976) The crystal structure of a post-synaptic neurotoxin from sea-snake at 2.2 Å resolution. FEBS Lett. 68: 1–4

Tu, A. T., Hong, B.-S. (1971) Purification and chemical studies of a toxin from the venom of *Lapemis hardwickii* (Hardwick's sea snake). J. Biol. Chem. 246: 2772–2779

Tu, A. T., Toom, P. M. (1971) Isolation and characterization of the toxic component of *Enhyrina schistosa* (common sea snake) venom. Ibid. 246: 1012–1016

Tu, A. T., Hong, B. S., Solie, T. N. (1971) Characterization and chemical modifications of toxins isolated from the venoms of the sea snake *Laticauda semifasciata*, from Philippines. Biochemistry 10: 1295–1304

Tu, A. T., Lin, T. S., Bieber, A. L. (1975) Purification and chemical characterization of the major neurotoxin from the venom of *Pelamis platurus*. Ibid. 14: 3408–3413

Weber, K., Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406–4412

Yang, C. C., Chang, C. C., Liou, I. F. (1974) Studies on the status of arginine residues in cobrotoxin. Biochim. Biophys. Acta 365: 1–14