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The Effect of Tryptophan Modification on the Structure and Function of a Sea Snake Neurotoxin

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SUMMARY

A neurotoxin has been isolated from the venom of the sea snake *Lapemis hardwickii*. This neurotoxin contains only one tryptophan residue which was chemically modified with two different reagents, 2-hydroxy-5-nitrobenzyl bromide and *N*-bromosuccinimide. Following modification, the neurotoxin almost completely lost its acetylcholine receptor-binding activity. To examine the effect tryptophan modification had on the conformation of the neurotoxin, circular dichroic and Raman spectra were taken of the modified neurotoxin and compared with those of the native toxin. The overall conformation of the modified neurotoxin was very similar to the native conformation. The Raman data also indicated some side chain conformations in the modified toxin were very similar to those of the native toxin. These data suggest that the tryptophan in sea snake venom short chain neurotoxins may have a direct role in the acetylcholine receptor binding process as well as a role in stabilizing the neurotoxin's active site in the proper conformation for optimum binding.

INTRODUCTION

Snake venoms are typically mixtures of many different components, each with its own function and degree of toxicity. Sea snake venom is extremely toxic because of the neurotoxins present in the venom (1, 2). In order to study structure-function relationships, several neurotoxins have been chemically modified and their toxicities investigated (3–8). Among the many amino acid residues in neurotoxins, tryptophan is thought to be important since modification of this residue results in a loss of toxicity (3–5). Also, this residue is highly conserved in all known snake neurotoxins (9). Many studies have determined the effect of chemical modification by measuring the change in toxicity. However, the best way to study neurotoxicity is to examine the specific binding of the neurotoxin to the acetylcholine receptor. Therefore, we have examined the effect of chemical modification of tryptophan in *Lapemis hardwickii* (Hardwick's sea snake) neurotoxin on the binding of this toxin to the acetylcholine receptor. In the past, conformational differences resulting from modification procedures were not well investigated, so we have examined the conformation of the tryptophan-modified toxin using circular dichroism and Raman spectroscopy. We selected the *Lapemis hardwickii* major toxin (*Lapemis* toxin)[1] for several reasons. One clear advantage of *Lapemis* toxin is that it contains only 1 mol of tryptophan, allowing a clearer interpretation of the data than if more than 1 mol of tryptophan were present. Another reason is that the amino acid sequence and conformation of *Lapemis* toxin have been extensively studied in our laboratory and the conformation of modified toxin can be readily compared (10–12). This study clearly shows that the tryptophan residue in *Lapemis* major toxin is important for its binding to the acetylcholine receptor.

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[1] Abbreviations and trivial names used are: *Lapemis* toxin, major neurotoxin isolated from *L. hardwickii* sea snake; LD_{50}, lethal dosage which kills 50% of the experimental group (expressed in micrograms of toxin/g of body weight); HNBB, 2-hydroxy-5-nitrobenzyl bromide; NBS, *N*-bromosuccinimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; α-Bgtx, α-bungarotoxin; AcChR, acetylcholine receptor.

MATERIALS AND METHODS

Materials. Sephadex G-50 was purchased from Pharmacia Fine Chemicals Co. and CM Bio-Gel A from Bio-Rad Laboratories (Richmond, CA). *Torpedo californica* electroplax, excised and then quick frozen in liquid nitrogen, was obtained from Pacific Bio-Marine Laboratories (Venice, CA) and stored at −70° until needed. ^{125}I-α-Bungarotoxin and Econofluor were purchased from New England Nuclear. 4-*N*-Methanesulfonic acid and sodium tetrathionate were purchased from Pierce Chemical Co. All other chemicals were purchased from Sigma Chemical Co.

Venom. Sea snakes, *L. hardwickii*, were captured in the Gulf of Thailand in 1976 and the venom was extracted as previously described (4).

Neurotoxin. The major neurotoxin was isolated by a modification of the method of Tu and Hong (4) using a two-step gel chromatography procedure at 4°. One g of crude venom was loaded onto a Sephadex G-50-80 column (2.5 × 100 cm) which had been previously equilibrated with a 0.01 M potassium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The toxin was eluted with the same buffer at a flow rate of 14 ml/hr and the eluate was collected in 3-ml aliquots. The absorbance of each fraction at 280 nm was determined with a Beckman DB-G spectrophotometer.

The tubes which included protein peak III were pooled, dialyzed for

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48 hr against water, and lyophilized. The lyophilized fraction was dissolved in 2–3 ml of buffer containing 0.01 M Tris-HCl (pH 8.0) and loaded onto a CM Bio-Gel A column (1.5 × 30 cm) which had been previously equilibrated with the same buffer. The toxin was eluted with a linear gradient of NaCl from 0.0 to 0.05 M in 0.01 M Tris buffer. The tubes containing the toxic protein were pooled, dialyzed for at least 48 hr, and lyophilized.

Toxicity was also 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 which was twice the LD_{50} of crude venom. After CMBA chromatography, five groups of five mice each were injected with different dosage levels and the LD_{50} was determined by the method of Litchfield and Wilcoxon (13).

The homogeneity of the toxin was checked using isotachophoresis and polyacrylamide gel electrophoresis. Capillary isotachophoresis was performed on a LKB Tachophor Model 2127 using a cationic system. The cationic system used 5 mM KOH adjusted to pH 7.0 with cacodylic acid as the leading electrolyte and creatinine (113 mg/100 ml) as the trailing electrolyte. Ampholine (1%), pH range 3.5 to 10, was used as a spacer ion. A constant current of 75 μA and a variable voltage of 3–12 kV were used. Polyacrylamide gel electrophoresis was done using the β-alanine acetate system as described previously (6).

Modification of tryptophan. In separate experiments the single tryptophan in *Lapemis* toxin was modified with HNBB or NBS. HNBB modification was done using the method of Horton and Koshland (14) as described by Tu and Hong (4). NBS modification was done by the method of Freisheim and Huennekens (15) as described by Tu and Hong (4).

Acetylcholine receptor isolation. Acetylcholine receptor was isolated from *T. californica* electroplax tissue using the method of Saitoh and Changeux (16) with the following modifications. The buffer used in all steps contained 50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinine, 5 μg/ml pepstatin, and 0.02% sodium azide. The homogenized pellet from the 7,000 rpm spin was adjusted to 32% sucrose (w/w) and then layered onto a 41.5% sucrose solution (w/w) in buffer and centrifuged in a Beckman 60-Ti rotor for 50 min at 50,000 rpm.

Neurotoxin-acetylcholine receptor binding. Toxin binding to receptor was determined using the method of Schmidt and Raftery (17). All assays were done in *Torpedo* Ringer's buffer containing 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.0), and 0.1% bovine serum albumin at room temperature in a total volume of 125 μl for 1 hr. Two Whatman DE-81 filter discs were placed on a Millipore filter holder connected to a vacuum flask and were rinsed with a wash buffer containing 100 mM NaCl, 0.1% Triton X-100, and 10 mM sodium phosphate (pH 7.4). Then 50 μl of the incubation mixture was applied to the DE-81 filter discs and allowed to soak in for 10 min. The discs 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. Background counts due to nonspecific binding were determined by doing the same assay in the presence of a 100-fold excess of unlabeled α-Bgtx (18).

The binding of *Lapemis* toxin was measured by determining the decrease in ^{125}I-α-Bgtx binding to receptor following preincubation of the receptor with varying amounts of *Lapemis* toxin for 1 hr. In other words, ^{125}I-α-Bgtx was used as a nonreversible back-titrant to measure specific binding of a ligand competing for the same receptor (19, 20). Following preincubation of receptor with varying amounts of *Lapemis* toxin, a saturating concentration of ^{125}I-α-Bgtx was added. This mixture was then incubated for 1 hr, and the counts/min determined as above.

Amino acid analysis. Amino acid analysis was done using the method of Simpson *et al.* (21). This method involves 4 *N*-methanesulfonic acid hydrolysis, which does not destroy tryptophan. This method also allows differentiation between free cysteine and half-cysteine since the former is carboxymethylated and the latter sulfonated. All analyses were performed on a JEOL JLC-6AH analyzer.

Spectroscopic assays. CD spectra were measured with a Jasco Model J-41C spectropolarimeter at 25°. Protein samples were dissolved in distilled water (pH 6.8) at a concentration of 0.1 mM.

Raman spectra were obtained by excitation with the 514.5-nm line (Spectra Physics, Model SP-164 argon ion laser) with a green interference filter. The spectra represent an average of 10 scans and were recorded using a Spex Ramalog 5 Raman spectrophotometer and a Spex SCAMP data acquisition processor. All spectra were done with solid protein samples and were recorded from 400 to 3300 cm^{-1} with a 4 cm^{-1} spectral width resolution.

UV spectra were taken with a Cary model 118CX spectrophotometer.

RESULTS

Purity of toxin. The homogeneity of the *Lapemis* toxin was established by two independent methods, acrylamide gel electrophoresis and analytical isotachophoresis. A single band was observed on acrylamide gels after applying 25 μg of *Lapemis* toxin. Isotachophoresis, which is more sensitive than the acrylamide gel method, also showed that only one protein was present. The LD_{50} of the neurotoxin was similar to the value reported previously (4).

Chemical modification. Chemical modification of the tryptophan residue in *Lapemis* toxin was done using two separate methods. The extent of modification of the tryptophan using the first reagent, HNBB, was determined spectrophotometrically (14). The absorption spectrum of the HNBB-modified toxin from 220 to 500 nm was determined in 1 M NH_4OH (pH > 10), and the spectrum was very similar to one published previously (4). No tryptophan was detected in the modified toxin by amino acid analysis. There were no other differences detected in the amino acid content of the native and HNBB-modified toxins.

HNBB will also modify cysteine, although at a much slower rate (14). The single cysteine in native and modified *Lapemis* toxin was not detectable by amino acid analysis, so Raman spectra were taken of the native and modified toxin from 2550 to 2600 cm^{-1} to determine if the cysteine was modified (11). The S-H stretching vibration was detected in the native and modified toxins at 2580 and 2574 cm^{-1}, respectively, indicating that the S-H group had not been modified by HNBB.

The absorption spectra of the NBS-modified toxin from 230 to 350 mm was compared with the native toxin spectra. The peak at 278 nm almost completely disappeared in the NBS-modified toxin and a new peak appeared at 255 nm, indicating that the tryptophan side chain had been oxidized to oxindole (Fig. 1). Amino acid analysis of the NBS-modified toxin was done previously and indicated that there were no other changes in the amino acid content of the toxin following NBS modification (4, 22).

AcChR binding. In order to test that the AcChR isolated was biologically active, the binding of radiolabeled α-Bgtx to the AcChR was measured (Fig. 2). Specific ^{125}I-α-Bgtx binding to AcChR leveled off at a concentration of about 40 nM, indicating saturation of binding sites.

As can be seen in Fig. 3, *Lapemis* toxin completely inhibited ^{125}I-α-Bgtx binding when it was preincubated

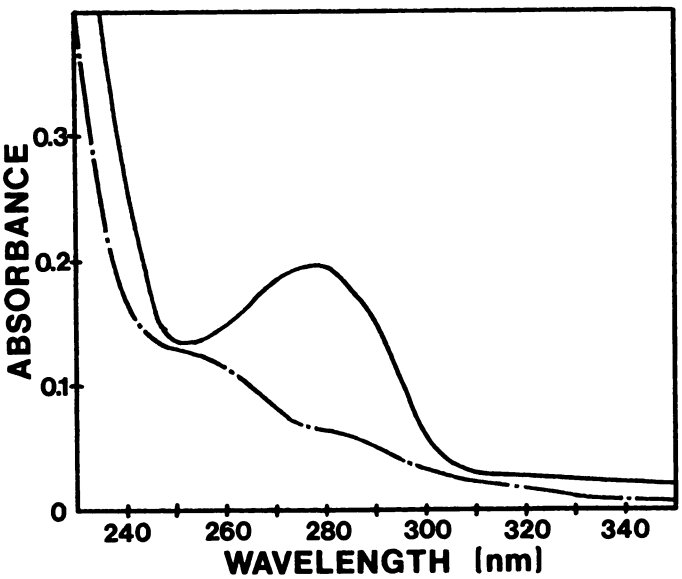

FIG. 1. *UV absorption spectra of the native (——) and NBS-modified (– · –) Lapemis neurotoxin*

Spectra were taken with a 0.2-cm pathlength and concentrations of the native and modified toxins were 0.05 and 0.10 M, respectively.

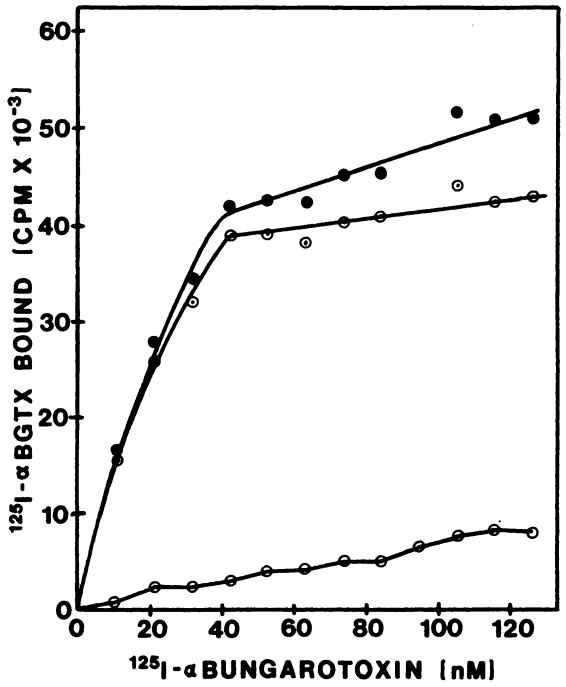

FIG. 2. *The total (●), nonspecific (○), and specific (⊙) binding of ^{125}I-α-Bgtx to the AcChR (2.5 μg) measured by the DEAE disc assay*

with AcChR at a concentration greater than or equal to the amount of ^{125}I-α-Bgtx added. A saturating concentration of ^{125}I-α-Bgtx was used in these experiments as determined by the binding experiments shown in Fig. 2. In contrast to native toxin, modified *Lapemis* toxin caused only a very slight decrease in ^{125}I-α-Bgtx binding to AcChR, indicating that the tryptophan modification nearly abolished the binding activity of *Lapemis* toxin.

Conformation of Lapemis toxin following modification. The conformation of native and modified *Lapemis* toxins was studied using CD spectroscopy. The CD spectrum of

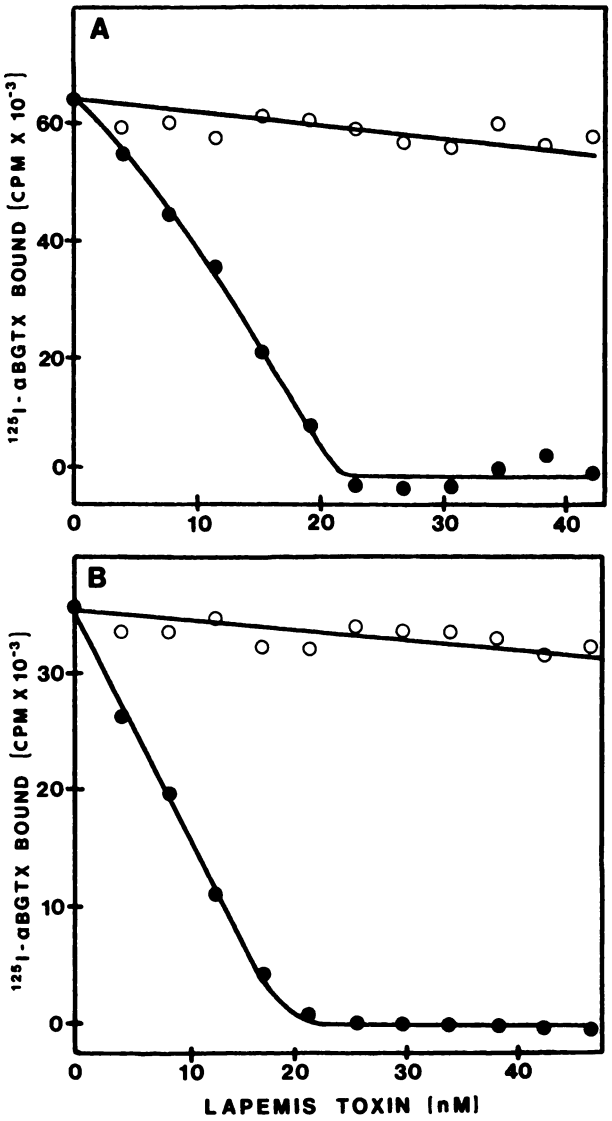

FIG. 3. *The binding of Lapemis neurotoxin to the AcChR measured as a decrease in ^{125}I-α-Bgtx binding following preincubation of AcChR (2.5 μg) with varying amounts of native (●) or modified (○) Lapemis neurotoxin*

A, native and HNBB-modified toxin binding to AcChR. B, native and NBS-modified toxin binding to AcChR.

the native toxin in the near UV region (Fig. 4) shows a large negative band at 278 nm with a shoulder at 268 nm. These bands are probably due to aromatic groups with some contribution from the four disulfide groups as evidenced by the trailing off of the ellipticity above 300 nm (12, 23, 24).

The HNBB-modified toxin spectrum shows a large decrease in the ellipticity at 278 nm and the appearance of negative bands at 332 and 427 nm and a small positive band at 395 nm. The decrease at 278 nm is due to modification of the tryptophan and the new bands are probably due to the 2-hydroxy-5-nitrobenzyl group attached to the tryptophan. The NBS-modified toxin spectrum also shows a large decrease in the 278-nm band and the appearance of a new band at 255 nm, which is probably due to the oxindole formed during the modification (25).

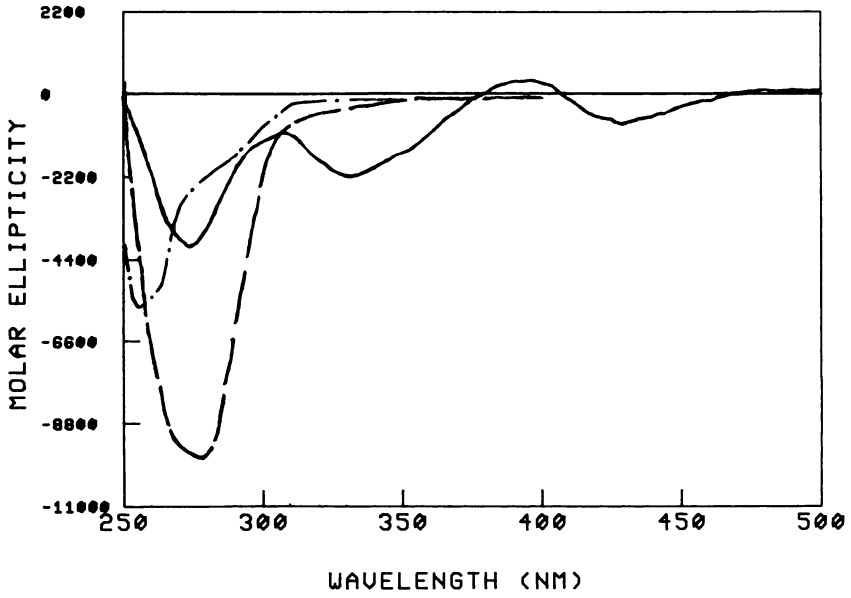

FIG. 4. *The CD spectra of the native and modified Lapemis neurotoxin in the near UV region*
Native toxin (– – –), HNBB-modified toxin (——), NBS-modified toxin (–·–).

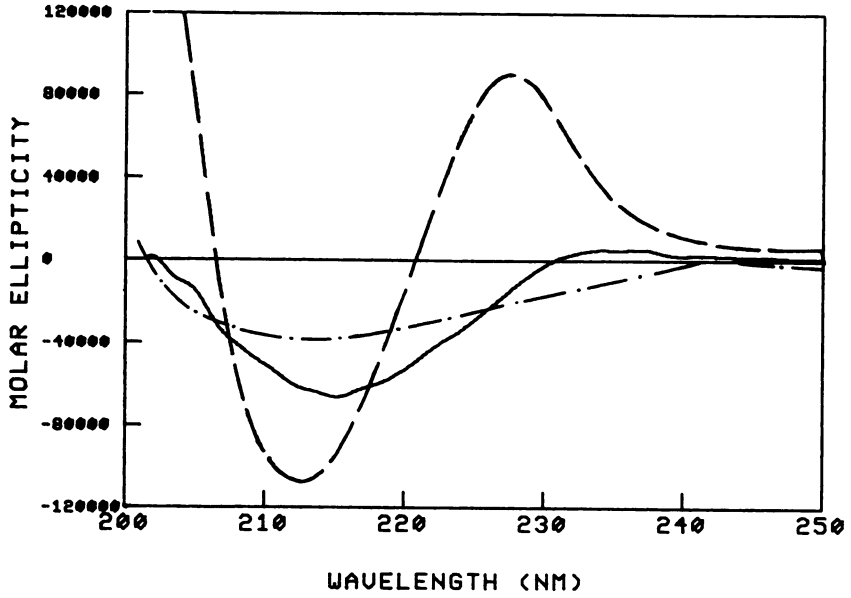

FIG. 5. *The CD spectra of the native and modified Lapemis neurotoxin in the far UV region*
Native toxin (– – –), HNBB-modified toxin (——), NBS-modified toxin (–·–).

In the far UV region (Fig. 5), the native toxin CD spectrum shows a negative band at 213 nm and positive bands at 228 nm and below 207 nm. Previously, this toxin has been reported to contain 20% β-sheet and 33% β-turn with no α-helical structure (12). The negative band at 213 nm and the positive band below 207 nm are indicative of β-sheet structure with some β-turn and unordered structure (12, 23, 26). The positive band at 228 nm is probably due to tryptophan with some contribution from the four disulfide bonds (12, 23, 26). Following modification of the toxin, the band at 213 nm is shifted slightly to 215 nm and becomes considerably broader. The positive ellipticity is still present below 202 nm, but it is weaker. The positive band at 228 nm disappears and becomes slightly negative. The NBS-modified toxin spectrum is similar, although the band at 213 nm becomes even broader. It is apparent that the rotational strength of the negative band at 213 nm is similar in the native and modified toxin spectra and that at 200 nm the native and modified spectra are both positive. This indicates that the modified toxin still retains considerable β-sheet structure. The disappearance of the positive band at 228 nm is probably due to modification of the tryptophan. However, the considerable broadening of the band at 213 nm could be due to structural changes in the toxin, the loss of tryptophan's contribution to the far UV region of the CD spectra, or both.

Therefore, we examined the conformation of the native and modified toxins using Raman spectroscopy. Raman

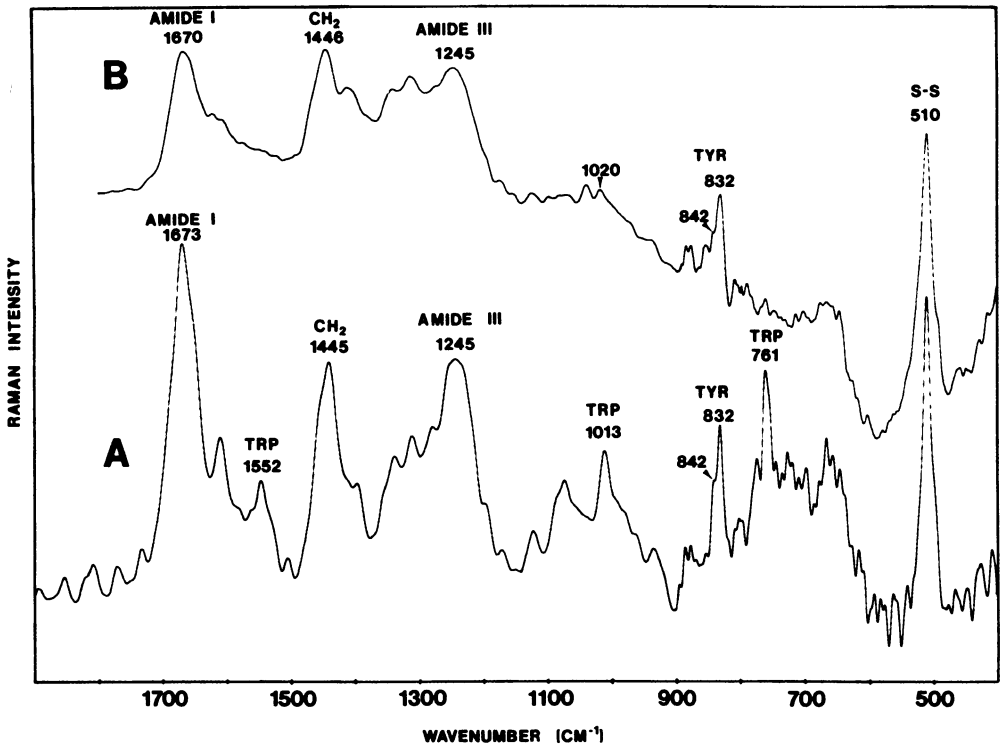

FIG. 6. *Raman spectra of the (A) native and (B) NBS-modified Lapemis neurotoxins*

spectra were obtained which show clearly the amide bands in the native and NBS-modified toxins (Fig. 6). The amide I band arises mainly from the coupled C=O stretching vibrations of peptide linkages and is useful in determining the peptide backbone structure. The amide III band of proteins arises mainly from the "in plane" N-H bending vibrations of the peptide group and can be influenced by many factors such as the strength of hydrogen bonds involved (10, 27, 28).

The best way to determine the peptide backbone conformation from Raman spectra is to determine the frequency of both amide I and amide III (27–29). Distinct amide I and amide III bands appeared at 1673 and 1245 cm^{-1}, respectively, in the native toxin Raman spectrum. The amide bands of native *Lapemis* toxin were observed at 1672 and 1240 cm^{-1} in an earlier study (10). The amide I and amide III bands of the NBS-modified toxin are seen at 1670 and 1245 cm^{-1}, respectively. The amide I and amide III bands are sensitive to conformational changes in the peptide backbone. The amide III band position of the modified toxin is identical to the native toxin amide III position and the amide I has been shifted by only 3 cm^{-1} relative to the native toxin amide I, indicating the overall structure has not been significantly modified. The intensity of the amide bands relative to the CH_2 deformation band at 1452 cm^{-1} is somewhat lower for the modified toxin than for the native toxin. Also, the amide I band of the modified toxin appears somewhat broader. Therefore, the native and modified toxin conformations may not be identical, but the fact that the amide bands have not been significantly shifted shows that the overall conformations are very similar.

It has been shown previously that the peaks at 832 and 842 cm^{-1} are due to tyrosine vibrations and that the ratio of intensities of the 842 and 832 cm^{-1} bands is related to the environment of the tyrosine side chain (27, 28). The relative intensity of the native toxin's 842 and 832 cm^{-1} bands indicates that the tyrosine is buried and probably involved in hydrogen bonding to other residues in agreement with structural data on similar toxins (9).

The tyrosine bands at 832 and 842 cm^{-1} are virtually identical in position and relative intensity in both the native and modified toxin spectra, indicating that the environment of the tyrosine has not been significantly affected by the tryptophan modification. This is especially significant as the tyrosine is only four residues distant from the tryptophan residue. This indicates that the conformation of some amino acid side chains in the vicinity of tyrosine and tryptophan have not been significantly altered by the modification and further supports the conclusion that the overall conformations of the native and modified toxins are very similar.

The lack of a distinct tryptophan peak at 1360 cm^{-1} in the native toxin spectrum indicates that the tryptophan is relatively exposed in agreement with NMR and fluorescence data (9, 30–32). The tryptophan bands at 761, 1013, and 1552 cm^{-1} were not found in the modified toxin spectrum, indicating that the modification was complete. Following the modification, the tryptophan band at 1013 cm^{-1} disappeared, and a new band at 1020 cm^{-1} appeared, which is probably due to oxindole (33).

The S-S stretching band was seen at 510 cm^{-1} in both the native and modified toxin spectra which shows that the disulfides had a similar conformation in both cases (27, 28). This is significant because the toxin contains four disulfides which largely control the overall backbone structure of the toxin.

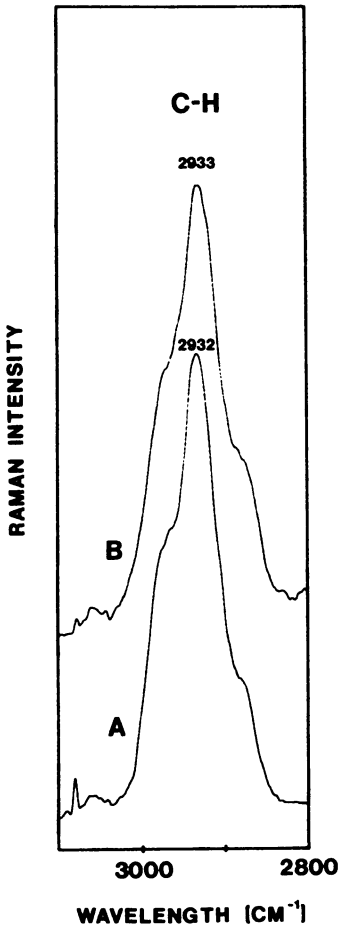

FIG. 7. *Raman spectra of the (A) native and (B) NBS-modified Lapemis neurotoxins in the C-H stretching region*

The C-H stretching band appeared at 2932 cm^{-1} in the native toxin spectrum and 2933 cm^{-1} in the modified toxin spectrum and was virtually identical in intensity (Fig. 7). This is further evidence that the modification did not significantly alter the conformation of the toxin since the C-H band intensity has been shown to increase greatly following denaturation of a protein (34).

DISCUSSION

Based on amino acid sequencing and X-ray diffraction data of many similar neurotoxins, loop 4 of *Lapemis* toxin contains Trp 27, Arg 31, and Gly 32 which appear in all the neurotoxins studied, and Lys 25, Asp 29, and Glu 36 which appear in greater than 66% of the neurotoxins studied (Fig. 8). All of these residues are oriented toward the same face of the molecule (9). These data indicate that those residues are all probably functionally important in *Lapemis* neurotoxin as well. Chemical modification studies also indicate the importance of these residues in the neurotoxic activity of these toxins. The tryptophan of *Lapemis* toxin was chemically modified in a previous study in this laboratory and shown to be essential for neurotoxicity in mice (4). This investigation has examined the effect of modification of the tryptophan residue of *Lapemis* toxin on neurotoxic activity at the molecular level and found it to be important for AcChR-binding activity.

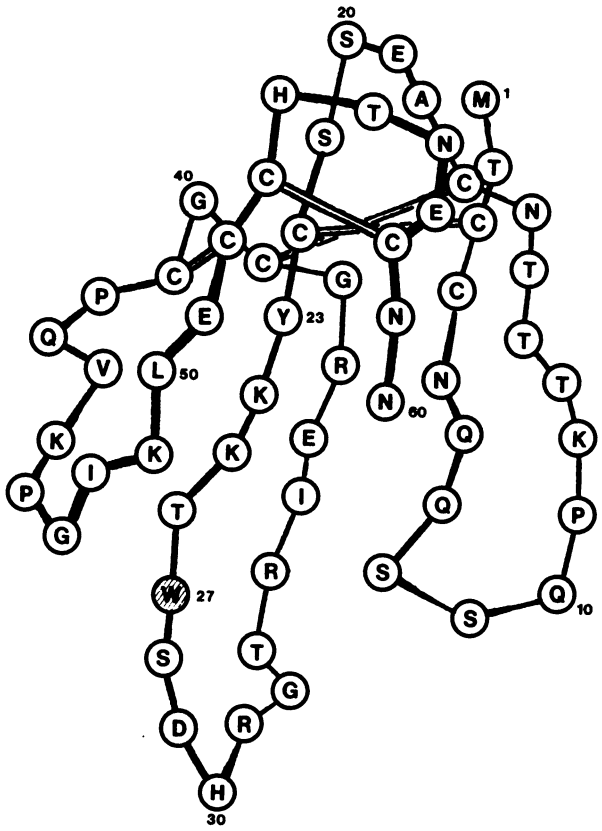

FIG. 8. *The amino acid sequence and structure of Lapemis neurotoxin*

The structure is based on the crystal structure of a similar neurotoxin, toxin b (36).

Amino acid analysis of the HNBB-modified toxin indicated that only tryptophan was modified. In addition, an S-H band was still observed at 2574 cm^{-1} in the Raman spectrum of the HNBB-modified toxin, indicating that the single cysteine had not been modified. Amino acid analysis of the NBS-modified toxin showed that only tryptophan was modified. There was no change in the lysine content of the toxin following the modification, indicating that no lysines were modified (4, 22). Detailed amino acid analyses before and after modification of tryptophan were made on a similar neurotoxin from *Laticauda semifasciata* and also showed no change in other amino acid residues following the modification (5). The S-H band at 2585 cm^{-1} was not detectable in the Raman spectrum of the NBS-modified toxin, indicating that the cysteine may have been modified. However, modification of this cysteine has been shown to have no effect on the binding of *Lapemis* toxin to AcChR (35). Therefore, the loss of *Lapemis* toxin binding to AcChR following HNBB or NBS modification is due to modification of the single tryptophan.

From Raman and NMR data, it is clear that the tryptophan is relatively exposed to the surface of short chain neurotoxins. The lack of a distinct Raman band at 1360 cm^{-1} indicates that the tryptophan is exposed and the presence of tryptophan band widths seen in NMR spectra implies that tryptophan is more flexible than the nearby tyrosine, also supporting the conclusion that

tryptophan is exposed (9, 30, 32). Therefore, it is possible that the tryptophan directly interacts with the AcChR upon binding.

Previously, it had not been clear whether tryptophan played a secondary role in stabilizing the conformation of the active site of short chain neurotoxins or if it was directly involved in the binding process. Therefore, the conformation of the modified toxin was examined to see if the modification of tryptophan had significantly disrupted the structure of the toxin. No major changes in the overall structure were found as indicated by the Raman spectra of the native and modified toxins (Figs. 6 and 7). Examination of the Raman tyrosine bands for the native and modified toxins indicated that the environment of the tyrosine side chain was not significantly changed by the modification. Based on NMR and X-ray crystallographic data from studies on similar neurotoxins, tyrosine interacts extensively with several residues in all three major loops of *Lapemis* toxin (Fig. 8). Hydrogen bonding occurs between Tyr 23 (O) and Asn 59 (γ-amide NH). The hydroxyl group of Tyr 23 forms a hydrogen bond with Glu 36 as the probably acceptor, accounting for its high phenolic pK_a (~12). Tyr 23 also participates in extensive hydrophobic interactions with Lys 25, Glu 36, Pro 42, and Leu 50 which, together with residues 38 to 41, effectively enclose tyrosine, accounting for its hindered side chain mobility (9). The Raman data lead us to believe that these interactions have not been significantly disrupted. Therefore, the available spectroscopic data indicate that the loss in AcChR binding is not due to major changes in the conformation of the toxin caused by the modification of tryptophan. Rather, the data indicate that tryptophan may play a more direct role in the AcChR-binding process such as π-bonding during complex formation. It will be necessary to determine the structure of the AcChR neurotoxin-binding site before a determination of the exact role of the sea snake venom neurotoxin's tryptophan residue in the binding process can be determined.

REFERENCES

1. Tu, A. T. Neurotoxins of animal venoms: snakes. *Annu. Rev. Biochem.* **42**:235–258 (1973).
2. Tu, A. T. *Venoms: Chemistry and Molecular Biology*. John Wiley & Sons, Inc., New York (1977).
3. Chang, C. C., and K. Hayashi. Chemical modification of the tryptophan residue in cobratoxin. *Biochem. Biophys. Res. Commun.* **37**:841–846 (1969).
4. Tu, A. T., and B.-S. Hong. Purification and chemical studies of a toxin from the venom of *Lapemis hardwickii* (Hardwick's sea snake). *J. Biol. Chem.* **246**:2772–2779 (1971).
5. Tu, A. T., B.-S. Hong, and T. N. Solie. Characterization and chemical modifications of toxins isolated from the venoms of the sea snake, *Laticauda semifasciata*, from Philippines. *Biochemistry* **10**:1295–1304 (1971).
6. Tu, A. T., T. S. Lin, and A. L. Bieber. Purification and chemical characterization of the major neurotoxin from the venom of *Pelamis platurus*. *Biochemistry* **14**:3408–3413 (1975).
7. Chicheportiche, R., J.-P. Vincent, C. Kopeyan, H. Schweitz, and M. Ladzunski. Structure-function relationship in the binding of snake neurotoxins to the *Torpedo* membrane receptor. *Biochemistry* **14**:2081–2091 (1975).
8. Calvo-Fernandez, P., and M. Martinez-Carrion. A modification of α-bungarotoxin amino group residues with retention of binding properties to isolated and membrane-bound acetylcholine receptor. *Arch. Biochem. Biophys.* **208**:154–159 (1981).
9. Dufton, M. J., and R. C. Hider. Conformational properties of the neurotoxins and cytotoxins isolated from Elapid snake venoms. *CRC Crit. Rev. Biochem.* **14**:113–171 (1983).
10. Yu, N.-T., T. S. Lin, and A. T. Tu. Laser Raman scattering of neurotoxins isolated from the venoms of sea snakes *Lapemis hardwickii* and *Enhydrina schistosa*. *J. Biol. Chem.* **250**:1782–1785 (1975).
11. Fox, J. W., M. Elzinga, and A. T. Tu. 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**:217–220 (1977).
12. Fox, J. W., and A. T. Tu. Conformational analysis of a snake neurotoxin by prediction from sequence, circular dichroism, and Raman spectroscopy. *Arch. Biochem. Biophys.* **193**:407–414 (1979).
13. Litchfield, J. T., Jr., and F. Wilcoxon. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **96**:99–113 (1949).
14. Horton, H. R., and D. E. Koshland, Jr. A highly reactive colored reagent with selectivity for the tryptophan residue in proteins: 2-hydroxy-5-nitrobenzyl bromide. *J. Am. Chem. Soc.* **87**:1126–1132 (1965).
15. Freisheim, J. H., and F. M. Huennekens. Effect of *N*-bromosuccinimide on dihydrofolate reductase. *Biochemistry* **8**:2271–2276 (1969).
16. Saitoh, T., and J.-P. Changeux. Phosphorylation *in vitro* of membrane fragments from *Torpedo marmorata* electric organ: effect on membrane solubilization by detergents. *Eur. J. Biochem.* **105**:51–62 (1980).
17. Schmidt, J., and M. A. Raftery. A simple assay for the study of solubilized acetylcholine receptors. *Anal. Biochem.* **52**:349–354 (1973).
18. Leprince, P., R. L. Noble, and G. P. Hess. Comparison of the interactions of a specific neurotoxin (α-bungarotoxin) with the acetylcholine receptor in *Torpedo californica* and *Electrophorus electricus* membrane preparations. *Biochemistry* **20**:5565–5570 (1981).
19. Fulpius, B. W., R. Miskin, and E. Reich. Antibodies from myasthenic patients that compete with cholinergic agents for binding to nicotinic receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:4326–4330 (1980).
20. Juillerat, M. A., B. Schwendimann, J. Hauert, B. W. Fulpius, and J. P. Bargetzi. Specific binding to isolated acetylcholine receptor of a synthetic peptide duplicating the sequence of the presumed active center of a lethal toxin from snake venom. *J. Biol. Chem.* **257**:2901–2907 (1982).
21. Simpson, R. J., M. R. Neuberger, and T.-Y. Liu. Complete amino acid analysis of proteins from a single hydrolysate. *J. Biol. Chem.* **251**:1936–1940 (1976).
22. Hong, B.-S. Studies on the toxic principles in the venoms of sea snakes. Colorado State University, Ph.D. thesis (1971).
23. Sears, D., and S. Beychok. Circular dichroism, in *Physical Principles and Techniques of Protein Chemistry* (S. J. Leach, ed.), Part C. Academic Press, New York, 445–593 (1970).
24. Strickland, E. H. Aromatic contributions to circular dichroism spectra of proteins. *CRC Crit. Rev. Biochem.* **2**:113–115 (1974).
25. Strickland, E. H., M. Wilchek, and C. Billups. Circular dichroism of modified tryptophan residues β-3-oxindolyl-L-alanine. *Biochim. Biophys. Acta* **303**:28–35 (1973).
26. Chen, Y.-H., J.-C. Tai, W.-J. Huang, M.-Z. Lai, M.-C. Hung, M.-D. Lai, and J. T. Yang. Role of aromatic residues in the structure-function relationship of α-bungarotoxin. *Biochemistry* **21**:2592–2600 (1982).
27. Yu, N.-T. Raman spectroscopy: a conformational probe in biochemistry. *CRC Crit. Rev. Biochem.* **4**:229–280 (1977).
28. Tu, A. T. *Raman Spectroscopy in Biology: Principles and Applications*. John Wiley & Sons, Inc., New York (1982).
29. Peticolas, W. L. Applications of Raman spectroscopy to biological macromolecules. *Biochimie* **57**:417–428 (1975).
30. Yu, N.-T. Comparison of protein structure in crystals, in lyophilized state, and in solution by laser Raman scattering. III. α-Lactalbumin. *J. Am. Chem. Soc.* **96**:4664–4668 (1974).
31. Grinvald, A., and I. Z. Steinberg. The fluorescence decay of tryptophan residues in native and denatured proteins. *Biochim. Biophys. Acta* **427**:663–678 (1976).
32. Inagaki, F., T. Miyazawa, and R. J. P. Williams. The dynamic structures of proteins: short and long neurotoxins as examples. *Biosci. Rep.* **1**:743–755 (1981).
33. Schmidt, H., and L. Bieker. Raman spectra of chemically modified lysozyme. Oxindole derivatives. *Arch. Biochem. Biophys.* **195**:205–210 (1979).
34. Verma, S. P., and D. F. H. Wallach. Changes of Raman scattering in the CH-stretching region during thermally induced unfolding of ribonuclease. *Biochem. Biophys. Res. Commun.* **74**:473–479 (1977).
35. Ishizaki, H., M. Allen, and A. T. Tu. Effect of sulfhydryl group modification on the neurotoxic action of a sea snake toxin. *J. Pharm. Pharmacol.* **36**:36–41 (1984).
36. Tsernoglou, D., and Petsko, G. A. The crystal structure of a postsynaptic neurotoxin from sea snake at 2.2 Å resolution. *FEBS Lett.* **68**:1–4 (1976).

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