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Effect of sulfhydryl group modification on the neurotoxic action of a sea snake toxin

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Pelamis toxin *a* is a major neurotoxin isolated from the venom of *Pelamis platurus* (yellow-bellied sea snake). The effect of sulfhydryl group modification by *NN'*-1,4-phenylenedimaleimide on the neurotoxic action of Pelamis toxin *a* has been investigated. The cross-linked toxin having a molecular weight of 11 000 was formed without significant structural changes in the toxin. Lethality tests on the modified toxin indicated that it retained considerable toxicity, although its potency was weaker than that of the native toxin. Binding studies with the acetylcholine receptor isolated from the electroplax of *Torpedo californica* indicated that the modified toxin binds to the receptor but less effectively than the native toxin. These results suggest that the decreases in toxicity and binding to the receptor are due to a decrease in accessibility of cross-linked neurotoxin to the receptor. This leads us to the conclusion that the region of the neurotoxin containing the sulfhydryl group is not essential for its biological activity. Analysis of the structure and function relationships of the modified toxin suggests that the neurotoxin-acetylcholine receptor interaction requires the proper orientation of the neurotoxin molecule.

It was well established that neurotoxins of the postsynaptic type isolated from Elapidae and sea snake (Hydrophiidae) venoms bind to acetylcholine receptors with extremely high binding constants (Changeux et al 1970; Eldefrawi et al 1972). Practically all investigations used lethality in mice as the biological assay of neurotoxic action. Since neurotoxins have high toxicity, determination of lethality (LD50) does reflect neurotoxic activity; however, in a strict sense lethality and neurotoxic action are not the same. Therefore, determination of the binding ability of neurotoxin to the acetylcholine receptor as well as the LD50 are necessary in the biological assay of neurotoxins.

To find what part of the neurotoxin is essential for neurotoxic action, structure-function relationships of neurotoxins have been a subject of extensive study (Tu 1973). It was well established that tryptophan, tyrosine, lysine and arginine residues played a role in the neurotoxic action of snake toxins (Lee 1972; Tu 1977). Sea snake postsynaptic neurotoxins have four disulfide bonds and some have an additional single sulfhydryl group (Wang et al 1976; Fox et al 1977). Pelamis toxin *a* was originally found to consist of 55 amino acid residues (Tu et al 1975). Subsequently, the amino acid sequence of the toxin from *Pelamis platurus* was identified, and it was shown to contain 60 residues (Wang et al 1976). The discrepancy is quite common for results obtained from amino acid composition data compared to the sequence study. According to the sequence study the toxin contained one free sulfhydryl group which is located at position 4 from the N-terminal. However, it is still not clear whether the single sulfhydryl group present in some neurotoxins is important for neurotoxic action.

We have investigated the effect of sulfhydryl group modification on the neurotoxic action of Pelamis toxin *a* using *NN'*-1,4-phenylenedimaleimide, a modifying reagent specific for sulfhydryl groups (Chang & Flaks 1972; Moore & Ward 1956). The cross-linked neurotoxin was purified, characterized, and its toxicity and binding to acetylcholine receptor were examined.

MATERIALS AND METHODS

Sea snakes, *Pelamis platurus*, were captured off the Pacific coast of Costa Rica in 1977. Venom was extracted as previously described (Tu & Hong 1971). Pelamis neurotoxin *a* was isolated from crude venom by Sephadex G-50 and carboxymethyl-cellulose column chromatography as described by Tu et al (1975). Homogeneity of Pelamis toxin *a* was established by SDS-phosphate polyacrylamide gel electrophoresis. *NN'*-1,4-Phenylenedimaleimide was purchased from Aldrich Chemical Company. α-Chymotrypsinogen A, myoglobin, cytochrome C, insulin B chain, and

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BSA (bovine serum albumin) were obtained from Sigma Chemical Company. All other chemicals were of reagent grade.

Chemical modification of the sulfhydryl group

Pelamis toxin *a* (6·2 mg) in 0·05 M Tris-HCl buffer, pH 7·8, containing 0·05 M KCl and 0·5 M urea was reacted with *NN'*-1,4-phenylenedimaleimide, which was first dissolved in acetone and then solubilized in buffer for 48 h at 24 °C. The final Pelamis toxin *a* and *NN'*-1,4-phenylenedimaleimide concentrations were 5·0 and 2·5 mM, respectively. Immediately after the reaction the modified Pelamis toxin *a* was purified on a column (1·6 × 87 cm) of Sephadex G-50 (superfine) eluted with 0·01 M potassium phosphate buffer, pH 7·0, containing 0·1 M NaCl. Fractions of 2·0 ml were collected at a flow rate of 5·3 ml h^{-1}, and absorbance at 280 nm was measured spectrophotometrically (Fig. 1).

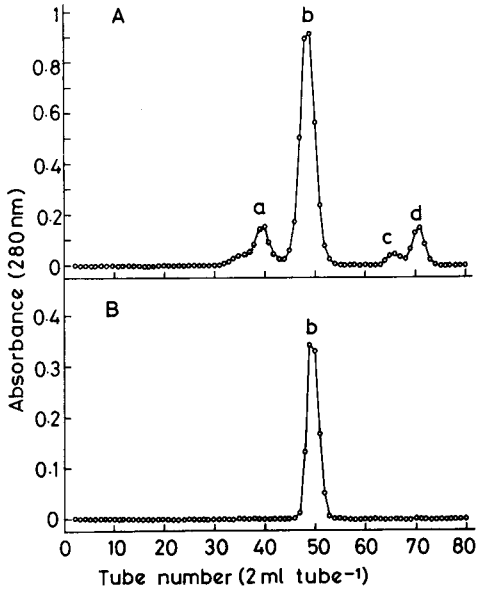

FIG. 1. (A) Elution profile of the reaction mixture on a Sephadex G-50 column. Peak a is modified Pelamis toxin *a*; shaded area indicates fractions collected. Peak b is unmodified Pelamis toxin *a*. Peaks c and d are excess reagent. (B) Elution profile of native Pelamis toxin *a* on the same column as used in A. Note the position of the native toxin (B, peak b) compared with that of the unmodified toxin (A, peak b).

Raman spectra were obtained with a Spex Industries Ramalog 5 Raman spectrometer equipped with a computer (Spex SCAMP). Samples (lyophilized powder) were illuminated using the 514·5-nm line excitation of an argon ion laser (Spectrophysics model SP-164) at 4 cm^{-1} spectral width resolution and with a green interference filter. The final spectra represent an average of 10 scans obtained by a SCAMP data acquisition processor (Fig. 2).

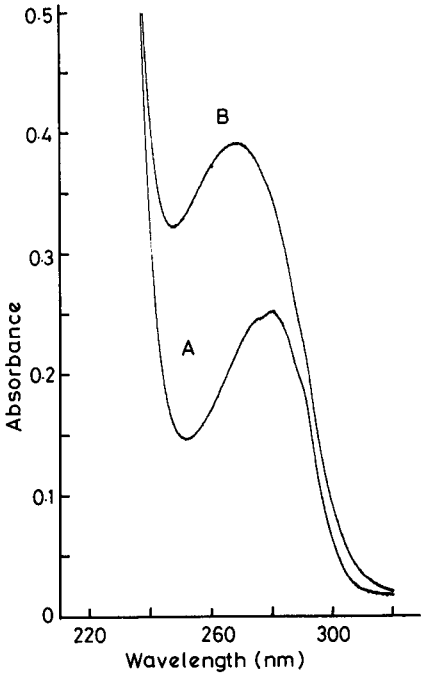

FIG. 2. Absorption spectra of (A) native Pelamis toxin *a* (0·33 mg ml^{-1}); (B) chemically modified Pelamis toxin *a* (0·20 mg ml^{-1}). Note the slight blue shift in the absorption maximum.

The molecular weight of the modified toxin was estimated by Sephadex G-50 (superfine) column chromatography using 0·1 M potassium phosphate buffer, pH 7·0, containing 0·1 M NaCl as elution buffer. The column (1·6 × 87 cm) was calibrated with the following protein standards: α-chymotrypsinogen A (25 700), myoglobin (17 200), cytochrome *c* (12 400), Pelamis toxin *a* (6200), and insulin B chain (3500).

Lethality test

Toxicity was assayed with white Swiss-Webster mice of 18 to 22-g. Toxin in 100 µl of 0·9% NaCl (saline) was injected by intravenous route. The LD50 was determined with 5 to 10 mice per dose level, allowing a 24 h interval according to the method of Litchfield & Wilcoxon (1949).

Neurotoxicity assay

Acetylcholine receptor (AChR) was purified from *Torpedo californica* electroplax (Pacific Biomarine

Labs) by the method of Saitoh & Changeux (1980) with the following changes: the buffer used in all steps of the purification contained 50 mM Tris-HCl (pH 7·5), 3 mM EDTA (ethylene diamine tetraacetic acid), 1 mM EGTA (ethyleneglycol-bis(β-amino-ethyl ether) *NN'*-tetracetic acid), 0·1 mM PMSF (phenylmethyl sulphonyl fluoride), 0·02% azide and 5 μg ml^{-1} each of aprotinine and pepstatin to inhibit proteolysis. Following homogenization of the pellet and adjustment to 32% (w/w) sucrose, about 12 ml of this suspension was layered on top of 14 ml of a 41·5% (w/w) sucrose solution and centrifuged for 50 min at 50 000 rev min^{-1} in a Beckman Ti-60 rotor (Lindstrom et al 1980).

The ^{125}I-labelled α-bungarotoxin (^{125}I-αBgtx) was purchased from New England Nuclear (NEN). Toxin binding was determined by the DEAE disc assay of Schmidt & Raftery (1973). The assays were done in Torpedo Ringer's buffer containing (in mM) NaCl 250, KCl 5, $CaCl_2$ 4, $MgCl_2$ 2, sodium phosphate 5 (pH 7·5), and 0·1% BSA to decrease non-specific binding. Varying amounts of Pelamis toxin *a* or modified toxin were pre-incubated with a set amount of AChR in Torpedo Ringer's buffer at room temperature for 1 h. Then a saturating amount of ^{125}I-αBgtx was added to the assay mixture to give a final volume of 125 μl, and this was incubated for another hour. Two Whatman DE81 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 DE81 filter discs and allowed to soak in for 10 min. The discs were then rinsed 5 times with 5 ml of buffer. The filters were finally dried under a heat lamp, placed in 5 ml of Econoflour (NEN), and counted in a Beckman LS 7800 liquid scintillation counter. Background counts were determined by doing the same assay in the presence of a 100-fold excess of unlabelled αBgtx.

Protein concentrations were determined by the method of Hartree (1972). BSA was used as a protein standard.

RESULTS

Pelamis toxin a

Homogeneity of the native Pelamis toxin *a* was established by SDS-phosphate polyacrylamide gel electrophoresis. Since Raman spectroscopy can detect the stretching vibration of the S–H bond, it is a useful analytical technique for detection of sulfhydryl groups. The S–H stretching vibration band was detected at 2568 cm^{-1}, confirming the presence of the sulfhydryl group (Fig. 3A). The S–H bond stretching vibrations of alkyl thiols usually appear in the 2670 to 2580 cm^{-1} region of the Raman spectra (Silverstein & Bassler 1966; Dollish et al 1973; Tu 1982).

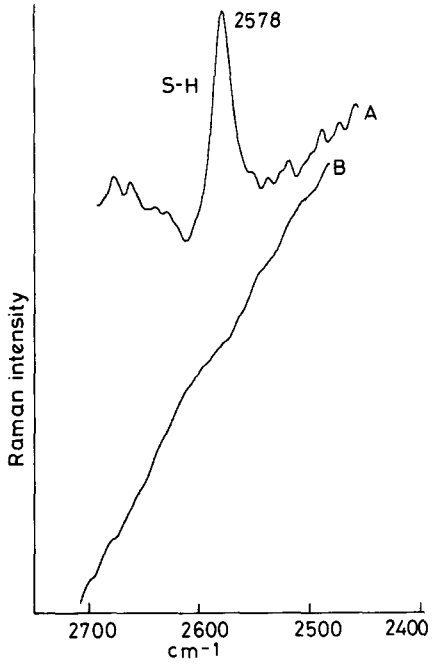

FIG. 3. Raman spectra in the region of the S–H stretching vibration at 2578 cm^{-1}.
(A) Unmodified Pelamis toxin *a*. The band at 2578 cm^{-1} indicates the presence of a sulfhydryl group.
(B) Pelamis toxin *a* after chemical modification to form a dimer. No S–H stretching vibration band is seen, indicating that the S–H group is conjugated by the bifunctional reagent. Experimental conditions are the same as in Fig. 6, except that radiant power of 250 mW (A) and 300 mW (B) was used.

The Raman spectrum of the native toxin from 400 to 1750 cm^{-1} was measured (Fig. 4A) and it agrees well with previously published data (Tu et al 1976), which indicated that the native toxin consists primarily of antiparallel β-sheet and β-turn structures. Fig. 2A shows the absorption spectrum of the native toxin, which has a maximum at 280 nm and shoulders centered at 275 and 290 nm.

Modification of Pelamis toxin a

After chemical modification of Pelamis toxin *a* with *NN'*-1,4-phenylenedimaleimide, the reaction mixture was placed on a Sephadex G-50 (superfine) column to separate modified from unmodified toxin

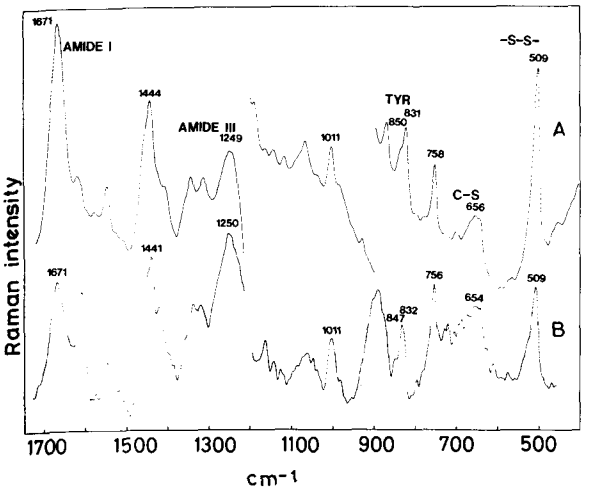

FIG. 4. Raman spectra of (A) native Pelamis toxin *a*; (B) chemically modified Pelamis toxin *a*. Experimental conditions: radiant power, 60 mW (A), 80 mW (B); integration time, 0·5 s; scale, 1000 pulse s^{-1} (A), 2000 pulse s^{-1} (B); slit width, 500 μm; scan increment, 0·5 cm^{-1}; number of scans, 10.

and excess reagent (Fig. 1A). The second fraction (fraction b) was the unmodified Pelamis toxin *a*, since in a separate experiment the native Pelamis toxin *a* eluted at the same place (Fig. 1B). The molecular weight of fraction a in Fig. 1A was found to be 11 000, about twice that of the native Pelamis toxin *a*. The modified toxin was found to be homogeneous by SDS-phosphate polyacrylamide gel electrophoresis. The yield of the cross-linked toxin was calculated to be about 12·5%.

Absorption spectra of the native and modified toxin are compared in Fig. 2. The spectrum of the modified toxin showed a maximum at 270 nm (Fig. 2B). This blue shift in the absorption maximum is slightly different from that of the unmodified toxin. This seems reasonable since the modifying agent, which absorbs in the near uv region, attaches to a sulfhydryl group.

The Raman spectrum of the modified toxin does not show the S–H bond stretching vibration at 2578 cm^{-1}, indicating that the sulfhydryl group is modified (Fig. 3B). Although the free sulfhydryl group disappears upon modification, there is no effect on the disulfide bonds, as shown by the fact that the S–S bond stretching vibration at 509 cm^{-1} can be clearly seen in the Raman spectrum of the modified toxin (Fig. 4B). The 509-cm^{-1} line for both native and modifed toxin indicates that the C–C–S–S–C–C conformation in the disulfide bridges is of the gauche-gauche-gauche form (Sugeta et al 1972, 1973).

The relative intensities of the bands of 831 and 850 cm^{-1} for both native and modified toxins indicate that the single tyrosine residue is buried and is not accessible to solvents (Yu et al 1973). The C–H bond stretching regions of the Raman spectra (2800 ~ 3100 cm^{-1}) for native and modified toxins (not shown) are essentially the same, suggesting that there is little change in the microenvironment of the hydrophobic side chains.

Both amide I and III bands are used for elucidation of the protein backbone conformation. The amide I and III bands of native and modified toxins appear at 1671 and 1249–1250 cm^{-1}, respectively (Fig. 4A and 4B). This suggests that essentially no change in the peptide backbone conformation occurs after the neurotoxin is cross-linked to form a dimer.

Lethality

The toxicity of the modified toxin was determined and compared with that of the native toxin. The LD50 was determined statistically from the number of mice which died from 9 different doses with five to ten mice used for each dose. The LD50 of the modified toxin is 0·97 μg g^{-1}, whereas the LD50 for native toxin is 0·13 μg g^{-1}. This indicates that the modified toxin retains considerable toxicity, although it is weaker than native toxin.

Acetylcholine receptor binding studies

Binding of native and modified Pelamis toxin *a* was studied using acetylcholine receptor isolated from the electroplax of *Torpedo californica* and ^{125}I-αBgtx. Binding studied using ^{125}I-αBgtx and acetylcholine receptor indicated that there were 1·2 mmoles of toxin-binding sites per gram of AChR protein. To measure the binding of Pelamis toxin *a* to the acetylcholine receptor, varying amounts of native toxin and receptor were pre-incubated for 1 h, after which a saturating amount of ^{125}I-αBgtx was added and the mixture was incubated for another hour. As shown in Fig. 5 (bottom curve), the more Pelamis toxin *a* which was bound to the acetylcholine receptor, the less ^{125}I-αBgtx was bound. When the receptor was pre-incubated with an equal or greater amount of Pelamis toxin *a*, all the toxin-binding sites of receptor were occupied by Pelamis toxin *a* and no ^{125}I-αBgtx was bound (Fig. 5, bottom curve).

However, a concentration of modified toxin greater than that of the native toxin was required to fill the same amount of toxin-binding sites, as shown in Fig. 5 (top curve). The concentration of modified toxin required to inhibit ^{125}I-αBgtx binding by 50% (IC50) was 20 nM compared with 12 nM for native

toxin, indicating that the modified toxin is less neurotoxic than the native toxin. All binding studies were repeated three times with similar results, proving that these results are significant. Fig. 5 is one representative experiment. Therefore, the toxicity and AChR-binding data agree since both decrease following modification, although the correlation is not linear as the toxicity decreases several fold more than the AChR-binding ability. The correlation is closer when one considers that in-vivo probably only one dimer binds per receptor, which would make the LD50 of modified toxin one-half the value found, as will be discussed later.

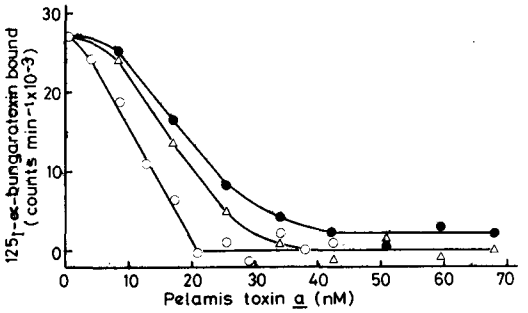

FIG. 5. Comparison between the binding of native and modified neurotoxin to acetylcholine receptor. In this assay the acetylcholine receptor preparation was incubated with increasing amounts of native for 1 h (○——○) or modified toxin for 1 h (●——●), and 4 h (△——△). Then a saturating amount of ^{125}I-αBgtx was added to the preparation and incubated for 1 h more. The total volume of each assay was 125 μl.

When the native toxin was pre-incubated with the receptor for 24 h under the same experimental conditions, no increase in binding to the receptor was observed. However, when modified toxin was pre-incubated for 4 h, the IC50 decreased from 20 to 17 nM, indicating that binding to the receptor had increased. No further increase in binding to receptor was observed when pre-incubation was carried out for 12 h. Although the binding of modified toxin to receptor increased with a longer pre-incubation period, the modified toxin bound less effectively than did native toxin, as can be seen in Fig. 5 (middle curve).

DISCUSSION

Two molecules of Pelamis toxin *a* reacted with *NN'*-1,4-phenylenedimaleimide since the reagent contains two reacting groups. Thus the modified toxin is a dimer cross-linked by the reagent (Fig. 6).

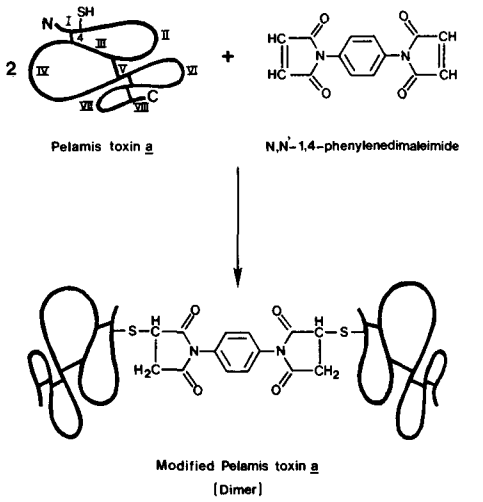

FIG. 6. Diagram of the reaction of 2 moles of Pelamis toxin *a* with *NN'*-1,4-phenylenedimaleimide to form a dimer through conjugation of the sulfur atoms. The four bars connecting Pelamis toxin *a* represent four disulfide bonds. The single cysteinyl residue in the toxin is represented by –SH, and the number 4 refers to the position of the –SH group. The Roman numerals represent the loop numbers of the neurotoxin.

Compared to the LD50 of 0·13 μg g^{-1} for the native toxin, the LD50 of the modified toxin was 0·97 μg g^{-1}, indicating that the chemically modified toxin without a free sulfhydryl group retained considerable toxicity. From the binding studies of native and modified toxin using acetylcholine receptor isolated from the electroplax of *Torpedo californica*, it was found that the modified toxin binds to the receptor less effectively than the native toxin. When the toxin becomes cross-linked, it seems possible that only one-half of a dimer could bind to a receptor in-vivo; therefore, the LD50 of 0·97 μg g^{-1} for the cross-linked toxin is really equivalent to 0·48 μg g^{-1}. This makes the correlation of toxicity data to the receptor binding better.

Since the modified toxin retains a considerable amount of the structure associated with the native toxin, it is suggested that because the toxicity and the ability to bind to the receptor decreased but were not lost completely, that the change is due to the cross-linked neurotoxin being less accessible to the receptor. This leads to the conclusion that the region of the neurotoxin containing the sulfhydryl group is not involved in its biological activity and therefore it is not located in the vicinity of the active site of the neurotoxin molecule.

According to X-ray diffraction and chemical modification data, loop IV is essential for acetylcholine receptor binding activity (Dufton & Hider 1983).

Since the sulfhydryl group is located in loop II next to the disulfide bridge (between residues 3 to 22), our results agree well with previous studies (Fig. 6).

The time course studies on the binding of the native and modified toxin to the acetylcholine receptor indicated that, whereas the binding of native toxin to the receptor did not increase with a longer pre-incubation time, the binding of modified toxin to the receptor increased. This result suggests that the proper orientation of the modified toxin is essential for strong interaction of the toxin with the receptor. Since the modified toxin is a cross-linked molecule, the number of toxin-receptor interactions which would result in strong binding would be limited compared with the interactions of native toxin with the receptor. Thus more modified toxin can achieve the proper orientation during a longer pre-incubation period. The present study suggests that strong interactions of the neurotoxin with the acetylcholine receptor requires the proper orientation of the neurotoxin molecule with respect to the toxin-binding sites on the receptor.

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