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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

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ABSTRACT: The role of the "C-terminal tail" segment of long neurotoxins has been investigated. The C-terminal four to five residues of α-bungarotoxin and Laticauda colubrina b have been cleaved off by carboxypeptidase P. The effect of such deletion on the toxin conformation has been monitored in proton nuclear magnetic resonance spectra and circular dichroism spectra. The removal of the C-terminal residues primarily affects the chemical shifts of proton resonances of the residues close to the cleavage site and does not induce a major conformational change. Therefore, the C-terminal tail of long neurotoxins does not appear to be important in maintaining the specific polypeptide chain folding. On the other hand, competition binding with tritium-labeled toxin α to *Narke japonica* acetylcholine receptor has revealed that cleavage of the C-terminal residues reduces the binding activity of α-bungarotoxin or Laticauda colubrina b to acetylcholine receptor. Thus it is likely that (the basic amino acid residues in) the C-terminal tail is directly involved in the binding of long neurotoxins to electric organ (and muscle) acetylcholine receptor.

Venoms of proteroglyphous snakes (cobras and sea snakes) contain small curaremimetic proteins called neurotoxins [reviewed by Dufton and Hider (1983)]. The neurotoxin binds specifically to the nicotinic acetylcholine receptor (AChR)[1] in the postsynaptic membranes of vertebrate muscle and fish electric organs, prevents the binding of chemical neurotransmitter acetylcholine, and thereby blocks the nerve impulses. At present, amino acid sequences of about 80 neurotoxins are available, and they form a large family of homologous proteins, which can be further divided into two subgroups, namely, short neurotoxins and long neurotoxins. Long neurotoxins have an

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; α-Bgt, α-bungarotoxin from *Bungarus multicinctus*; Lc b, Laticauda colubrina b from *Laticauda colubrina*; Tx α, toxin α from *Naja nigricollis*; CPase, carboxypeptidase; NMR, nuclear magnetic resonance; CD, circular dichroism; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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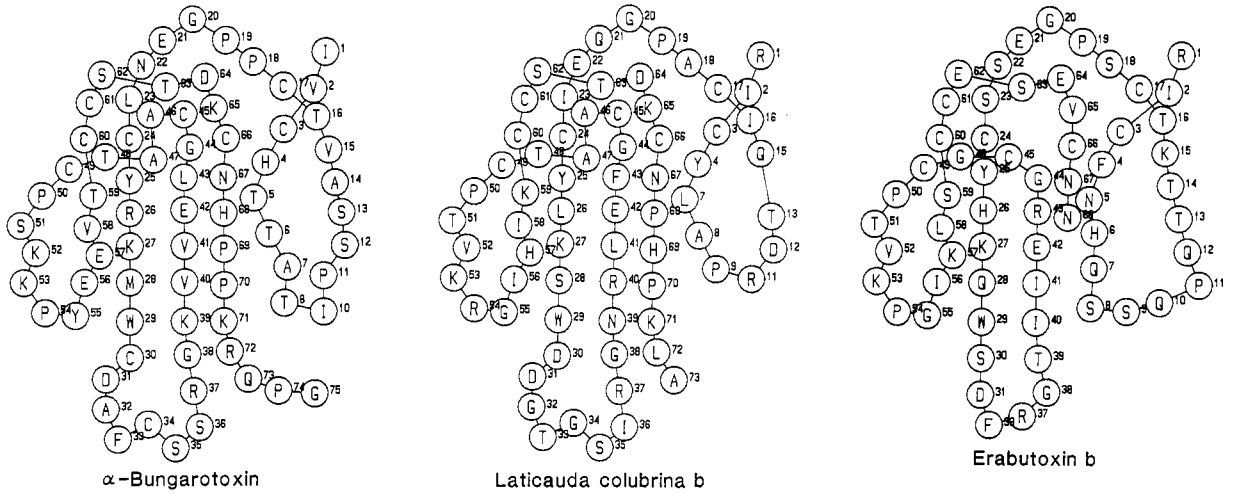

FIGURE 1: Amino acid sequences of α-Bgt (Mebs et al., 1972), Lc b (Kim & Tamiya, 1982), and erabutoxin b (Sato & Tamiya, 1971). The sequences are drawn schematically on the polypeptide chain folding of erabutoxin b (Low et al., 1976; Tsernoglou & Petsko, 1976; Bourne et al., 1985), α-cobratoxin (Walkinshaw et al., 1980), and α-Bgt (Agard & Stroud, 1982; Stroud, 1982).

extra "C-terminal tail" segment with five to nine amino acid residues [except for Laticauda semifasciata III from *Laticauda semifasciate* (Ls III) with only two extra residues] when compared with short neurotoxins. The amino acid sequences of α-bungarotoxin (α-Bgt) and Laticauda colubrina b (Lc b), long neurotoxins from *Bungarus multicinctus* and *Laticauda colubrina*, respectively, and erabutoxin b, a short neurotoxin from *Laticauda semifasciata*, are schematically shown in Figure 1.

In this study, the role of the C-terminal tail in the molecular conformation and function of long neurotoxins has been examined. The C-terminal residues of α-Bgt and Lc b have been removed by carboxypeptidase (CPase) digestion, and the effect of such deletion on the toxin conformation has been monitored in proton nuclear magnetic resonance (NMR) spectra and circular dichroism (CD) spectra. Then, binding activity to AChR from the electric organ of *Narke japonica* has been compared between native neurotoxins and those without C-terminal tail residues. Contrary to the previous view of the neurotoxin–receptor interactions, direct involvement of the C-terminal tail in binding to electric organ (and muscle) AChR is now suggested. Short neurotoxins may bind to muscle AChR probably because they have alternative binding site(s) for the receptor.

Materials and Methods

Materials. α-Bgt and Lc b were isolated from the venoms of *B. multicinctus* and *L. colubrina*, respectively, according to the methods described previously (Mebs et al., 1972; Kim & Tamiya, 1982). Tritium-labeled toxin α (Tx α; 19 Ci/mmol), a short neurotoxin from *Naja nigricollis*, was a kind gift from Dr. A. Ménez. CPase P from *Penicillium janthinellum* was obtained from Peptide Institute Inc. (Osaka, Japan).

AChR was purified from the electric organ of *N. japonica*. Purified detergent-solubilized AChR was prepared according to the methods reported previously (Hayashi et al., 1981; Kaneda et al., 1982). The final standard buffer contained 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.1% Tween 20, and 0.02% NaN_3. The concentration of AChR was determined by monitoring the fluorescence change upon binding of a neurotoxin (Endo et al., 1986). Concentrations of α-Bgt and Lc b before and after enzymatic digestion were determined by spectrophotometry with a molar absorption coefficient of 9300 at 280 nm.

NMR and CD Measurement. Proton NMR spectra were recorded on a Bruker WH-270 spectrometer. Chemical shifts are given in parts per million from the internal standard sodium 4,4-dimethyl-4-silapentane-1-sulfonate. pH values are uncorrected meter readings of ^2H_2O solutions. The pH dependence of proton chemical shifts was analyzed by a least-squares fitting, and pK_a values for the chemical shift changes were obtained. CD spectra were recorded on a Jasco J20A spectropolarimeter at 26 °C, with a cell of 2-mm path length.

CPase Digestion. CPase P digestion of neurotoxins was carried out directly in NMR sample tubes. The pH of the 2.0 mM ^2H_2O solution of a neurotoxin (0.1 M NaCl) was at first adjusted to pH 4.3 (α-Bgt) or 4.0 (Lc b). Then 15 or 10 μL of CPase solution (1 mg/mL) was added to α-Bgt or Lc b solution, respectively, and enzymatic digestion of a neurotoxin was carried out at 37 °C. At appropriate time intervals in the course of digestion, proton NMR spectra were recorded to monitor the effect of the enzymatic cleavage of C-terminal amino acid residues. Spectral changes were followed by plotting the intensities of perturbed resonances that were calibrated with the intensity of unaffected resonances, and a half-time of the proteolytic reaction t_d was obtained.

As the proteolysis proceeded, the rate of digestion became slower, and when the proteolytic reaction reached the stage at which the reaction rate was too slow to be followed, the sample was subjected to ultrafiltration by using cutoff membrane for M_r 10000 to remove CPase P. Then, free amino acids were separated from the neurotoxin by dialyses in 0.1% acetate, and the amino acid composition was analyzed by a Hitachi amino acid analyzer, Model 835. The separated neurotoxin derivatives without C-terminal tail residues were lyophilized and used for further NMR analyses, CD analyses, and competition binding experiments to AChR.

Competition Binding Experiment of Neurotoxins to AChR. The relative dissociation constants of neurotoxins with and without C-terminal tail residues were obtained by competition binding experiments of toxins with tritium-labeled Tx α to AChR (Ishikawa et al., 1977). A volume of 0.05 mL of AChR solution (final concentration 2 nM) was added to 0.12 mL of the solution containing tritium-labeled Tx α (final concentration 5 nM) and a cold neurotoxin (final concentration 2 nM–1 μM). The reaction mixture was incubated at pH 7.4 and 30 °C for 14 h to attain the equilibrium state of the reaction. Then, an aliquot of the mixture was filtered through (diethylaminoethyl)cellulose filter paper disk DE-81 (What-

man) (Schmidt & Raftery, 1973), and the amount of labeled Tx α bound to AChR was evaluated by counting the radioactivity of the disks by a Packard Tri-Carb (Model 3255) liquid scintillation counter.

In the competition binding experiment of a labeled toxin (T*) and a cold neurotoxin (T) with AChR (R), the association reaction is described as

$$R + T^* \rightleftharpoons RT^* \quad (1)$$

$$R + T \rightleftharpoons RT \quad (2)$$

where RT* and RT are complexes of AChR and a labeled toxin or cold toxin, respectively. The dissociation constants K_d^* and K_d of a labeled toxin and a cold toxin, respectively, are defined as

$$K_d^* = \frac{[T^*][R]}{[RT^*]} = \frac{([T^*]_0 - [RT^*])([R]_0 - [RT^*] - [RT])}{[RT^*]} \quad (3)$$

$$K_d = \frac{[T][R]}{[RT]} = \frac{([T]_0 - [RT])([R]_0 - [RT^*] - [RT])}{[RT]} \quad (4)$$

Then

$$\frac{[T]_0}{[R]_0 - [RT^*] - K_d^* [RT^*]/([T^*]_0 - [RT^*])} = \left(\frac{K_d}{K_d^*}\right)\left(\frac{[T^*]_0 - [RT^*]}{[RT^*]}\right) + 1 \quad (5)$$

Under the present experimental conditions of $[R]_0$ and $[T^*]_0$, the term $K_d^*[RT^*]/([T^*]_0 - [RT^*])$ is negligible as compared to $[R]_0 - [RT^*]$, since K_d^* has a value on the order of 10^{-11} M (Faure et al., 1983). Then

$$\frac{[T]_0}{[R]_0 - [RT^*]} = \left(\frac{K_d}{K_d^*}\right)\left(\frac{[T^*]_0 - [RT^*]}{[RT^*]}\right) + 1 \quad (6)$$

The plot of the left-hand side against $([T^*]_0 - [RT^*])/[RT^*]$ gives a straight line with a slope of K_d/K_d^* and an intercept at 1. A least-squares fit with eq 6 was applied to the results of the competition binding experiment, and thereby a relative dissociation constant K_d/K_d^* was obtained.

Results

CPase P Digestion of α-Bgt. Proton NMR spectra of α-Bgt (0.1 M NaCl, pH 4.3, 37 °C) have been recorded at different stages of the enzymatic cleavage of the C-terminal tail residues by CPase P (Figure 1 in supplementary material; see paragraph at end of paper regarding supplementary material). The enzymatic cleavage occurs in two distinct steps. First, at t_d = 100–150 min of incubation, the resonances from the free amino acids glycine, proline, glutamine, and arginine appear. These amino acids should correspond to those cleaved off from the C-terminal residues of α-Bgt, Gly-75, Pro-74, Gln-73, and Arg-72. The simultaneous cleavage of the four residues at the C-terminus (Arg-72–Gly-75) means that the removal of Gly-75 is a rate-limiting step for the cleavage of the other three C-terminal residues. The proteolytic cleavage of Arg-72–Gly-75 is also accompanied by changes in the chemical shifts of the C-2 and C-4 proton resonances of His-68, the γ-methyl resonance of Val-41, and another methyl resonance (1.07 ppm). The second step of the proteolysis at t_d = 300–400 min results in the removal of Lys-71, since the ϵ-methylene proton resonance from the free amino acid lysine appears at this stage. In this step of the cleavage, the C-2 and C-4 proton resonances of His-68, the C-2 proton resonance of His-48, and two methyl proton resonances (0.88 and 1.02 ppm) also exhibit chemical shift changes. The plot of proton resonance intensity against incubation time shows that, at the end of proteolytic reaction, C-terminal residues Lys-71–Gly-75 are nearly completely removed but the proteolysis does not proceed further. Quantification by amino acid analysis of free amino acids that were separated by dialysis supports these observations also: only the free amino acids glycine, proline, glutamine, arginine, and lysine were found in the dialysate in the mole ratio of 1.0:0.8:0.7:0.8:0.9. The resonances from the free amino acids glycine, proline, glutamine, arginine, and lysine were not observed in the spectrum of the digested α-Bgt recorded after removing the free amino acids by dialysis.

α-Bgt contains six lysine residues in positions 27, 39, 52, 53, 65, and 71. Five or six ϵ-methylene proton resonances from these lysine residues were found from the pH titration behavior in the alkaline pH region and patterns of spin coupling as illustrated in Figure 2: a broad resonance at 2.65 ppm, a triplet at 2.88 ppm, two or three triplet resonances at 2.99 ppm, and a quartetlike resonance at 3.10 ppm. When the ϵ-methylene proton resonance of the cleaved free lysine appears at 3.03 ppm, one of the lysine ϵ-methylene resonances at 2.99 ppm reduces its intensity and another lysine ϵ-methylene resonance at 3.10 ppm changes its apparent spin multiplet structure from quartet to triplet (Figure 2). The former resonance probably arises from Lys-71. The latter is likely assigned to Lys-39 since Lys-39 is in the β-sheet structure (Inagaki et al., 1985) and its side chain is expected to be oriented in the direction toward the C-terminal tail. The broad lysine ϵ-methylene resonance at 2.65 ppm is probably assigned to invariant Lys-27 residue because Lys-27 ϵ-methylene proton resonance is broad and lies at an unusually high field in such neurotoxins as erabutoxin b (Inagaki et al., 1980), neurotoxin II from *Naja naja oxiana* (Tsetlin et al., 1979), and neurotoxin III from *Naja mossambica mossambica* (Arseniev et al., 1981). The pH dependence of the chemical shifts of the lysine ϵ-methylene proton resonances is shown in Figure 2 together with those of the Gly-75 C_α proton resonance and the Arg-72 δ-methylene proton resonance.

There are also several resonances whose chemical shifts are affected by the cleavage of the C-terminal residues (Figure 1 in supplementary material; see paragraph at end of paper regarding supplementary material). However, on the basis of their chemical shift values (Wüthrich, 1976; Bundi & Wüthrich, 1979), these resonances can be tentatively assigned to residues 71–75 that are being cleaved off. Most of the proton resonances other than those mentioned above are free from spectral perturbation caused by the C-terminal tail digestion.

CPase P Digestion of Lc b. Proton NMR spectra of Lc b (0.1 M NaCl, pH 4.0, 37 °C) have been recorded at various stages in the course of proteolytic cleavage by CPase P (Figure 2 in supplementary material; see paragraph at end of paper regarding supplementary material). Appearance of proton resonances from the free amino acids and concomitant spectral changes show that the proteolysis proceeds in four distinct steps. Within 5 min of proteolytic reaction, C-terminal Ala-73 is cleaved off. Then, Leu-72, Lys-71, and Pro-70 are cleaved off at t_d = 10–20, ~50, and 150–200 min, respectively. It was confirmed that the free amino acids alanine, leucine, lysine, and proline in fact existed in dialysate. The C-2 and C-4 proton resonances of His-69 shift at the stage when Leu-72 is cleaved off and also at the stage when the cleavage of Pro-70 leaves His-69 as the C-terminal residue. When Pro-70 is

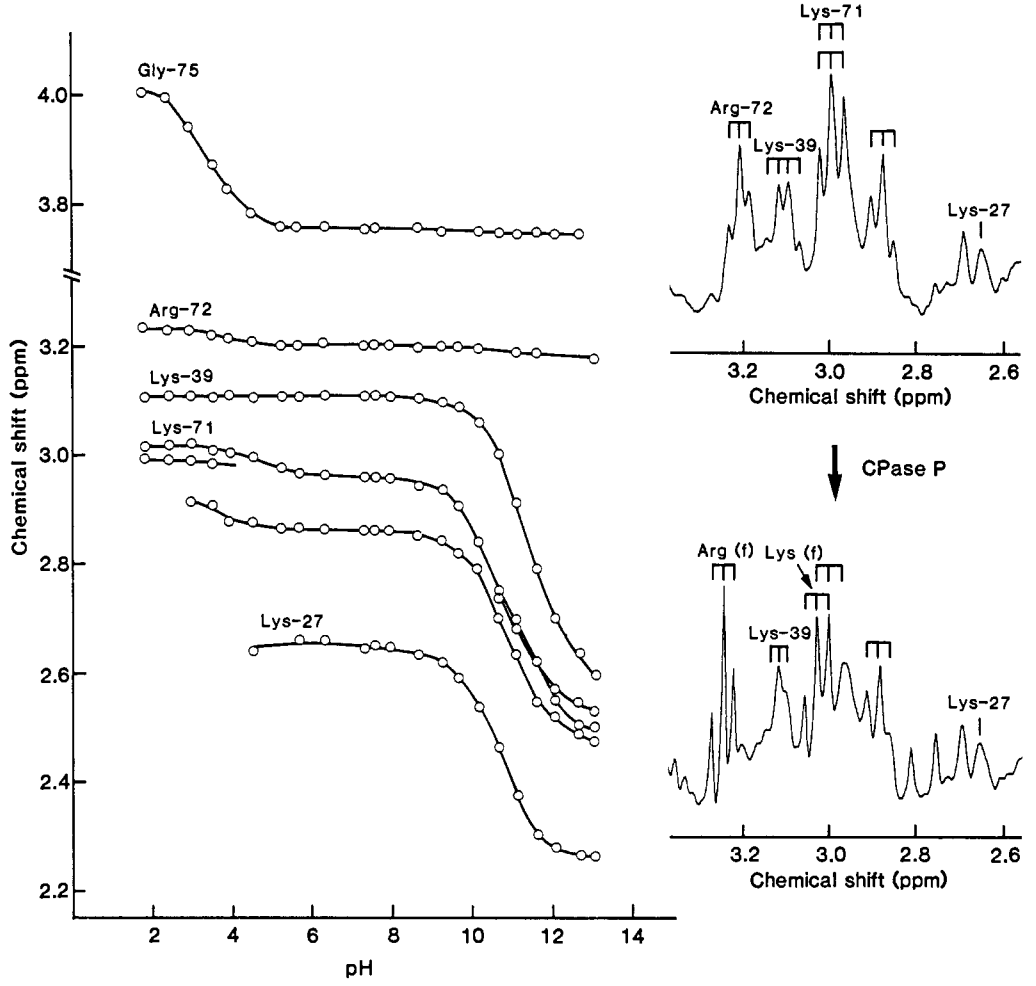

FIGURE 2: pH dependence of proton chemical shifts of the Gly-75 Cα proton resonance, Arg-72 δ-methylene proton resonance, and ε-methylene proton resonances of lysine residues of α-Bgt in ^2H_2O solution (0.1 M NaCl, 25 °C). The assignments of the Arg-72 δ-methylene proton resonance and ε-methylene proton resonances of lysine residues before and after enzymatic cleavage are shown in the right. (f) means the resonances from the free amino acids. The spectral resolution is enhanced.

cleaved off, two pairs of Leu/Val methyl resonances (0.70 and 0.68 ppm, and 0.51 and 0.12 ppm) and another Ala/Thr methyl proton resonance (0.14 ppm) show chemical shift changes. Most of the other resonances shifting with the proteolysis are assigned to residues 70–74 on the basis of their chemical shifts. As in the case of α-Bgt, plots of proton resonance intensity against incubation time and amino acid analysis show that, at the end of proteolytic reaction, the C-terminal four residues are nearly completely removed but the proteolysis does not proceed further.

CD Analysis of α-Bgt and Lc b before and after Enzymatic Cleavage. Hider and co-workers have measured the CD spectra of various short and long neurotoxins and found that the CD bands below 240 nm are sensitive to minor changes in the main chain conformation induced by change in temperature or pH (Drake et al., 1980; Hider et al., 1982; Dufton & Hider, 1983; Inagaki et al., 1985). Thus in this study, the CD spectra of α-Bgt and Lc b were compared at acidic and neutral pHs before and after the cleavage of the C-terminal residues and the effects of CPase digestion on the backbone conformation were examined (Figure 3). Intact α-Bgt and Lc b exhibit CD spectra that are characteristic of the spectra of long neurotoxins (Mênez et al., 1976; Dufton & Hider, 1983); the CD maximum at 195–200 nm and the CD minimum at 210–215 nm due to the transitions of peptide chromophores were observed. These spectral features are also observed for α-Bgt and Lc b without C-terminal four to five residues, although a slight increase in intensity of the CD maximum at 195–200 nm suggests the possibility that the β-sheet content has little increased after CPase digestion. From the comparison of the intensity change in the CD band of α-Bgt and Lc b with that observed for long neurotoxins upon varying pH and temperature (Drake et al., 1980; Hider et al., 1982; Dufton & Hider, 1983; Inagaki et al., 1985), it is concluded that the overall main chain conformation is not altered by the cleavage of the C-terminal residues in α-Bgt or Lc b. Figure 3 also indicates that the main chain conformation of α-Bgt and Lc b does not differ significantly between the acidic and the neutral pH regions.

Table I: Relative Dissociation Constant K_d/K_d^* [a] for AChR Binding of α-Bgt and Lc b before and after CPase Digestion

neurotoxin	K_d/K_d^* before digestion	K_d/K_d^* after digestion
α-Bgt	12.4 ± 1.7	190 ± 40
Lc b	0.57 ± 0.05	125 ± 20

[a] K_d/K_d^* is the ratio of the dissociation constants of toxin–receptor complex (K_d) and labeled Tx α–receptor complex (K_d^*).

Binding Ability of α-Bgt and Lc b to Electric Organ AChR before and after CPase Digestion. Binding ability to AChR from the electric organ of *N. japonica* was estimated from competition binding experiments between tritium-labeled Tx α and α-Bgt or Lc b before and after CPase P digestion of the C-terminal residues. The results are shown in Figure 4, together with the curves obtained with the best fit parameters. The relative dissociation constant K_d/K_d^* was estimated from

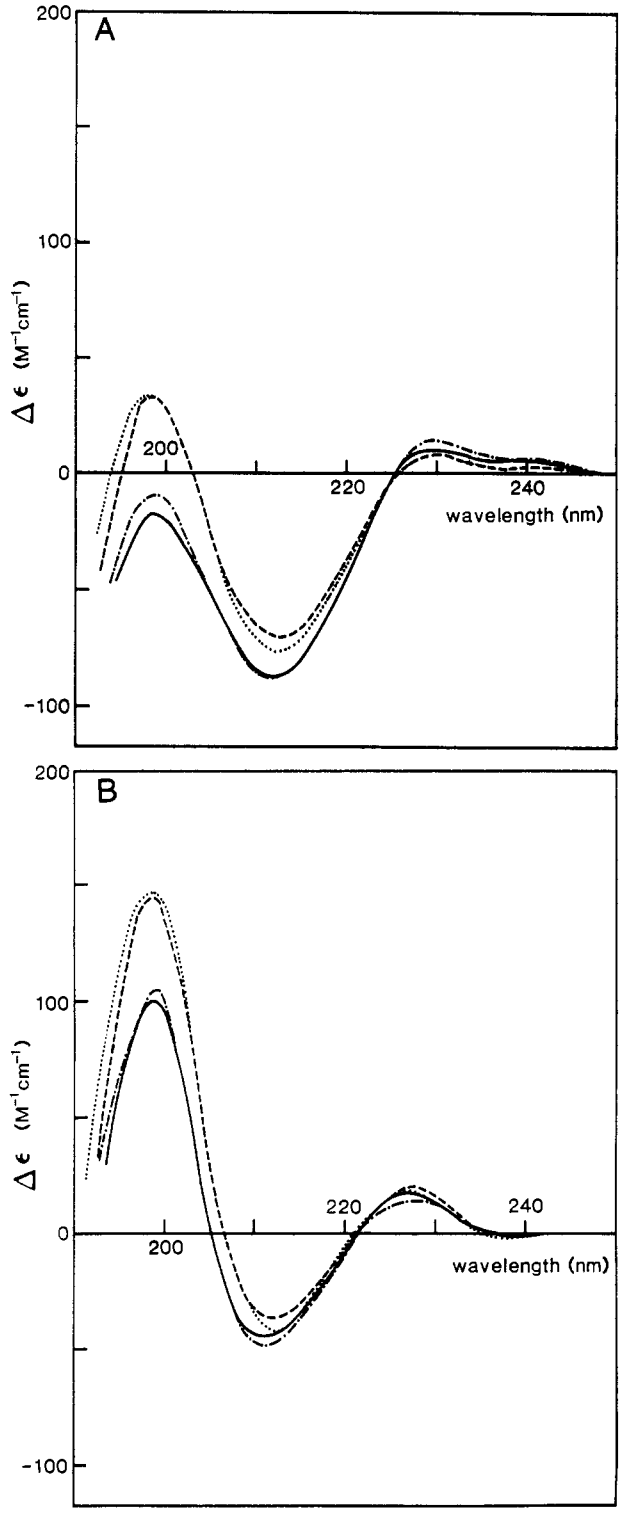

FIGURE 3: CD spectra of α-Bgt (A) and Lc b (B) before and after CPase digestion (26 °C). CD spectra were recorded for α-Bgt at pH 4.6 (-·-) and 7.4 (—) before digestion and at pH 4.5 (···) and 7.2 (---) after digestion. CD spectra were recorded for Lc b at pH 4.8 (-·-) and 7.5 (—) before digestion and at pH 4.8 (···) and 7.3 (---) after digestion. The toxin concentration is 15 μM.

the treatment described under Materials and Methods as shown in Table I.

Discussion

Role of C-Terminal Tail in the Conformation of Long Neurotoxin. (a) Removal of C-Terminal Tail Does Not Induce a Major Conformational Change. As compared with short neurotoxins, long neurotoxins have an additional C-terminal tail segment as long as nine residues. Crystal structures are at present available for a short neurotoxin, erabutoxin b from *L. semifasciata* (Low et al., 1976; Tsernoglou & Petsko, 1976; Bourne et al., 1985), and for two long neurotoxins, α-cobratoxin from *Naja naja siamensis* (Walkinshaw et al., 1980) and α-Bgt (Agard & Stroud, 1982; Stroud, 1982). Overall arrangement of the main chain folding in α-cobratoxin (*N. naja siamensis*) and α-Bgt is similar to that found in erabutoxin b (Figure 1): the main chain is folded into three loops bound by four disulfide bridges, and a three-stranded antiparallel β-sheet structure is formed. The β-sheet structure is distorted in α-Bgt in crystal (Stroud, 1982), but in solution, the β-sheet structure similar to those in α-cobratoxin (*N. naja siamensis*) and erabutoxin b was found for α-Bgt (Endo et al., 1981; Inagaki et al., 1985). In the crystal structure of α-cobratoxin (*N. naja siamensis*), the C-terminal tail segment, residues 67–75, hangs down around the central loop and the last four residues of the tail (residues 72–75) cannot be followed in continuous electron density, suggesting that the tail segment is statistically disordered or has high thermal mobility (Walkinshaw et al., 1980). The C-terminal tail in α-Bgt is found to be swung to the side of the central loop and is close to the first loop consisting of residues 5–16 in crystal (Agard & Stroud, 1982).

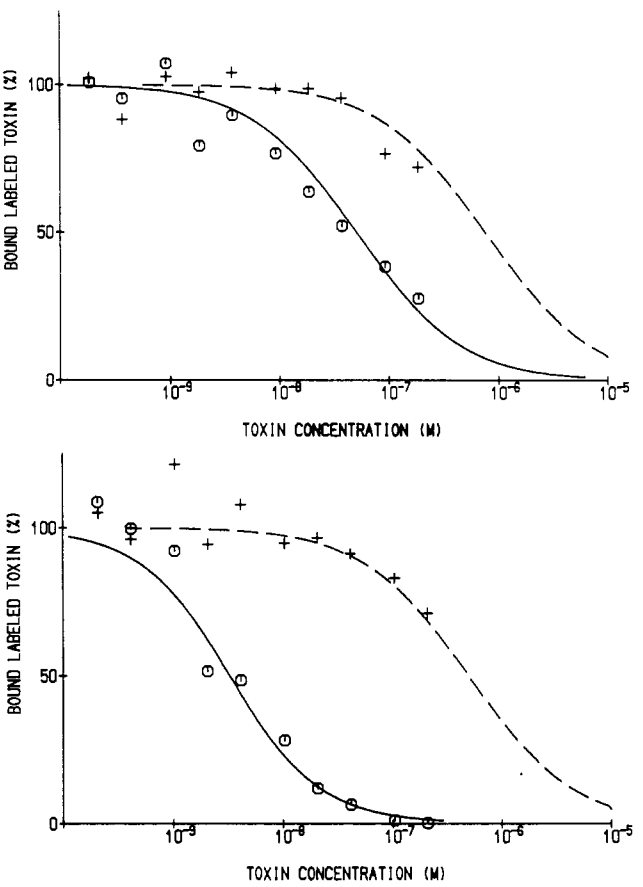

FIGURE 4: Binding of tritium-labeled Tx α to AChR in the presence of varying amounts of α-Bgt (A) or Lc b (B) before (O) and after (+) CPase cleavage. The curves are drawn with best fit values of relative dissociation constants K_d/K_d^*, which were obtained by applying a least-squares fitting to the experimental data.

In this study, C-terminal residues 71–75 in α-Bgt and residues 70–73 in Lc b were cleaved off by CPase P, and the effects of such deletion on the NMR spectra were analyzed. First, the proteolysis proceeds to four to five residues in the tail, suggesting that these residues are accessible to the enzyme and they are not fully buried in the molecule. Second, the

cleavage of the C-terminal residues did not affect most of the proton resonances in α-Bgt and Lc b. This means that the removal of the C-terminal residues does not induce a global conformational change in the molecule and it affects only a limited region of the molecule. CD spectra of α-Bgt and Lc b before and after CPase digestion also indicate that a significant change in the polypeptide chain folding does not occur upon the cleavage of the C-terminal tail. This means that the C-terminal tail does not play a critical role in maintaining the specific polypeptide chain folding in α-Bgt and Lc b (and likely in other long neurotoxins). The line widths of proton NMR signals do not change upon the enzymatic cleavage, suggesting that the cleavage of the C-terminal residues does not induce self-association of the molecule.

(*b*) *Salt Bridge or Hydrogen Bond Is Not Formed between Residue 39 and C-Terminus.* As described above, at least in crystal, the C-terminal tail in long neurotoxins is flexible and is not involved in interactions with the rest of the molecule for maintaining the biologically active conformation. On the other hand, it has been proposed that, in aqueous solution, a salt link or hydrogen bond between residue 39 and the C-terminal carboxylate/amide group may be formed (Maeda & Tamiya, 1978). It would be of interest to examine whether the proposed salt bridge or hydrogen bond between residue 39 and the C-terminus is present or not. The pK_a of the ε-amino group of Lys-39 in α-Bgt was found to be 11.35 ± 0.03 from the pH dependence of the chemical shift of the Lys-39 ε-methylene resonance (Figure 2). The pK_a values of the α-carboxyl group at the C-terminus in native α-Bgt and Lc b were obtained as 3.44 ± 0.03 and 3.40 ± 0.06, respectively, from the pH dependence of the chemical shifts of the Gly-75 Cα proton resonance of α-Bgt (Figure 2) and Ala-73 β-methyl proton resonance of Lc b (data not shown). Although the pK_a of the ε-amino group of Lys-39 is slightly higher than that for model compound *N*-acetyllysine amide (10.68 in ^2H_2O solution at 23 °C; T. Endo and T. Miyazawa, unpublished results), the pK_a of the C-terminal α-carboxyl group is normal; for example, the C-terminal carboxyl group in the tetrapeptide Gly-Gly-Gly-Ala exhibits a pK_a value of 3.56 in ^2H_2O solution at 24 °C (Bundi & Wüthrich, 1979). The pH dependence of the chemical shift of the Lys-39 ε-methylene proton resonance does not reflect the titration of the C-terminal carboxyl group, and the Gly-74 Cα proton resonance shifts only by 0.015 ppm in the alkaline pH region (Figure 2). Therefore, in the present case of α-Bgt and Lc b, a salt link or a hydrogen bond is not likely formed between the side chain of Lys-39 or Asn-39 and the C-terminus.

(*c*) *C-Terminal Tail May Take an Ordered (Helical) Conformation.* By the combined use of CPase digestion and NMR measurements of the C3a protein (the 77-residue fragment obtained from the α-chain of the third component of complement), Muto et al. (1985) have shown that information is obtained not only on the interaction between the C-terminal segment and the rest of the molecule but also on the conformation of the C-terminal segment itself. In the present case of α-Bgt, the C-2 and C-4 proton resonances of His-68 in the C-terminal tail shift upon the cleavage of Arg-72–Gly-75. This means that the deletion of residues 72–75 directly affects the microenvironment of His-68 or that appearance of a new C-terminus at residue 71 influences His-68. The C-2 and C-4 resonances of His-68 furthermore shift upon the cleavage of Lys-71. The pH dependence curve of the Lys-71 ε-methylene proton resonance reflects the ionization of His-68 with a pK_a of 4.99 (Endo et al., 1981) and vice versa (Figure 2; Endo et al., 1981). Appreciably low pK_a of His-68 in intact α-Bgt (4.99) is due to the positive charge of Lys-71 that is removed by CPase digestion, because, in CPase-digested α-Bgt without the C-terminal five residues, His-68 exhibits an intrinsic pK_a value of 6.61 ± 0.01. Therefore, His-68 is in proximity to Lys-71 and/or residues 72–75 in α-Bgt. The δ-methylene proton resonance of Arg-72 in α-Bgt shifts at pH 3.65 ± 0.08, probably reflecting the ionization of the C-terminal carboxyl group of Gly-75. In Lc b, the cleavage of Ala-73 changes the chemical shift of His-69 C-2 and C-4 proton resonances, suggesting the proximate relation of His-69 and Ala-73. The removal of Pro-70 in Lc b leaves the C-terminus at His-69, resulting in the chemical shift change of His-69 C-2 and C-4 proton resonances. Thus in both α-Bgt and Lc b, the effects of cleavage of the C-terminal residues reach to the residues that are separated by three to four residues from the cleavage sites. The effects of ionization on the proton chemical shifts also suggest the proximate relations between the residues separated by a three to four residue distance. These observations mean that the C-terminal tail segment is not extended but takes an ordered conformation such as a helical structure. In the crystal of α-cobratoxin (*N. naja siamensis*) or α-Bgt, such an ordered structure may be lost probably due to the effect of molecular packing.

Role of C-Terminal Tail in Binding to AChR. (*a*) *Removal of C-Terminal Tail Greatly Reduces the Affinity of Long Neurotoxins to AChR.* The specific binding and tight binding of neurotoxins to AChR are thought to be achieved through interactions with multiple point attachment probably involving invariant residues, e.g., Trp-29, Asp-31, Arg-37, and Lys-53 (Dufton & Hider, 1983). Long and short neurotoxins bind to AChR in a competitive manner with cholinergic agonists, and long and short neurotoxins also compete with each other in AChR binding.

In this study, dissociation constants for solubilized and purified AChR from *N. japonica* electric organ were obtained for α-Bgt and Lc b and compared both before and after the cleavage of the C-terminal four to five residues. The results (Table I and Figure 4) clearly show that the cleavage of the C-terminal tail residues reduces the affinity greatly as compared with those of native toxins in both cases. Thus it is evident that the C-terminal tail in α-Bgt and Lc b plays a critical role in binding to electric organ (and probably muscle) AChR. Since the C-terminal tail is not essential for maintaining the specific polypeptide chain folding in α-Bgt and Lc b as described above, it is very likely that the C-terminal tail is directly involved in AChR binding. Examination of the amino acid sequences of long neurotoxins reveals that the C-terminal tail always contains one to three basic amino acid residues (lysine or arginine). α-Bgt has Lys-71 and Arg-72 and Lc b has Lys-71 in the C-terminal tail segment, and α-Bgt and Lc b lack these residues after enzymatic cleavage of the C-terminal residues. Therefore, the basic residues in the C-terminal tail in long neurotoxins probably provide a binding site for a negative subsite in the electric organ and muscle AChR. This idea may be argued against by the fact that the side chains of invariant Trp-29, Asp-31, Arg-37, and Lys-53 are oriented in the direction opposite to the side of the molecule where the C-terminal sequence starts. However, it has been suggested that the neurotoxin–receptor interaction is not a simple surface-to-surface one and that the side chains of some residues on the side where the C-terminus is located are involved in the receptor binding (Tsetlin et al., 1982; Endo et al., 1986).

Our present observation of the effect of the removal of the C-terminal tail on the AChR binding ability is consistent with

the report on the lethal toxicity of a long neurotoxin with shortened C-terminal tail, Ls III (Maeda & Tamiya, 1974), but does not agree with those of CPase-digested α-cobratoxin (*N. naja siamensis*) (Karlsson et al., 1972) and toxin B (M. Ohta and K. Hayashi, personal communication). The latter disagreement may be related to the suggestion by Ishikawa et al. (1977) that toxin B binds to AChR in a somewhat different manner from α-Bgt.

(*b*) *Consideration of Binding of Short Neurotoxins to AChR.* Although long neurotoxin binds to AChR in a competitive manner with cholinergic agonists or short neurotoxins, long neurotoxins do not necessarily contain the *same* set of receptor-binding sites as cholinergic agonists or short neurotoxins. Long and short neurotoxins likely share several common binding sites for the receptor (e.g., Trp-29, Asp-31, Arg-37, and Lys-53), but the rest of the binding sites may be different between long and short neurotoxins. If long neurotoxins bind to AChR, blocking some of the binding sites for short neurotoxins in the receptor, the binding of short neurotoxins to the receptor will be sterically prevented and thereby competition binding will be observed. The possible difference in the binding sites between long and short neurotoxins may be the reason why, if one accepts the involvement of the C-terminal tail of long neurotoxins in receptor binding, short neurotoxins without the C-terminal tail can still bind to AChR with high affinity. Indeed, the tryptophan fluorescence change in AChR upon binding of neurotoxins has suggested that long neurotoxins interact with the receptor in a somewhat different manner from short neurotoxins (Endo et al., 1986). Short neurotoxins have several invariant residues that are not found in long neurotoxins; such residues include Asn-5, Ser/Thr-9, Thr/Ser-39, and Arg-43. Thus some of these residues in short neurotoxins may well provide alternative receptor binding sites, and these binding sites may enhance the affinity of short neurotoxins with AChR.

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Supplementary Material Available

Proton NMR spectra of α-Bgt at different stages of proteolysis by CPase P with resonance assignments (Figure 1) and proton NMR spectra of Lc b at different stages of proteolysis by CPase P with resonance assignments (Figure 2) (8 pages). Ordering information is given on any current masthead page.

Registry No. α-Bungarotoxin, 11032-79-4; acetylcholine, 51-84-3.

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