Role of Indole and Amino Groups in the Structure and Function of *Naja nigricollis* Toxin α†

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ABSTRACT: Seven pure derivatives of *Naja nigricollis* toxin α modified at a single residue were prepared, using ion-exchange chromatography and high-performance liquid chromatography. Five derivatives were obtained, which were monoacetylated at lysine residues 15, 27, 47, and 51 and at the N terminus. The other two derivatives result from modifications at either the N_1 (by formylation) or the C_2 (by nitrophenylsulfenylation) position of tryptophan-29. Circular dichroism analysis indicates that derivatization does not alter the overall conformation of the toxin α backbone. However, examination of the tryptophan-29 environment by means of emission and excitation fluorescence spectroscopy reveals the following: (i) acetylation at residue 15 or 27 induces an increase in the energy transfer efficiency from Tyr-25 to Trp-29 by 10% and 30%, respectively, as compared to that occurring in native toxin α; (ii) acetylation at residue 51 induces a 16% increase in the emission fluorescence intensity; (iii) acetylation at either residue 1 or residue 47 does not affect the fluorescence properties of toxin α. Binding competition experiments using acetylcholine receptor rich membranes isolated from *Torpedo marmorata* electric organs were performed with ^{3}H-labeled toxin α and the seven toxin derivatives. They reveal the following equilibrium dissociation constants (K_D): native toxin α, 2×10^{-11} M; [1-N^{α}-monoacetylleucine]toxin α, 3×10^{-11} M; [29-N^1-formylindole]toxin α, 7×10^{-11} M; [15-N^{ϵ}-monoacetyllysine]toxin α, 9×10^{-11} M; [51-N^{ϵ}-monoacetyllysine]toxin α, 9×10^{-11} M; [27-N^{ϵ}-monoacetyllysine]toxin α, 29×10^{-11} M; [47-N^{ϵ}-monoacetyllysine]toxin α, 52×10^{-11} M; [29-C^2-[(nitrophenyl)sulfenyl]indole]toxin α, 59×10^{-11} M. As a result of the structural and biological studies reported here, we conclude that the three residues at positions 27, 29, and 47 are important but not essential sensu stricto for the specific binding of toxin α to the receptor while those at positions 1, 15, and 51 are not.

Toxin α isolated from *Naja nigricollis* venom is a single chain polypeptide with 61 amino acids cross-linked by four disulfide bridges (Eaker & Porath, 1967; Kopeyan et al., 1973). When injected into mammals, the toxin produces a nondepolarizing muscular block due to a tight and specific binding to the nicotinic acetylcholine receptor (Tazieff-Depierre & Pierre, 1966; Meunier et al., 1972). The dissociation constant of the toxin–receptor complex is close to 2×10^{-11} M, indicating that toxin α interacts with the receptor through multiple contacts (Weber & Changeux, 1974) which, at the present time, are not clearly identified.

One possible way to clarify the role of any particular residue in the expression of the biological properties of a given protein is to selectively modify this amino acid and to analyze for the consequence on the structure and function of the protein. The present paper describes the role of indole and amino groups of toxin α in the formation of the toxin–receptor complex. Toxin α possesses a single tryptophan (at position 29) and seven amino groups localized at positions 1, 15, 26, 27, 47, 51, and 59. We report here (i) the modification of Trp-29 using two methods, formylation and nitrophenylsulfenylation, and (ii) the specific monoacetylation of amino groups with acetic anhydride.

The structural consequences of the introduced modifications have been studied in two different ways. First, the overall structure of each modified compound is compared to that of native toxin α by circular dichroism, and second, the local environment of Trp-29 in the native toxin and monoacetylated derivatives has been examined by emission and excitation fluorescence spectroscopy. In particular, the energy transfer occurring between Tyr-25 and Trp-29 has been evaluated. The determination of the binding affinities of the derivatives for the acetylcholine receptor is also reported. The results obtained are compared with those reported in the literature for homologous toxins (Hori & Tamiya, 1976; Tsetlin et al., 1979; Karlsson, 1979).

Materials and Methods

Materials

Toxin α isolated from *Naja nigricollis* (Pasteur Institute) was prepared as described previously (Fryklund & Eaker, 1975). Tritium gas and [^{14}C]acetic anhydride were purchased from CEA (Saclay France). 2-Nitrophenylsulfenyl chloride was obtained from Fluka (Buchs, Switzerland), formic acid (99% pure) was from Merck (Darmstadt, Germany), Biorex 70 and Bio-Gel P2 were from Bio-Rad (Richmond CA), and trypsin was from Worthington Inc. (Freehold, NJ). The high-pressure liquid chromatography (HPLC) C_{18} μBondapak column was purchased from Waters Associates (Milford, MA). All solvents were obtained from Merck and used without further purification. Acetylcholine receptor rich membranes were prepared from *Torpedo marmorata* according to the method described previously (Sobel et al., 1977). Amino acid compositions were analyzed by using a Technicon automatic analyzer. Circular dichroism spectra were recorded with Jobin Yvon CD III dichrograph. Excitation and emission fluorescence spectra were recorded with a Fica 55000 differential absolute spectrofluorometer.

Methods

Chemical Modifications. Formylation of toxin α was performed according to the method of Coletti-Previero et al. (1969). Toxin α (5 mg) was dissolved in 2.5 mL of formic acid saturated with HCl for 75 min at room temperature. The

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reaction mixture was freeze-dried, dissolved in water, and freeze-dried again. Sulfenylation of toxin α was carried out according to the method of Fontana et al. (1966). 2-Nitrophenylsulfenyl chloride (0.4 mg) in 2 mL of pure acetic acid was added to 14 mg of toxin in 2 mL of pure acetic acid, and the mixture was left at room temperature overnight. The reaction mixture was filtered through a Bio-Gel P2 column equilibrated in 10% acetic acid. Acetylation of toxin α with ^{14}C-labeled acetic anhydride (11 mCi/mmol) was performed as follows: acetic anhydride (2 μmol) in benzene/dioxane (1:1 v/v, 0.25 mL) was added to sodium phosphate buffer (0.02 M, pH 7.2, 1.5 mL) containing 2 μmol of toxin α. The mixture was left at room temperature for 90 min with stirring. The reaction mixture was desalted by filtration through a Bio-Gel P2 column (20 cm × 1 cm) equilibrated in 10% acetic acid. The acetylated protein was freeze-dried.

Purification of the Monomodified Derivatives. Desalted mixtures obtained after formylation or sulfenylation were directly purified on a C_{18} μBondapak column equilibrated in 0.1 M triethylamine adjusted to pH 3.2 with pure formic acid. Elution of the derivatives was made by using acetonitrile as the secondary solvent. The 140-min binary gradient profile was composed of eight linear segments between 0 and 20% acetonitrile and was terminated when the acetonitrile concentration reached 60%. The acetylated toxin α mixture was initially chromatographed on a Biorex 70 column (1 cm × 18 cm) equilibrated in 0.05 M ammonium acetate, pH 6.5, followed by a linear gradient from 0.05 to 0.4 M ammonium acetate, pH 6.5. All fractions containing one [^{14}C]acetyl group per mole of toxin were rechromatographed on a C_{18} μBondapak column under elution conditions similar to those described above unless otherwise indicated.

Identification of the Monoacetylated Amino Groups. Each monoacetylated derivative (0.7 mg) was dissolved in 0.3 mL of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 8 M urea. Reduced dithiothreitol (64 mM) was added, and the solution was allowed to stand for 75 min at 20 °C. Free sulfhydryl groups were blocked with iodoacetic acid (20% molar excess) in 0.5 M phosphate buffer (pH 8) for 15 min. The protein was desalted by filtration through a Bio-Gel P2 column equilibrated with 10% acetic acid and freeze-dried. The S-carboxymethylated derivative (0.50 mg) was dissolved in 0.5 mL of phosphate buffer (pH 8) and hydrolyzed with trypsin at the substrate/enzyme ratio of 50/1 (w/w) for 6 h at 37 °C. The reaction was quenched by the addition of formic acid (5 μL). The tryptic digest was chromatographed on a C_{18} μBondapak column by using a triethylammonium formate/acetonitrile system (pH 3.2). Eluted peptides were detected by UV absorption at 235 nm and hydrolyzed in 6 M HCl at 110 °C for 24 h, and their amino acid composition was determined.

Receptor Binding Assays. Binding affinities of monomodified derivatives for acetylcholine receptor rich membranes were determined from competition experiments with ^{3}H-labeled toxin α. In brief, solutions (0.1 mL) containing varying amounts of either native toxin (standard curve) or monomodified derivatives were added to 0.02 mL of ^{3}H-labeled toxin α (45 Ci/mmol, 5×10^{-9} M final concentration) in Ringer's solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7), followed by the addition of 0.05 mL of acetylcholine receptor (2×10^{-9} M final concentration). After 2-h incubation at room temperature, an aliquot of the mixture was filtered through two Millipore filters and rinsed with 30 mL of cold Ringer's solution. The radioactivity retained on the filters was counted by using an Intertechnique scintillation counter. The affinity constant for each derivative was derived from the curves obtained according to the procedure previously described (Ishikawa et al., 1977).

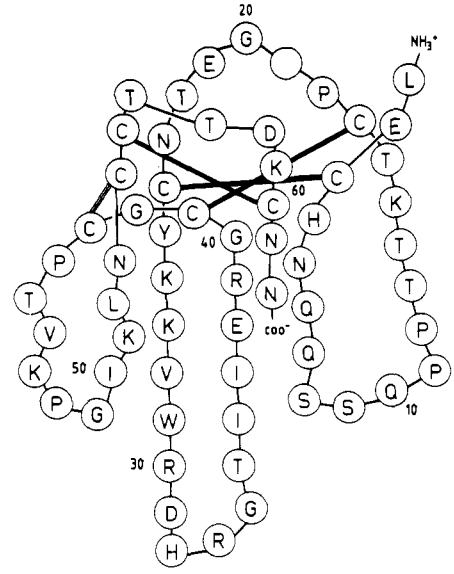

FIGURE 1: Amino acid sequence of *Naja nigricollis* toxin α (Eaker & Porath, 1967; Kopeyan et al., 1973).

Fluorescence and Circular Dichroism. Fluorescence emission and excitation spectra were recorded at 8 °C with dilute protein solutions of 2.5×10^{-5} and 2.5×10^{-6} M, respectively. The appropriate buffer was placed in the reference compartment. Excitation and emission slits were adjusted to 7.5 and 2.5 nm, respectively, for emission spectra and inversely for excitation spectra. Spectra recorded with a Fica 55000 apparatus were automatically corrected for the wavelength dependence of lamp intensity, monochromator transmission, and photomultiplier response. Energy transfer efficiencies were derived from the analysis of the excitation spectra according to the method initially described by Eisinger (1969) and subsequently modified (Ménez et al., 1980), using L-Tyr-L-Trp as reference. CD measurements were performed at 20 °C at a peptide concentration close to 3.5×10^{-5} M with cells of 0.05–0.2-cm path length. The CD spectra are expressed as molar ellipticities (θ) in deg·cm^2 dmol.$^{-1}$

Results

Chemical Modifications of Tryptophan-29. Figure 1 shows the amino acid sequence of *Naja nigricollis* toxin α (Eaker & Porath, 1967; Kopeyan et al., 1973). Modification of the single tryptophan of toxin α has been achieved by two distinct procedures. Introduction of a formyl group at the NH position of the indole ring was made according to the method previously described (Coletti-Previero et al., 1969). The absorption change monitored at 298 nm indicated that the reaction terminates after 75 min with a half-time close to 10 min. Reverse-phase liquid chromatography of the freeze-dried material revealed the presence of several byproducts, the major component being formylated toxin α which elutes at 43% acetonitrile (Figure 2A). Introduction of the more bulky 2-nitrophenylsulfenyl moiety at position 2 of the indole side chain was made according to Fontana et al. (1966). As is shown in Figure 2B, the use of stoichiometric amounts of reagent (2-nitrophenylsulfenyl chloride) and toxin α minimizes the formation of byproducts as only two major peaks were observed in the reverse-phase chromatography profile. These were

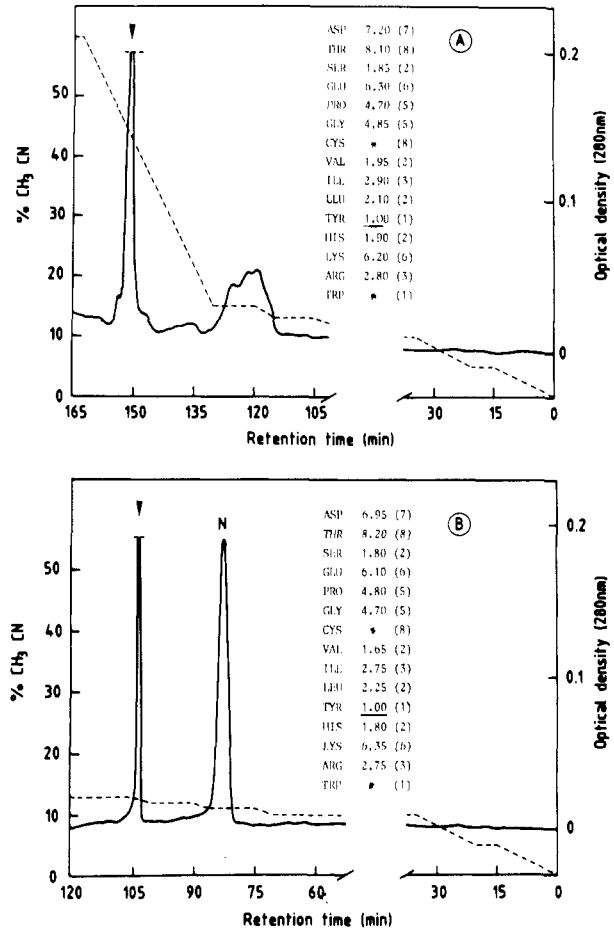

FIGURE 2: Chromatography of Trp-29-modified toxin α (A) after formylation and (B) after reaction with 2-nitrophenylsulfenyl chloride. In both cases, fractionation of the desalted reaction mixture was performed on a C_{18} μBondapak column (0.39 cm × 39 cm) equilibrated in triethylamine adjusted to pH 3.2 with formic acid. A binary gradient of triethylamine/acetonitrile was then applied (---). Arrows show the position of pure monomodified derivatives. N represents unreacted toxin. The inset shows the amino acid composition of each derivative. The numbers in parentheses correspond to the values obtained for the native toxin. Asterisks indicate that no values were determined.

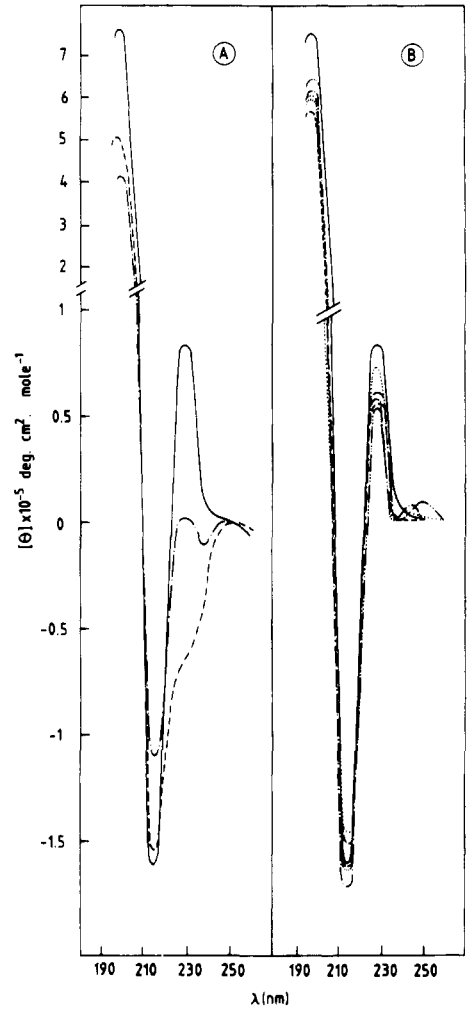

FIGURE 3: Circular dichroism spectra of native and modified toxins. (A) Native toxin (—); [N^1-formyl-Trp^{29}]toxin (---); [C^2-[(2-nitrophenyl)sulfenyl]-Trp^{29}]toxin (·--). (B) Native toxin (—); monoacetylated derivatives at residues 1 (···), 15 (-··-), 27 (-···-), 47 (·--), and 51 (---). In all cases, the protein concentration was approximately 3.5×10^{-5} M, and the cell path lengths were either 0.2 or 0.05 cm. The compounds were dissolved in water.

monosulfenylated toxin and unreacted toxin α which elute at 13% and 11% acetonitrile, respectively. Both monoformylated and monosulfenylated toxins were homogeneous as judged by gel electrophoresis and display an amino acid composition similar to that of native toxin α (Figure 2A,B). The circular dichroic spectra of these derivatives were compared to that of native toxin α (Figure 3A). In all three spectra, two intense signals at 216 and 197 nm corresponding to the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the peptide chromophores were observed (Ménez et al., 1976). The similarity of these signals for native and modified toxins indicates that derivatization of the indole ring does not alter the overall structure of the toxin polypeptide chain. It should also be stressed that the circular dichroic spectrum of native toxin α displays an additional band centered at 228 nm which disappears when the indole ring 29 is formylated or sulfenylated (Figure 3A). This observation supports the previous conclusions which suggested that the indole chromophore 29 contributes to the formation of this signal (Ménez et al., 1976, 1978). Modifications of this residue clearly perturb its intrinsic and/or vicinal electronic distribution.

Modification of tryptophan with 2-nitrophenyl induces a substantial binding affinity decrease of the toxin for the acetylcholine receptor, but the formylation of tryptophan does not give significant differences in the acetylcholine receptor binding curves (Figure 4).

Chemical Modification of Amino Groups. (*a*) *Isolation of Monoacetylated Toxin α Derivatives.* Acetylation of toxin α with ^{14}C-labeled acetic anhydride yielded a complex mixture which, after being desalted, was chromatographed on Biorex 70 (Figure 5). The first eluted fraction consisted of a population containing three or more acetyl groups per toxin molecule. The three subsequent fractions mainly contained diacetyl derivatives whereas fractions I–IV contained monoacetylated derivatives. The last peak was not radioactive and was eluted with native toxin α. Fractions I–IV were rechromatographed on a reverse-phase column to which a binary gradient of triethylammonium formate/acetonitrile was applied. Fractions II–IV each contained a single monoacetylated derivative. In contrast, fraction I was further resolved into three components (Figure 6); fractions Ia and Ib contain monoacetylated derivatives while the minor component (Ic) was identified as a diacetylated toxin. Thus, five monoacetylated derivatives were isolated, all of which were demonstrated to be homogeneous by gel electrophoresis (results not shown).

(*b*) *Location of the Acetyl Groups.* Disulfide bonds of native toxin α and monoacetylated derivatives were reduced, and the

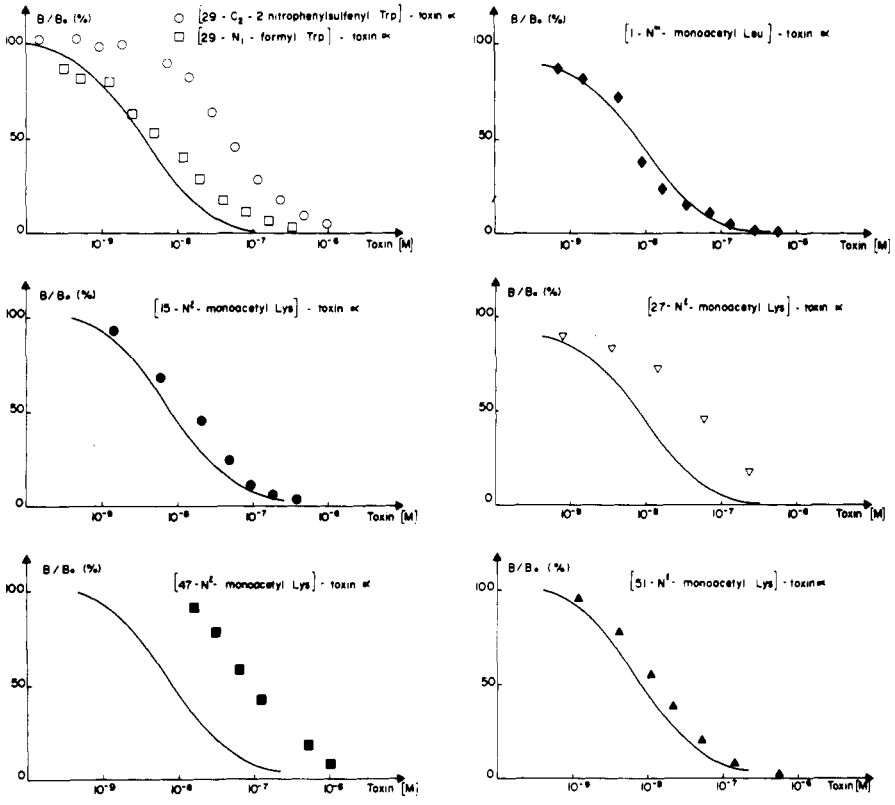

FIGURE 4: Binding of ^{3}H-labeled toxin α to AcChR in the presence of varying amounts of a range of toxin derivatives. In all cases, the continuous curve corresponds to the inhibition observed in the presence of native toxin. B and B_0 are the bound radioactivity in the presence and absence of derivatives, respectively.

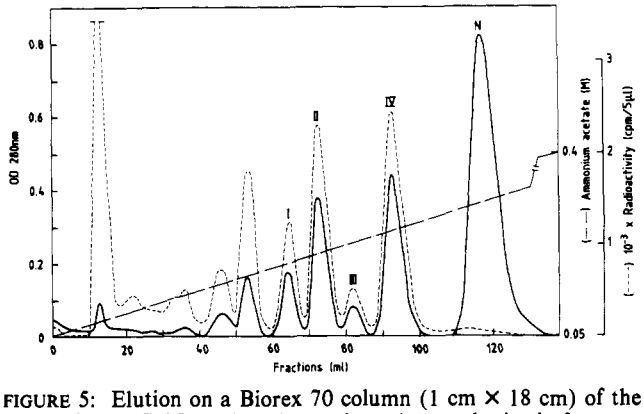

FIGURE 5: Elution on a Biorex 70 column (1 cm × 18 cm) of the desalted (Bio-Gel P2 column) reaction mixture obtained after acetylation of 2 μmol of toxin α by 2 μmol of [^{14}C]acetic anhydride. Specific radioactivity = 11 mCi/mmol. Fractions I–IV are monoacetylated components.

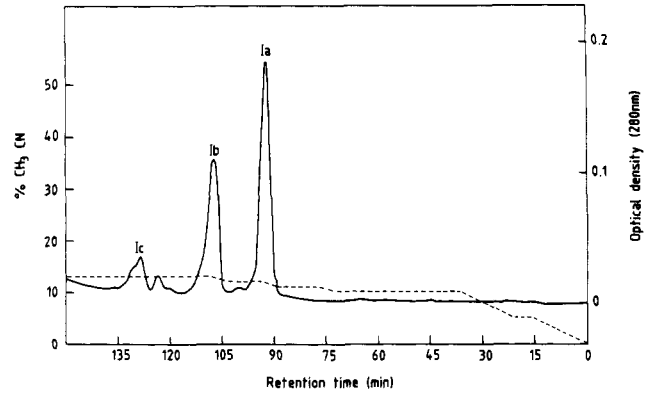

FIGURE 6: Elution on a C_{18} μBondapak column (3.9 × 30 cm) of the monoacetylated component I obtained after chromatography on Biorex 70 (see Figure 5). A binary gradient of triethylamine/acetonitrile (triethylamine acidified to pH 3.2 by formic acid) was applied on the column (- - -).

free thiol groups were blocked with iodoacetic acid. S-Carboxymethylated compounds were then hydrolyzed with trypsin, and the resulting enzyme digests were chromatographed on a reverse-phase column. The six elution profiles obtained are shown in Figure 7. Native toxin α gave rise to seven well-resolved peaks which correspond successively to segments 28–30, 27–30, 1–15, 52–62, 16–26, 16–25, and 40–51, respectively, as indicated by their amino acid composition (Table I). Fragment 34–39 was also present in peak 3. The highly polar tripeptide 31–33 was not detected. Modification of a single amino group altered this profile: one or more of the seven peaks disappeared whereas one or two new radioactive peaks emerged at high acetonitrile concentrations. Analysis of the amino acid composition of these fractions (Table I) enabled us to unambiguously identify the amino group which had been selectively acetylated. Thus, components Ia, Ib, II, III, and IV correspond to the derivatives of toxin α, monoacetylated at Lys-51, Lys-47, Lys-27, Lys-15, and the N-terminal amino group, respectively. No derivative selectively modified at either Lys-26 or Lys-59 was found in our preparations, irrespective of the experimental conditions.

(*c*) *Structural Analysis of the Monoacetylated Derivatives.* CD spectra of the monoacetylated derivatives were monitored in the far-ultraviolet region and compared to that of native toxin α (Figure 3B). Their shape was found to remain remarkably constant; the intensities of the signals at 216 and 197 nm remain virtually unchanged, indicating that selective acetylation of the single amino groups at positions 1, 15, 27, 47, or 51 has no effect on the overall structure of the toxin polypeptide chain. The positive signal at 228 nm which consists of contributions from both Trp-29 and Tyr-25 (Ménez

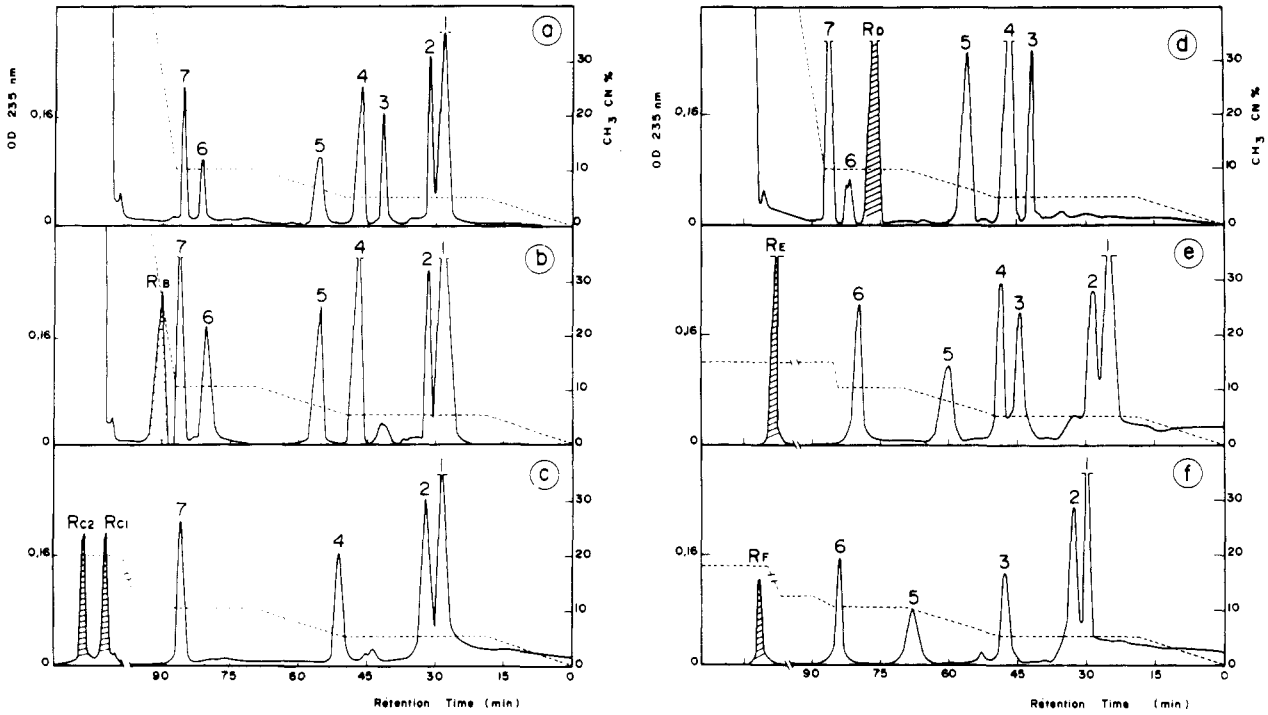

FIGURE 7: Elution patterns of tryptic digests of native toxin α and its monoacetylated derivatives on a C_{18} μBondapak column to which a binary gradient of triethylamine (pH 3.2)/acetonitrile was applied. Patterns a, b, c, d, e, and f were obtained with native toxin and with components IV, III, II, Ib, and Ia, respectively (Figures 5 and 6). The amino acid compositions of peaks 1–7 as well as those, radioactive, which are referred to as R_B, R_{C1}, R_{C2}, R_d, R_E, and R_F are given in Table I.

et al., 1976; Drake et al., 1977; 1980) is only weakly affected by monoacetylation, the most pronounced diminution being observed for the Lys-27-modified derivative.

Fluorescence emission spectra of native toxin α and monoacetylated derivatives in aqueous solution at neutral pH are represented in Figure 8. Previous studies have shown that the broad peak centered around 342 nm is due to Trp-29 only (Ménez et al., 1980). In all cases, excitation at 275 and 295 nm shows no difference in the 305-nm range, indicating the lack of tyrosine emission. Clearly, the Lys-27-modified derivative displays a maximum emission shift toward short wavelengths (λ = 338 nm), indicating that the Trp-29 environment becomes more hydrophobic with the loss of the Lys-27 positive charge. The maximum emission of the other derivatives, including native toxin α, is similarly centered at 342 nm. In general, the fluorescence intensity of the monoacetylated derivatives does not change appreciably (less than 8%) on derivatization, the sole exception being the Lys-51-modified derivative which displays an increase in intensity of approximately 16%. This observation suggests that the environment of Trp-29 is also affected slightly by the selective acetylation of the Lys-51 amino group.

Energy transfer measurements were performed at neutral pH in aqueous solution by comparing the excitation spectra recorded with the emission wavelength at 341 nm for native and modified toxins on the one hand and for model compounds on the other hand. An energy transfer efficiency of 100% was assumed in the case of the dipeptide L-Tyr-L-Trp and 0% for N-Ac-Trp-NH_2. It should be stressed that toxin α possesses a single tyrosine residue (located at position 25) and that 50% of the energy absorbed by this residue is transferred to the unique tryptophan (at position 29) (Ménez et al., 1980). This result is confirmed by the present experiments (see Figure 9). In addition, they show that acetylation at the N-terminal amino group, Lys-47, or Lys-51 does not modify this transfer whereas, in contrast, modification of either Lys-15 or Lys-27 induces an increase in transfer efficiency by 10% and 30%,

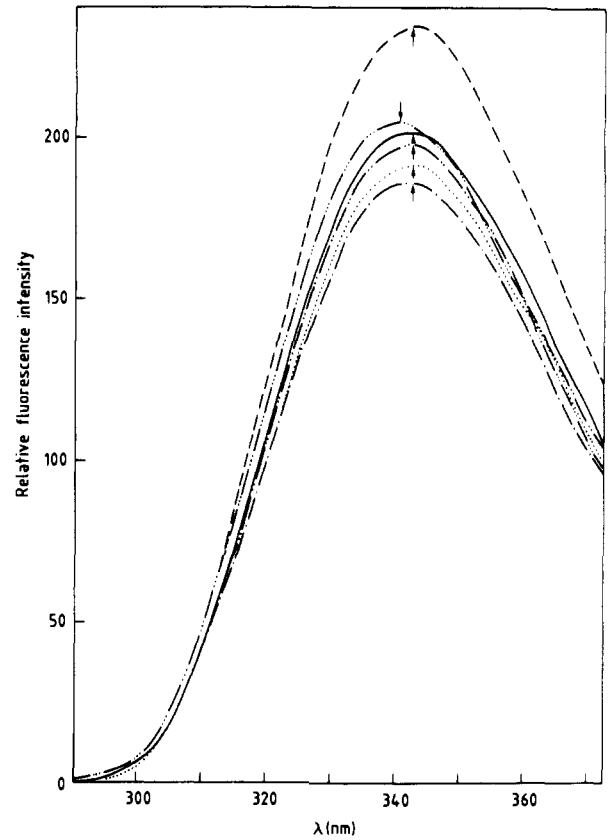

FIGURE 8: Emission fluorescence spectra of native toxin α (—) and its derivatives monoacetylated at residues 1 (···), 15 (-··-), 27 (-···-), 47 (-··-), and 51 (---). The compounds were dissolved in 10^{-3} M cacodylate buffer, 10^{-3} M NaCl, and 2×10^{-5} M EDTA, pH 7. The protein concentrations were approximately 10^{-5} M. The excitation wavelength was 280 nm.

respectively. This result demonstrates that modification of either of these two residues alters the environment of Trp-29.

Table I: Amino Acid Composition of Peptides Generated by Trypsin Hydrolysis and Fractionated by High-Performance Liquid Chromatography[a]

amino acids	peak 1	2	3	4	5	6	7	R_B	R_{C1}	R_{C2}	R_D	R_E	R_F
lysine		1_+^+ (1)	1.1 (1)	0.8 (1)	0.9 (1)		1.8 (2)	1.1 (1)	2_+^+ (2)	1_+^+ (1)	1.2 (1)	1.7 (2)	2.9 (3)
histidine			1.3 (1)					1.0 (1)	0.8 (1)	0.8 (1)			
arginine	1.2 (1)	1.1 (1)	1.2 (1)								1_+^+ (1)		
CM-cysteine			+	+	+	+	+	+	+	+		+	+
aspartic acid			1.0 (1)	3.7 (4)	0.9 (1)	1.2 (1)		1.1 (1)	1.8 (2)	1.7 (2)			3.8 (4)
threonine			3.0 (3)	2.1 (2)	1.8 (2)	1.6 (2)	1.2 (1)	1.8 (2)	3.2 (4)	3.8 (4)		1.0 (1)	2.1 (3)
serine			1.8 (2)					1.9 (2)	2.2 (2)	1.9 (2)			
glutamic acid			5.5 (5)	traces (0)	1.0 (1)	1.0 (1)		4.1 (4)	5.0 (5)	4.8 (5)			
proline			1.8 (2)	traces (0)	0.9 (1)	1.1 (1)	2.1 (2)	2.0 (2)	2.9 (3)	2.6 (3)		2.6 (2)	2.0 (2)
glycine			1.3 (1)	traces (0)	1_+^+ (1)	1.2 (1)	3.0 (3)	traces (0)	1.2 (1)	1.0 (1)		3_+^+ (3)	2.9 (3)
valine	1_+^+ (1)	0.6 (1)					1_+^+ (1)				0.6 (1)	0.8 (1)	0.7 (1)
isoleucine			1.8 (2)				0.9 (1)					0.7 (1)	0.6 (1)
leucine			1_+^+ (1)	1_+^+ (1)				1_+^+ (1)	0.8 (1)	0.8 (1)			1_+^+ (1)
tyrosine					0.9 (1)	1_+^+ (1)			0.8 (1)	0.8 (1)			
tryptophan	+	+									+ +		
corresponding peptides (see Figure 1)	28–30	27–30	1–15 (34–39)	52–62	16–26	16–25	40–51	1–15	1–26	1–25	27–30	40–51	40–62

[a] Peak nomenclature is that described in the legend of Figure 7. The presence of Trp and carboxymethylcysteine is indicated by +. In agreement with previous observations (Eaker & Porath, 1967), no tryptic cleavage is observed after Lys-47 and Lys-59.

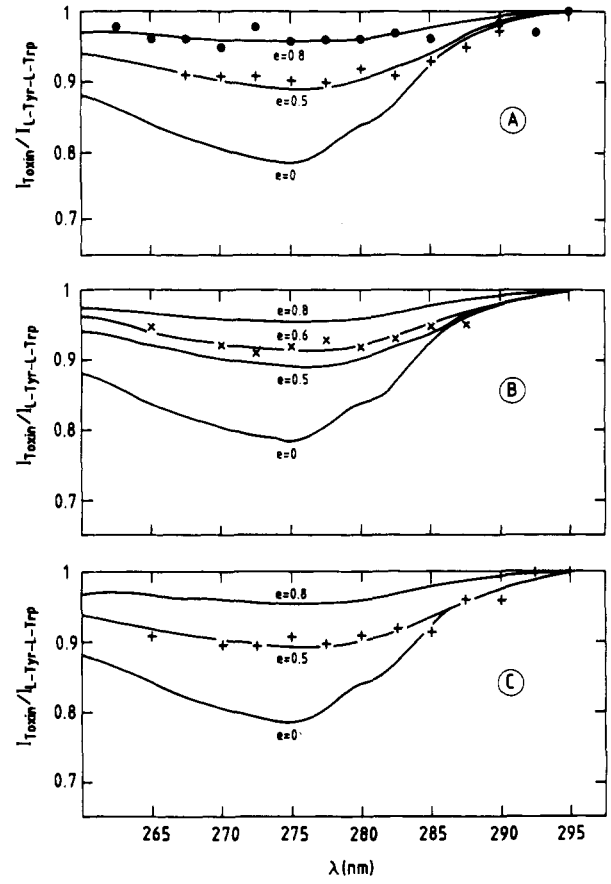

FIGURE 9: Determination of excitation energy transfer efficiencies from tyrosine-25 to tryptophan-29 in toxin α and its N-monoacetylated derivatives. Curves without symbols correspond to theoretical values of efficiencies and were calculated as previously described (Eisinger, 1969; Ménez et al., 1980), and curves with symbols correspond to fluorescence intensity ratios of the various toxins and the dipeptide L-Tyr-L-Trp vs. the excitation wavelength, in water. (A) Native toxin α (+); N-acetylated Lys-27 toxin α (●). (B) N-Acetylated Lys-15 toxin α (×). (C) N-Acetylated Leu-1 toxin α, N-acetylated Lys-47 toxin α, and N-acetylated Lys-51 toxin α. All three compounds in (C) give rise to identical transfer efficiencies ($e = 0.5$), and consequently, experimental values are given for only one, namely, monoacetylated Lys-51 toxin α (+). Intensities have been normalized at 295 nm with spectral shifts to shorter wavelengths of 1.5 nm for native toxin α and monoacetylated Lys-15 toxin and 2 nm for derivatives acetylated at the N-terminal amino function, Lys-47 and Lys-51.

(d) Biological Properties of the Monoacetylated Derivatives. Binding affinities to acetylcholine-rich membranes isolated from *Torpedo marmorata* (Sobel et al., 1977) were estimated from competition experiments between tritium-labeled toxin (45 Ci/mmol) and the derivatives under study (Figure 4). The dissociation constants derived from the experiments were then estimated according to previously described procedures (Ishikawa et al., 1977). The values obtained are shown in Table II. Clearly, binding affinity is not altered when the N-terminal amino group is acetylated while it is strongly influenced by acetylation of either Lys-27 or Lys-47. A slight but significant decrease in binding affinity is observed when Lys-15 or Lys-51 is acetylated.

Discussion

The present paper describes the preparation of seven homogeneous derivatives of toxin α isolated from *Naja nigricollis* venom, modified at a single amino acid of the polypeptide chain. Five of them are monoacetylated derivatives involving the residues 1 (N terminal), Lys-15, Lys-27, Lys-47, or Lys-51. The other two correspond to derivatives modified at two dis-

Table II: Equilibrium Dissociation Constants of Native Toxin α and Monomodified Derivatives[a]

modification	none	b	b	b	b	b	formylation	nitrophenylsulfenylation
modified residue	none (native toxin α)	51 (Ia)	47 (Ib)	27 (II)	15 (III)	1 (IV)	29	29
$K_D \times 10^{11}$ (M)	2	9	52	29	9	3	7	59
yield of recovery (%)		1.5	1.1	8	3	12	37	45

[a] Values were estimated according to Ishikawa et al. (1977). Numbers in parentheses correspond to those used in Figures 4 and 5.
[b] Acetylation.

tinct positions of the indole ring, namely, [N^1-formyl-Trp^{29}]toxin α and [C^2-[(2-nitrophenyl)sulfenyl]-Trp^{29}]toxin α. The isolation, purification, and identification of these compounds were greatly facilited by the use of reverse-phase high-performance liquid chromatography. This technique is obviously appropriate for the rapid separation of toxin isomers (see, for example, Figure 6), the resolution being markedly less when ion-exchange chromatography is used.

Modification of the Tryptophan Residue. Trp-29 is present in all potent postsynaptically acting cobra toxins (more than 60), and considerable work has been carried out with the view of clarifying its role. Conflicting conclusions are reported in the literature. It has been claimed that this residue is essential for an exhibition of toxicity of erabutoxin b (Seto et al., 1970) and also that it is excluded from the active site of a homologous Indian cobra neurotoxin (Ohta & Hayashi, 1974). More recently, on the basis of NMR (Tsetlin et al., 1979) and fluorescence experiments (Ménez et al., 1980), it was observed that Trp-29 interacts with several functionally important residues, possibly generating an ideal orientation of these groups for an adequate fit of the toxin to the receptor. The results reported here support this proposal. Incorporation of the 2-nitrophenylsulfenyl group at position C_2 of the indole group modifies the binding affinity of the toxin for the acetylcholine receptor while no overall conformational change of the toxin backbone is seen as judged from CD studies. We conclude, therefore, that Trp-29 is involved in the acetylcholine receptor (AcChR) binding site of toxin α. Two alternatives exist which are capable of interpreting this binding affinity decrease: (i) a local structural change occurs in the Trp-29 vicinity following its modification, and/or (ii) intrinsic alteration of the hydrophobic character of Trp-29 abolishes interactions occurring between this group and a specific partner present on the AcChR. In order to distinguish between these two possibilities, we selectively incorporated a polar formyl group at the indole NH moiety according to Coletti-Previero et al. (1969). A weak affinity decrease is thus observed, indicating that (i) the hydrophobic character of Trp-29 plays a minor role in the interaction between the toxin and the receptor, in agreement with previous conclusions of Chicheportiche et al. (1972), and that (ii) there is no critical hydrogen bond between the indole NH of the toxin and the receptor. Then it is reasonable to conclude that the substantial change in affinity which is observed after its modification with bulky reagents such as 2-nitrophenylsulfenyl chloride may partially result from an alteration of its environmental structure in the toxin. In agreement with previous suggestions (Chicheportiche et al., 1972; Ménez et al., 1980), the indole side chain 29 is likely to play some structural role in the stabilization of an adequate geometry for the AcChR binding site of toxin α.

Modification of Amino Groups. Due to the presence of seven amino groups in the toxin molecule, we expected to obtain an equivalent number of monoacetylated derivatives. However, two were not detected. In view of the power of the fractionation method used here, it seems unlikely that these species were present in appreciable quantity. This finding is probably related to the lack of reactivity of the lysine residues at positions 26 and 59. The other five amino groups reacted to varying extents in a manner which, in general, is similar to that reported by Hori & Tamiya (1976) using erabutoxin b and by Tsetlin et al. (1979) using *Naja naja oxiana* toxin II. There is, however, one marked difference between the modification of the N-terminal leucine amino group of the latter toxin and that of *Naja nigricollis* toxin: acetylation of this group was readily performed in the present case while it failed with toxin II. This result indicates that in spite of their strong structural homology as demonstrated by fluorescence and NMR investigations (Bystrov et al., 1978), the two toxins present conformational differences in the vicinity of their N-terminal amino group. This is in agreement with recently reported immunological data (Boulain et al., 1982). The sequences of these two toxins differ by eight residues. Seven of these residues, namely, 28, 30, 45, 50, 51, 56, and 59, are far removed from N-terminal amino group as judged from the X-ray structure of the homologous erabutoxin b molecule (Tsernoglou & Petsko, 1976; Low et al., 1976). A likely origin for the local differences is, therefore, the single substitution occurring at position 18, Pro in toxin α and Ser in toxin II.

As amino group acetylation does not affect the overall structure of toxin α, as revealed by CD measurements, we conclude that (i) the substantial loss in binding affinity which is observed with acetylation of Lys-27 or Lys-47 indicates that the positive charge of each of these two residues is involved in the AcChR binding area of the toxin and that (ii) the lack of effect on the toxin binding affinity after acetylation of the N-terminal group demonstrates that the N-terminal positive charge of toxin α is not involved the AcChR binding site.

Despite the clear similarities between the overall structures of the native toxin and its five monoacetylated derivatives, more subtle changes, not detected by CD investigations, may occur as a result of acetylation. In an attempt to monitor such local changes, we examined the environment of Trp-29 in each monoacetylated derivative by means of fluorescence spectroscopy. This environment as indicated above is probably located in the AcChR binding site of toxin α. Moreover, it is involved in a relatively flexible region of the toxin (Ménez et al., 1976; Drake et al., 1977, 1980). Furthermore, the fluorescence of Trp-29 is sensitive to environmental perturbations (Bukolova-Orlova et al., 1974; Ménez et al., 1980; Surin et al., 1981). We observed that selective abolition of the Lys-27 positive charge induced a blue shift of the maximum emission and a 30% increase in energy transfer occurring between Tyr-25 and Trp-29. The shift itself reflects an increase in the local hydrophobicity which probably results from the proximity of the aromatic side chain of residue 29 and the Lys-27 side chain as previously revealed by NMR (Bystrov et al., 1978), fluorescence (Ménez et al., 1980), and X-ray analyses (Tsernoglou & Petsko, 1976; Low et al., 1976). In principle, the 30% energy transfer increment could be interpreted as indicating that the neutralization of the Lys-27 positive charge, which is located between Tyr-25 and Trp-29 (Figure 1), decreases the distance separating these aromatic residues. Assuming a quantum yield of 0.1 for tyrosine, it can be calculated that in native toxin α this distance is approxi-

mately 13 Å (Ménez et al., 1980), in agreement with crystallographic observations (Kimball et al., 1979), whereas in the monoacetylated Lys-27 toxin it would approach 9.5 Å. Such a change necessitates a major alteration of the β-sheet structure bearing both Tyr-25 and Trp-29, a change inconsistent with CD observations. Alternatively, neutralization of the Lys-27 positive charge may simply facilitate the energy transfer as a result of a change in the orientation factor (K^2) (Eisinger, 1969) between the donor (Tyr) and the acceptor (Trp). This could be achieved by changing the environmental side chain organization, no alteration of the backbone being necessary. This second alternative is in agreement with CD results. As a result, we suggest that the substantial decrease in binding affinity which is observed for the Lys-27-modified derivative is due, at least in part, to a local perturbation of surrounding amino acid side chains including Tyr-25 and Trp-29.

Acetylation of either Lys-15 or Lys-51 induces a 10% energy transfer increase and a 16% emission intensity enhancement, respectively. As both residues are spatially remote from Trp-29, their selective acetylation has clearly induced minor conformational changes in the vicinity of the indole side chain which are reflected in their affinity decrease for the receptor (Table II). Acetylation of the N-terminal group does not modify the fluorescence properties of Trp-29, an observation which is in agreement with the nativelike affinity of this derivative.

Lys-47 behaves like the N-terminal group on acetylation in that no change in fluorescence was detected. This is extremely interesting as (i) Lys-47 is only separated from Trp-29 and from Lys-27 by 10 and 7.4 Å, respectively (Kimball et al., 1979), and (ii) Lys-47 and Lys-27 enter in repulsive interactions (Ménez & Tamiya, 1982). Thus, despite Lys-47 being in close proximity to two residues believed to be involved in the AcChR binding site, acetylation of its positive charge does not apparently alter the molecular organization of this region as judged by fluorescence and CD studies. The substantial weakening in affinity which is observed with acetylation of this residue is most conveniently explained as being directly related to the suppression of this positive center.

In general, our results which point out the participation of Lys-27, Trp-29, and Lys-47 in the AcChR binding site of *N. nigricollis* toxin α are in agreement with those reported for related toxins (Hori & Tamiya, 1976; Tsetlin et al., 1979; Karlsson, 1979). They also agree well with the fact that these three residues are highly conserved in the same position in more than 60 homologous neurotoxins (Karlsson, 1979; Ménez et al., 1978). However, it must be stressed that the loss in binding affinity which is observed with derivatization of Lys-27 or Trp-29 (by nitrophenylsulfenylation) may not be exclusively the result of the alteration of the group itself but also of some environmental perturbation. As a result, it is likely that both Lys-27 and Trp-29 contribute less, in terms of direct binding, to the association of toxin to AcChR than is indicated by the apparent affinity decrease.

Recently, Arseniev et al. (1981) concluded that the positive charge of Lys-15 and the N terminus are involved in the AcChR binding site of *N.n. oxiana* toxin II. The present findings indicate, in contrast, that this is not the case for the corresponding functions of the homologous *N. nigricollis* toxin α. We reported that a monoclonal toxin α specific immunoglobulin directed to an antigenic site involving Lys-15 and the N terminus may bind transiently to toxin α while the latter is simultaneously associated with its specific receptor, thus destabilizing the toxin α–AcChR complex (Boulain & Ménez, 1982). This observation agrees well with the present data and furthermore indicates that the two residues 1 and 15 are accessible to the surrounding media while the toxin is bound to the nicotinic receptor.

There are 27 residues present in toxin α which are also shared by more than 60 homologous neurotoxins, 15 of which are also being conserved in the other structurally homologous snake venom components such as cardiotoxins (Karlsson, 1979). The conserved residues unique to neurotoxins are Ser- or Thr-8, Lys- or Arg-27, Trp-29, Asp-31, His/Phe- or Trp-32, Arg-33, Gly-34, Glu- or Asp-38, Val- or Ala-46, Lys- or Arg-47, Gly-49, and Leu/Ile- or Val-52. All these residues possibly represent the basic AcChR binding site common to potent postsynaptically acting neurotoxins (Karlsson, 1979; Low, 1979). As a result of this multiplicity of possible interacting sites, one can expect that modification of a single residue will diminish the overall binding affinity of the toxin without abolishing it totally. This is indeed observed in the present study.

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Assignment of Fluorine Nuclear Magnetic Resonance Signals from Rabbit Cyanomethemoglobin†

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ABSTRACT: A fluorine NMR study of cyanomethemoglobin prepared from hemoglobin isolated from rabbits maintained on a diet containing DL-*p*-fluorophenylalanine is described. The results indicate that substitution of fluorophenylalanine occurs essentially randomly at all phenylalanine positions of the α- and β-globin chains; a set of hybrid hemoglobins in which only the α- or only the β-chains contain the fluorinated amino acid was prepared and used to ascertain the fluorine NMR signals arising from each chain. The temperature and pH dependences of chemical shifts, spin-lattice relaxation times, $^{19}F\{^1H\}$ nuclear Overhauser effects, and the effect of chemical modification of the β-93 sulfhydryl groups were examined. When considered in light of presently available X-ray structures of human and horse hemoglobins, the available data permit a tentative assignment of most signals to particular fluorophenylalanine/phenylalanine positions in the globin sequences.

Fluorine nuclear magnetic resonance (NMR)[1] spectroscopy is a powerful tool for examining biological structures because of the high sensitivity of fluorine chemical shifts to the details of local molecular structure. Many organisms will incorporate fluorinated amino acids present in their growth medium or diet into proteins (Sykes & Weiner, 1980), and these proteins often exhibit a number of resolved resonances in their fluorine NMR spectra. In these cases, as in all high-resolution NMR spectra, maximum structural information is obtained when the observed signals can be assigned to particular molecular features, that is, when each fluorine resonance can be associated with a particular amino acid of the protein sequence. Assignments of the fluorine signals from 3-fluorotyrosine-labeled *lac* repressor have been made (Jarema et al., 1981), but beyond this elegant work, there have been few attempts to establish the correspondence between a particular fluorine resonance and a particular amino acid position in a protein which contains biosynthetically incorporated fluorinated amino acids.

Westhead & Boyer (1961) have reported that DL-*p*-fluorophenylalanine replaces a significant fraction of phenylalanine in proteins of the rabbit when the animal is maintained on a diet containing this fluoro amino acid. We have examined the hemoglobin formed by these animals when 0.3% (by mass) of fluorophenylalanine is present in the diet. Under these conditions about one replacement of phenylalanine per $\alpha_2\beta_2$ tetramer takes place, and the fluorine NMR spectrum of the cyanomet form of the protein is consistent with the presence of 16 resonances, one for each phenylalanine/fluorophenylalanine position in the sequences of rabbit α- and β-globins. In attempting to assign these signals to particular positions, the genetic approach of Jarema et al. (1981) is not yet open to us, and assignments based on a collection of observations including temperature and pH variation of chemical shifts, spin-labeling, spin-lattice and spin-spin relaxation rates, and fluorine-proton nuclear Overhauser effects are reported. We emphasize, at the outset, that the assignments are tentative and rest on a presumed close homology between the X-ray crystal structures of human or horse hemoglobins and the structure of rabbit hemoglobin in solution. The cyanomet form

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; ^1H NMR, proton NMR; UV-vis, ultraviolet-visible; NOE, nuclear Overhauser effect; CSA, chemical shift anisotropy; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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