Preparation and Activity of Guanidinated or Acetylated Erabutoxins

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1. Erabutoxins a, b and c, neurotoxic proteins of a sea snake *Laticauda semifasciata*, were guanidinated with *O*-methylisourea. The amino groups of all the lysine residues and those at the *N*-termini of the toxins were modified. The lethal activity of the toxins decreased to 50% (erabutoxins a and b) or 17% (erabutoxin c) of the original value on the modification. The c.d. (circular dichroism) maximum at 227 nm of the modified toxins became lower, whereas the whole profile of the c.d. curve remained unchanged.

2. The amino groups of erabutoxin b were acetylated with acetic anhydride. All the five monoacetyl derivatives were isolated from the reaction products by CM-cellulose and Bio-Rex 70 column chromatography. [1-*N*^ε-acetylarginine]-, [15-*N*^ε-acetyl-lysine]- and [51-*N*^ε-acetyl-lysine]-erabutoxin b retained the toxicity of the native toxin, whereas [27-*N*^ε-acetyl-lysine]- and [47-*N*^ε-acetyl-lysine]-erabutoxin b were 17 and 8% active respectively. The overall profile of c.d. spectrum of erabutoxin b remained unchanged on the monoacetylation.

Erabutoxins a, b and c are neurotoxic proteins from the venom of the sea snake *Laticauda semifasciata* and consist of 62 amino acid residues (Tamiya & Arai, 1966; Sato & Tamiya, 1971; Endo *et al.*, 1971; Tamiya & Abe, 1972).

The present paper describes the effects of guanidination and acetylation of the amino groups of erabutoxins on their activity. Erabutoxin c has three amino groups (lysine residues) at residue numbers 15, 27 and 47 in addition to the terminal amino group. Erabutoxins a and b have an additional amino group (lysine) at residue number 51.

Materials and Methods

Erabutoxins a, b and c were prepared as described previously (Tamiya & Arai, 1966; Tamiya & Abe, 1972). Trypsin (twice-crystallized and salt free, from Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was treated with diphenylcarbamoyl chloride (Erlanger *et al.*, 1966). *O*-Methylisourea sulphate (Tokyo Kasei Kogyo Co., Tokyo, Japan) was recrystallized from aq. acetone. [1-¹⁴C]Acetic anhydride (4.4 mCi/mmol) was from New England Nuclear Corp., Boston, Mass., U.S.A. Acetic anhydride and dioxan were redistilled before use. Scintillators [2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] were obtained from Beckman Instruments Inc., Fullerton, Calif., U.S.A.

Protein determination

The protein concentration was determined on sample solutions by measuring the extinction at

280 nm by Hitachi-Perkin-Elmer 139 spectrophotometer (Hitachi Co., Tokyo, Japan). The molar extinction coefficient of erabutoxin b was 8600 litre·mol⁻¹·cm⁻¹; a newly freeze-dried preparation of erabutoxin b contained about 17% water from the elementary analysis.

Radioactivity determination

The radioactivity of a protein solution was determined by a liquid-scintillation counter (Unilux IIA, Nuclear-Chicago, Des Plaines, Ill., U.S.A.). The scintillator solution was prepared as described by Bray (1960). The d.p.m. value was obtained by means of automatic standardization.

Circular-dichroism measurement

Circular dichroism (c.d.) of the erabutoxins and their derivatives was measured on 0.1–1.4 mg/ml of solution in 0.1 M-NaCl by a JASCO J-20A recording spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The c.d. data are expressed in terms of degrees dmol⁻¹·cm², on the basis of the average residue weight.

Disc electrophoresis

The polyacrylamide-gel disc electrophoresis of the modified toxins was carried out at pH 4.0 as described by Reisfeld *et al.* (1962), with slight modifications (Tamiya & Arai, 1966). The samples (8–25 µg) were loaded in 60% (w/v) sucrose in 0.06 M-potassium acetate buffer, pH 6.7 (0.1 ml).

Amino acid analysis

The peptides (0.03–0.06 μ mol) were hydrolysed with 6M-HCl (0.5 ml) at 110°C for 24 h in evacuated sealed glass tubes. The hydrolysates were dried *in vacuo* and analysed with a JLC-5AH automatic amino acid analyser (Japan Electron Optics Ltd., Tokyo, Japan).

Assay of free amino groups

The proteins (0.5–1.0 mg) were dissolved in 2% NaHCO₃ (2 ml) and their amino group contents were determined with 2,4,6-trinitrobenzene sulphonic acid as described by Habeeb (1967).

Toxicity measurement

The toxicity of the modified toxins was determined by intramuscular injection of the solution (0.08–0.20 ml) in 0.85% NaCl into mice (17–25 g) as described by Tamiya & Arai (1966).

Contraction experiment on isolated frog muscles

The action of guanidinated erabutoxins on the isolated rectus abdominis muscle of the frog *Rana nigromaculata* was studied as described by Tamiya & Arai (1966) with SB-1T force-displacement transducer and multipurpose recorder RH-20 (Nihon Kodens Co., Tokyo, Japan).

Results

Guanidination of erabutoxins

Guanidination of all the amino groups of erabutoxins a, b and c was carried out as described by Chauvet & Acher (1967). Erabutoxin a (20.3 mg) and *O*-methylisourea sulphate (492 mg) were dissolved in water (8.0 ml) and the pH of the solution was adjusted to 10.5 with 3.5M-NaOH and kept at the same pH with 1M-NaOH during the course of the reaction at 0°C for 7 days. After 4 days, 2M-*O*-methylisourea sulphate (1.5 ml) was added to boost the reaction. At certain intervals, a portion (0.5 ml) of the reaction mixture was neutralized with acetic acid and applied to a column (1.4 cm $\times$ 60 cm) of Sephadex G-25 (fine grade) in 0.1M-acetic acid. The desalted protein-containing part of the effluent was freeze-dried. Guanidination of erabutoxins b and c was carried out for 5 days under similar conditions except that the boost reagent was not added.

The time-course of the reaction with erabutoxin a was followed by the conversion of the lysine residues into those of homoarginine on acid hydrolysates (Fig. 1). Some 90% of the lysine residues was guanidinated in 2 days. The gradual decrease in arginine contents suggested the guanidination of the amino group of the *N*-terminal arginine of the toxin.

Fig. 1. Time-course of guanidination of erabutoxin a

Experimental details are given in the text. ●, Lysine contents; ○, arginine contents; ■, toxicity. Molar amino acid contents were calculated by assuming the total values of lysine and homoarginine to be 4.

The *N*-terminal guanidinated and non-guanidinated derivatives were separated from each other as described below.

The erabutoxins kept for 5 days at pH 10.5 without *O*-methylisourea as control experiments showed the same toxicity and amino acid contents as the corresponding native toxins.

The guanidinated erabutoxin a (11.1 mg) was dissolved in 0.01M-ammonium acetate buffer, pH 9.1 (5 ml), and applied to a column (1.4 cm $\times$ 17 cm) of CM-cellulose which had been equilibrated with 0.2M-ammonium acetate buffer, pH 9.1, and treated with the 0.01M-acetate buffer (20 ml). The elution was carried out with the 0.01M-acetate buffer (50 ml), followed by a linear concentration gradient elution with the 0.01M buffer (150 ml) in the mixing chamber and 0.04M-ammonium acetate buffer, pH 9.1 (150 ml), in the reservoir. The flow rate was 100 ml/h. Two components, aI and aII, were obtained at 160 and 210 ml respectively. The guanidinated erabutoxin b was separated into its components bI and bII under the same conditions as those above. The guanidinated erabutoxin c (4.2 mg) was also separated on a narrower column (1.0 cm $\times$ 24 cm) with more dilute buffer (0.005M) into components cI and cII, which were eluted at 30 and 50 ml respectively.

The amino group, lysine, arginine and homoarginine contents of the guanidinated derivatives are given in Table 1. All the other amino acid contents remained unchanged as compared with those of the native toxins. The results show that all the lysine residues in erabutoxins are converted into homoarginine residues in the above treatment. Component aII, bII or cII has one less arginine residues as compared with component aI, bI or cI; components II are the *N*-terminal guanidinated derivatives of the corresponding I components.

Table 1. *Compositions and toxicities of guanidinated erabutoxins*

The results are expressed in molar proportions of the amino acids and the amino group. The amino group contents were measured with 2,4,6-trinitrobenzenesulphonic acid as described by Habib (1967). LD₅₀ values by intramuscular injection are shown.

Toxins and derivatives	Amino acids (residues/molecule)			Amino group (mol/mol)	LD ₅₀ (μg/g)	Toxicity (%)
	Lysine	Homoarginine	Arginine			
Erabutoxin a	4	0	3	5	0.12	100*
aI	0.12	3.88	2.88	0.79	0.26	46
aII	0.05	3.95	1.94	0.03	0.25	48
Erabutoxin b	4	0	3	5	0.12	100*
bI	0.07	3.93	3.03		0.28	43
bII	0.14	3.86	2.26		0.24	50
Erabutoxin c	3	0	3	4	0.10	100*
cI	0.08	2.92	3.00		0.64	16
cII	0.08	2.92	2.10		0.76	13

* The LD₅₀ values of native toxins are taken as 100%.

Fig. 2. *Elution pattern of ¹⁴C-acetylated erabutoxin b derivatives from a CM-52 cellulose column*

¹⁴C-Acetylated erabutoxin b mixture (13.4mg, 2.36×10⁶d.p.m.) in 0.01M-phosphate buffer, pH6.4 (26ml), was chromatographed on a column (1.5cm×33cm) of CM-52 cellulose, which had been equilibrated with the buffer. A linear gradient elution was applied with the buffer (400ml) in the mixing chamber and the buffer containing 0.05M-NaCl (400ml) in the reservoir. The flow rate was 20ml/h and 5.0ml fractions were collected. E₂₈₀ (—), radioactivity (····) and NaCl concentration (----) were measured. Bar 1 denotes pooled peaks III–IV, bar 2 pooled peaks V–VI; for further details see the text.

Limited acetylation of erabutoxin b and the separation of monoacetyl derivatives

[1-¹⁴C]Acetic anhydride (4.4mCi/mmol, 22.7μmol) was dissolved in benzene (2.3ml) and the solution (0.1ml) mixed with the unlabelled anhydride (1.7μmol) in dioxan (0.1ml). The whole mixture was added to erabutoxin b (13.4mg, 1.95μmol) solution in 0.1M-phosphate buffer, pH7.2 (5.1ml). The reaction mixture was stirred for 90min at room temperature, then applied to a column (1.5cm×68cm) of Sephadex G-25 (fine grade) in 0.01M-phosphate buffer, pH6.4. The flow rate was 30ml/h. The

protein-containing part of the effluent (26ml), which incorporated 2.36×10⁶d.p.m. of the radioactivity, was applied to a column (1.5cm×33cm) of CM-cellulose (CM-52, Whatman Biochemicals Ltd., Maidstone, Kent, U.K.). By a linear gradient elution, seven protein peaks (I–VII) were observed (Fig. 2). The radioactivity/E₂₈₀ ratio showed that peaks I and II contained di (or more)-acetyl-, peaks III–VI monoacetyl- and peak VII unmodified erabutoxin b. The components in peaks I, II and VII were not treated further. The yield of the monoacetyl derivatives, which contained 1.20×10⁶d.p.m. of radioactivity, was 5.30mg. The desalted and freeze-

dried material from peaks III–IV (indicated by bar 1 in Fig. 2, 3.8mg, 8.7×10^5 d.p.m.) was mixed with corresponding non-radioactive material (7.2mg, see below), dissolved in 0.01M-ammonium acetate buffer, pH 6.5 (4.0ml), and chromatographed with the same buffer at a flow rate of 4ml/h on a column (1.2cm $\times$ 101cm) of Bio-Rex 70 (minus 400 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) which had been equilibrated with 0.2M-ammonium acetate buffer, pH 6.5, and treated with 0.01M buffer, pH 6.5 (50ml). Components III (0.73mg, 0.81×10^5 d.p.m.), IVa (5.03mg, 3.65×10^5 d.p.m.) and IVb (1.96mg, 1.29×10^5 d.p.m.) were eluted at 145, 162 and 186ml respectively.

The desalted and freeze-dried material (1.18mg, 2.5×10^5 d.p.m.) from peaks V–VI (indicated by bar 2 in Fig. 2) was dissolved in 0.01M-ammonium acetate buffer, pH 8.7 (15ml), applied to a column (1.5cm $\times$ 32cm) of CM-52 cellulose which had been equilibrated with the same buffer. The elution was carried out with the 0.01M buffer (34ml), followed by a linear gradient of 0.01–0.04M-ammonium acetate buffer, pH 8.8, with the 0.01M-acetate buffer (100ml) in the mixing chamber. The flow rate was 25ml/h. Components VI (0.21mg, 0.46×10^5 d.p.m.) and V (0.80mg, 1.7×10^5 d.p.m.) were eluted at 132 and 154ml respectively.

The experiment was repeated on ten times the scale with non-radioactive acetic anhydride and the same components were obtained. The physical and

biological properties were examined on these non-radioactive derivatives.

Location of the acetyl groups in monoacetylerabutoxin b derivatives

Radioactive monoacetylerabutoxin b derivatives were diluted with their corresponding non-radioactive components (except component V) to show a specific radioactivity of approx. 7×10^4 d.p.m./mg, and reduced and S-carboxymethylated in 6M-guanidine hydrochloride with dithiothreitol as described by Crestfield *et al.* (1963). The reduced and S-carboxymethylated derivatives (0.5–6.2mg) were hydrolysed in 0.1M- NH_4HCO_3 , pH 7.8 (0.5–1.6ml), with trypsin at a substrate/enzyme ratio of 50:1 (w/w) at 37°C for 7h. Each digestion mixture was chromatographed on a column (1.24cm $\times$ 101cm) of Sephadex G-25 (fine grade) with 0.1M-acetic acid. A single radioactive peak was obtained from the tryptic digest of each derivative. Radioactive fragments were obtained at the void volume (components IVb and VI) or at the position of salts (component V). The radioactive part of the effluent from component III or IVa was evaporated to dryness, dissolved in 0.02M- (component III) or 0.1M- (component IVa) NH_4HCO_3 buffer, pH 8.0 (2ml), and chromatographed on columns (0.95cm $\times$ 4cm) of DEAE-cellulose (DE-52, Whatman Biochemicals Ltd.), which had been equilibrated with the appropriate

Table 2. *Amino acid compositions of the radioactive fragments from monoacetylerabutoxin b derivatives*

The results are expressed in molar proportions of the amino acids. The values in parentheses give the expected molar proportions of the fragments from acetylated erabutoxin b derivatives.

Components	III (Lys-47-)	IVa (Lys-27-)	IVb (Lys-15-)	V (α -)	VI (Lys-51-)
Lysine	2.20 (2)	1.00†(1)	2.00‡(2)		2.00‡(2)
Histidine		0.94 (1)	2.08 (2)		
Arginine		0.98 (1)	0.60	1.00‡(1)	
Cysteic acid	0.43	0.24	0.11		0.59
CM-cysteine*	} 0.38†(2)	1.24 (2)	2.46 (3)	} 0.01†	} 4.55†(5)
Aspartic acid		1.07 (1)	1.15 (1)		
Threonine	1.02 (1)	0.93 (1)	2.79 (3)		1.07 (1)
Serine	0.25	3.74 (4)	4.57 (5)	0.01	2.08 (2)
Glutamic acid	0.19	1.96 (2)	3.99 (4)		2.14 (2)
Proline	1.55 (2)	1.16 (1)	2.33 (2)		1.85 (2)
Glycine	3.00‡(3)	1.06 (1)	1.29 (1)	0.01	3.05 (3)
Half-cystine	0.42	0.19	0.32		0.68
Valine	0.92 (1)		0.13		1.63 (2)
Isoleucine	0.81 (1)		0.76 (1)		0.84 (1)
Leucine			0.05		0.84 (1)
Tyrosine		0.92 (1)	0.72 (1)		
Phenylalanine		0.98 (1)	1.01 (1)		
Tryptophan§		+	(1)		

* S-Carboxymethylcysteine tends to give low value.

† The separation was incomplete.

‡ The contents of these amino acids were taken as standards.

§ Tryptophan was detected by spot test with Ehrlich reagent (Smith, 1953).

Fig. 3. C.d. spectra of erabutoxin c and its guanidinated derivatives

Guanidinated erabutoxin cI (—) or native erabutoxin c (····) was dissolved in 0.1M-NaCl. Above 235nm, protein concentration was 1.30mg/ml for cI or 1.44mg/ml for the native toxin and path-length 1.0cm. Below 235nm, protein concentration was 0.129mg/ml for cI or 0.133mg/ml for the native toxin and path-length 0.5cm. The c.d. curve of guanidinated erabutoxin cII was the same as that of cI.

NH_4HCO_3 buffer. The elution was carried out with the NH_4HCO_3 buffer (12ml) and followed by a linear gradient elution from 0.02 to 0.2M-(component III) or 0.1 to 0.5M-(component IVa) NH_4HCO_3 buffer, pH8.0 (total 100ml). Radioactive peptide was eluted at 45 ml (component III) or 51 ml (component IVa).

The amino acid compositions of the radioactive fragments are given in Table 2. From these results, components III, IVa, IVb, V and VI are identified as Lys-47-, Lys-27-, Lys-15-, N-terminal- and Lys-51-acetylerabutoxin b respectively. The value of specific radioactivity of acetylarginine from component V agreed with that of component V itself.

Properties of guanidinated and monoacetylated erabutoxin derivatives

Guanidinated erabutoxins aI, aII, bI, bII, cI and cII gave single bands at the same positions as the native toxins on disc electrophoresis at pH4.0. Table I shows that the guanidinated erabutoxins a and b are 50% as toxic as the native toxins, whereas the guanidinated erabutoxin c is less toxic. Guanidination of the terminal amino group does not affect the toxicity.

Guanidinated erabutoxins (0.08 μg per ml of Ringer solution) inhibited the contraction of the rectus abdominis muscle of the frog similarly to the native toxins, however, the inhibition is more easily removed by washing than is that caused by the native toxins.

The overall profile of the c.d. spectra of the guanidinated toxins remained unchanged. However, the positive maximum at about 227nm decreased

Fig. 4. Disc electrophoresis of acetyl erabutoxin b derivatives

The electrophoresis was performed at a constant current of 3mA/gel for 200min with 8–25 μg of samples in 60% (w/v) sucrose in 0.06M-potassium acetate buffer, pH 6.7 (0.1ml). The gel size was 0.45cm $\times$ 9.0cm for separation and 1.0cm for stacking. The relative mobility was determined by mixing with erabutoxins a and b as standards.

Table 3. Monoacetyl derivatives of erabutoxin b

The yield is expressed on the basis that the total amount of the monoacetyl derivatives is 100%. LD₅₀ value by intramuscular injection is shown. For toxicity measurements, the LD₅₀ value of erabutoxin b is taken as 100%.

Components	LD ₅₀ (μg/g)	Toxicity (%)	Yield (%)
III (47)	1.5	8	9
IVa (27)	0.70	17	48
IVb (15)	0.12	100	17
V (α)	0.12	100	21
VI (51)	0.12	100	5

significantly on guanidination. The largest decrease is seen in the case of erabutoxin c (Fig. 3).

Monoacetylated erabutoxin b derivatives gave discrete single bands on disc electrophoresis at pH 4.0 (Fig. 4). Table 3 shows that the *N*-terminal-, Lys-15- or Lys-51-acetyl toxin is fully toxic as compared with the native toxin, whereas the Lys-27- or Lys-47-acetyl toxin is less toxic.

The profile of the c.d. spectrum of all the monoacetyl derivatives remained unchanged.

Discussion

All the ϵ -amino groups of the lysine residues of erabutoxins were guanidinated. The toxicity of the guanidinated derivatives were 50–17% of those of the native toxins. The *N*-terminal amino group was guanidinated partially, and the guanidination did not affect the toxicity. The inhibition of the acetylcholine-induced contracture of isolated frog muscles with the guanidinated toxins can more easily be reversed by washing than those produced with native toxins. The results suggest the decrease in affinity of the toxins for the receptor sites on the modification.

Five monoacetyl derivatives of erabutoxin b were isolated. The toxicity of the *N*-terminal-, Lys-15- or Lys-51-acetyl toxins was the same as that of the native toxin, whereas that of the Lys-27- and Lys-47-acetyl toxins was 17 and 8% respectively. The finding agrees well with the fact that residues 15 and 51 are variant whereas residues 27 and 47 are conservative among the snake neurotoxins sequenced so far (cf. Maeda & Tamiya, 1974).

Chang *et al.* (1971) reported on *Naja naja atra* cobrotoxin that the guanidination of all the ϵ -amino

groups, the trinitrophenylation of the α -amino group of the *N*-terminal residue or of the ϵ -amino group of Lys-27 did not affect the toxicity, but the trinitrophenylation of both Lys-27 and Lys-47 extinguished the toxicity. Karlsson *et al.* (1972) reported on *Naja naja siamensis* toxin 3 that the guanidinated toxin was one-half as toxic and all the monoacetyl derivatives were two-thirds as toxic as the native toxin.

Weber & Changeux (1974), Y. Ishikawa & A. Menez (unpublished work) observed that the dissociation constants of *Naja nigricollis* toxin α and erabutoxins to the electroplax acetylcholine receptor of electric rays (*Torpedo marmorata*) are at the order of 0.01 and 1 nM respectively. The affinity of the toxins to the receptor sites can be explained only by the binding at a number of sites. The modifications of the amino groups described in the paper may affect the affinity to various extents and result in the apparent toxicity change of the modified derivatives.

The terminal *N*-acetyl derivatives of erabutoxins are fully active and easily separable. The modification provides a good method for labelling the toxins by radioactivity.

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