THE IDENTIFICATION OF THE SPECIAL GLUTAMIC ACID RESIDUE ESSENTIAL FOR ACTIVITY OF COBRATOXIN*

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SUMMARY: When the γ-carboxyl groups of the seven glutamic acid residues of cobratoxin were modified according to Koshland, six out of seven free carboxyl groups reacted in the absence of guanidine·HCl. The activity of the resulting modified venom was unaltered. When the remaining γ-carboxyl group was modified in the presence of 5 M guanidine·HCl, the resulting venom was devoid of activity. This "buried" carboxyl group was identified as belonging to Glu(21).

In 1965, cobratoxin was purified from the venom of the Formosan cobra (Naja naja atra) by Yang (1). The amino acid sequence and the position of the four disulfide bridges have recently been established (2,3,4).

We have found previously that Trp(29) (5) and Tyr(25) (6) are essential for full toxicity. In extending these studies we have now examined the importance of the free carboxyl groups for toxicity. Carboxyl groups were modified by the carbodiimide procedure developed by Koshland (7,8).

This communication identifies the location of the "buried" group in cobratoxin and shows that it is essential for activity.

MATERIALS

Cobratoxin was prepared from the venom of the Formosan cobra (Naja naja atra) as described previously (1). Cobratoxin was reduced and alkylated according to Crestfield et al. (9). 1-Ethyl-3-dimethylaminopropylcarbodiimide·HCl (EDC·HCl) was contributed from the laboratory of Dr. Koshland, University of

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California. Methyl ^{14}C-glycinate·HCl was prepared from ^{14}C-glycine (New England Nuclear) by esterification in HCl-saturated methanol and diluted with unlabeled reagent obtained from Nakarai Chemicals, Ltd. Trypsin and chymotrypsin came from Worthington Biochemical Corporation. Counting was performed on an Aloka Model PSC-4 gas flow counter. Amino acid analysis was carried out on a Hitachi Model KLA-3B automatic amino acid analyzer. Polyacrylamide gel electrophoresis at pH 2.3 was carried out as described by Ornstein (10). The protein bands in the gel were stained with solutions of Amido Black B.

METHODS AND RESULTS

Modification with EDC·HCl and Methyl Glycinate·HCl:Partially Carboxy-Modified Cobratoxin (Preparation 1). Cobratoxin (60 mg/3 ml) and methyl glycinate·HCl (1.0 M) were dissolved in water. The pH was adjusted to 4.75 and immediately the reaction initiated by the addition of solid EDC·HCl to a concentration of 0.2 M. The pH was maintained at 4.75 by addition of 1.0 N HCl. For completion the mixture was allowed to stand at room temperature for 3 hours. Excess reagents were then removed by passage through a column of Sephadex G-25, equilibrated with 1% AcOH, and the protein fraction was lyophilized. The resulting modified venom showed a single band on disc electrophoresis. The results of amino acid analysis (11) are shown in Table I.

Modification in the Presence of 5 M Guanidine·HCl with EDC·HCl and Methyl Glycinate·HCl:Completely Carboxy-Modified Cobratoxin (Preparation 2). Cobratoxin (10 mg/ml) and methyl glycinate·HCl (1.0 M) were dissolved in 5 M guanidine·HCl and the solution adjusted to pH 4.75 and reacted with solid EDC·HCl as described above. The modified venom was homogeneous by the criteria of disc electrophoresis. Amino acid composition is shown in Table I.

Modification of Preparation 1 in the Presence of 5 M Guanidine·HCl and ^{14}C-Methyl Glycinate·HCl (Preparation 3). Preparation 1 (50 mg/2 ml) and methyl ^{14}C-glycinate·HCl (1.0 M) were dissolved in 5 M guanidine·HCl. The subsequent procedures were performed as described for preparation 2.

Table I

Amino Acid Composition of Cobratoxin Modified at the γ-Carboxyl Groups

Amino Acid	Molar Ratio		
	Native Venom	Modified Venom 5 M Guanidine·HCl absent	present
Lysine	3	2.9	2.9
Histidine	2	1.9	1.9
Arginine	6	6.0	6.0
Tryptophan	1	1.0	1.0
Aspartic Acid	8	8.0	8.3
Threonine	8	8.1	8.0
Serine	4	4.1	4.0
Glutamic Acid	7	7.2	7.4
Proline	2	1.8	1.9
Glycine	7	13.1	13.9
Half-Cystine	8	7.9	8.0
Valine	1	1.1	1.1
Isoleucine	2	2.0	2.0
Leucine	1	0.9	0.9
Tyrosine	2	1.9	1.9
Alanine	0	0	0
Methionine	0	0	0
Phenylalanine	0	0	0

Reduction and Carboxymethylation of ^{14}C-Labeled Cobratoxin. To a solution of ^{14}C-labeled cobratoxin (ca. 30 mg) in 0.2 M Tris-HCl buffer, pH 8.2, containing 8 M urea, was added 0.1 ml of β-mercaptoethanol. After standing for 4 hours a freshly prepared solution of iodoacetic acid (300 mg) in 2.0 N NaOH was added with stirring and the pH of the solution maintained at pH 8.6 with the aid of 2.0 N NaOH. After 30 min the solution was desalted by passage through a column of Sephadex G-25. The ^{14}C-labeled reduced and S-carboxymethylated (RCM-) cobratoxin, which emerged in the void volume, was pooled and lyophilized.

Hydrolysis of ^{14}C-Labeled Cobratoxin by Trypsin. To the solution of the protein (10 mg) in 2 ml of 0.2 M NH_4HCO_3 buffer, pH 8.1, was added 0.2 ml of 1% trypsin solution in the same buffer. The mixture was kept at 37° for 15 hours. To isolate ^{14}C-peptide, the digest was fractionated by high voltage paper electrophoresis in pyridine-acetic acid-water (1:10:289 by volume), pH 3.6, on a strip of Toyo No. 50 at 35 volts per cm for 90 min. Radioactivity of 2-cm strips was measured by a gas flow counter. The radioactive fraction was eluted with 1% AcOH and further purified by descending paper chromatography on Toyo No. 50 paper in the solvent system 1-butanol-pyridine-acetic acid-water (15:10:3:12), pH 5.1, for 16 to 18 hours. The radioactive fraction was eluted with 1% AcOH and concentrated to dryness in vacuo. The "^{14}C-T-peptide" was hydrolyzed with 6.0 N HCl at 105° for 24 hours and subjected to amino acid analysis. The amino acid composition showed that the "^{14}C-T-peptide" was derived from Residues Leu(1) → Lys(26) of cobratoxin.

Hydrolysis of "^{14}C-T-Peptide" by Chymotrypsin. To a solution of "^{14}C-T-peptide" (ca. 3 mg) in 0.25 ml of 0.2 M NH_4HCO_3 buffer, pH 8.1, was added 0.05 ml of 1% chymotrypsin solution in the same buffer. The mixture was kept at 37° for 16 hours. The hydrolysate was worked up as described above. The "^{14}C-C-peptide" gave the following amino acid composition: CM-$Cys_{0.8}$, $Asp_{1.4}$, $Thr_{1.0}$, $Ser_{0.7}$, $Glu_{1.0}$, $Gly_{3.5}$.

N-Terminal Amino Acid of "^{14}C-C-Peptide." "^{14}C-C-Peptide" was subjected to Edman degradation. PTH-Gly was detected as the amino terminal. The remaining peptide, after one cycle, had the following composition: CM-$Cys_{0.8}$, $Asp_{1.2}$, $Thr_{1.0}$, $Ser_{1.0}$, $Glu_{1.0}$, $Gly_{2.5}$. Therefore, "^{14}C-C-peptide" contains the residues 16 to 23: H·Gly-Cys-Ser-Gly-Gly-Glu-Thr-Asn·OH.

Measurement of Activity. The lethality of the venom was measured by intraperitoneal injection of a series of 2-fold diluted venom solution into mice (16-18 g) as described previously (12). Four mice of both sexes were used for each dilution and the LD_{50} was calculated according to the 50% end point method of Reed and Muench (13).

DISCUSSION

There are seven free carboxyl groups in cobratoxin. Among the reagents introduced for the modification of carboxylic acid side chains of proteins the activation by carbodiimide and attachment of nucleophiles (7,8) appears to be sufficiently specific. The number of modified carboxyl group is determined by analysis for incorporated glycine.

In native cobratoxin six of the seven γ-carboxyl groups were modified, while the remaining γ-carboxyl group reacted in the presence of 5 M guanidine·HCl. The venom with six modified carboxyls showed no loss in activity, while modification of all seven γ-carboxyls led to complete loss of toxicity. In order to identify the "buried" carboxyl group, cobratoxin was treated first with non-radioactive methyl glycinate in aqueous solution in the absence of guanidine, and was then incubated with methyl ^{14}C-glycinate and carbodiimide in the presence of 5 M guanidine·HCl. The modified venom containing ^{14}C-glycine was reduced and S-carboxymethylated. The alkylated protein was hydrolyzed by trypsin to permit chromatographic isolation of a ^{14}C-labeled peptide, named "^{14}C-T-peptide. The amino acid analysis revealed that the peptide was derived from residues Leu(1) → Lys(26). The ^{14}C-T-peptide contains two free γ-carboxyl groups in Glu(2) and Glu(21). Thus, to determine which free carboxyl group existed in the buried state, the "^{14}C-T-peptide" was hydrolyzed by chymotrypsin and a radioactive peptide was separated by the combination of high voltage paper electrophoresis and descending paper chromatography. The radioactive peptide obtained was shown to be the peptide of residues Gly(16) to Asn(23). This was confirmed by determination of the N-terminal amino acid residue by Edman degradation.

Accordingly, the "buried" γ-carboxyl group in cobratoxin is Glu(21) in the sequence of Gly-Cys-Ser-Gly-Gly-$\overset{21}{\text{Glu}}$-Thr-Asn-. This carboxyl group is essential for the toxicity of cobratoxin.

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