Communication

γ-Carboxyglutamate in a Neuroactive Toxin*

(Rceived for publication, July 23, 1984)
J. Michael McIntosh, Baldomero M. Olivera, Lourdes J. Cruz, and William R. Gray
From the Department of Biology, University of Utah, Salt Lake City, Utah 84112

The venom of a fish-hunting cone snail (Conus geographus) contains a novel toxin, the "sleeper" peptide, which induces a sleep-like state in mice when injected intracerebrally. We demonstrate that this peptide contains 5 mol of γ-carboxyglutamate (Gla) in 17 amino acids.

The amino acid sequence of the sleeper peptide is Gly-Glu-Gla-Gla-Leu-Gln-Gla-Asn-Gln-Gla-Leu-Ile-Arg-Gla-Lys-Ser-Asn-NH2.

We have described the isolation and properties of a variety of peptide toxins from the venoms of two piscivorous snails, Conus geographus Linn. and C. magus Linn. (1-4). Most recently we reported the purification of a peptide from C. geographus that has a most remarkable biological activity, i.e. intracerebral injection of submicrogram amounts into young mice induces a prolonged sleep-like state. Unlike the other toxins we have found, this peptide lacks disulfide bridges and is extremely acidic rather than basic.

We report here the complete amino acid sequence of the sleeper peptide, conotoxin GV, which is as remarkable as the biological activity; it contains five residues of γ-carboxyglutamate in a total of 17 amino acids and has a net charge of -8 at pH 7.

MATERIALS AND METHODS

Isolation of Peptide—Purification was carried out by Method II of Olivera et al., using gel filtration on Sephadex G-25, and reverse-phase HPLC. Bioassays were also performed as described in that paper.

Amino Acid Analysis—Authentic DL-γ-carboxyglutamate was obtained from Sigma. A quantitative standard was prepared and analyzed on a Beckman 121C amino acid analyzer both before and after hydrolysis to glutamic acid (5.7 N HCl, 18 h, 110 °C).

Peptide samples were hydrolyzed in vacuo in 5.7 N HCl (18 h, 110 °C), or in 2 N KOH (22 h, 110 °C). In the latter case, samples were placed in small polypropylene tubes (Eppendorf) which were sealed in a sealed, evacuated desicator containing a reservoir of 2 N KOH. The volumes were kept below 0.15 ml so that no desalting was necessary prior to amino acid analysis. Samples were neutralized with 6 N HCl and diluted with the standard citrate buffer (1.5 ml). Amino-terminal Analysis—The dansylation procedure of Gray (5) was used.

Peptide Sequencing—Analysis of the intact peptide were carried out by sequential Edman degradation, initially by Tarr's manual method (6) and subsequently in a Beckman 8900D spinning cup sequenator, using 0.1 M Quadrol buffer and Polybrene carrier (7). Peptide fragments were analyzed by the manual method. PTH-derivatives were identified by HPLC on a Hewlett-Packard 1048B instrument, using a gradient of acetonitrile in 0.05 M sodium acetate, pH 4.5; the phase used was Zorbax ODS, 0.46 × 25 cm, 10 μm particle size, not end-capped.

Enzymatic Digestion—Samples of intact peptide (10-150 nmol) were dried and digested with the following enzymes in the volumes indicated: (a) staphylococcal protease, 20 μl of a 2 mg/ml solution in 0.1 M ammonium acetate, pH 4.0, containing 2 mM EDTA (37 °C, 18 h). (b) trypsin, 20 μl of a 2 mg/ml solution in 0.2 M ammonium acetate, pH 8.5, containing 5 mM CaCl2 (18 h at 37 °C); (c) pepsin, 30 μl of a 0.4 mg/ml solution in 5% (v/v) formic acid (25 °C, 1 h).

Digests were fractionated by HPLC on a 4.6 × 25-cm column of Vydac C-18, 5 μm particle size, not end-capped. A gradient of acetonitrile in 0.1% trifluoroacetic acid (8) was used, and the effluent was monitored at 205 nm. Peaks were collected manually in polypropylene tubes, and were dried under vacuum in a Speed-Vac (Savant Instruments).

High-voltage Paper Electrophoresis—Samples of peptides were applied to Whatman 3MM paper, and electrophoresis was carried out in 0.8 M pyridine acetate, pH 6.5 (2 h, 40 V/cm). Dns-OH and Dns-NH2 were included as internal mobility standards. After drying, the paper was stained with ninhydrin.

Decarboxylation—After prolonged storage of the toxin at −20 °C in dilute solution in 0.1% trifluoroacetic acid there is a progressive change in HPLC profile, with the appearance of many peaks of lower mobility. Some of these retain biological activity. In order to find out whether the Gla residues are essential for activity, we decarboxylated the peptide, following the method of Poser and Price (9). Approximately 40 nmol of peptide was dissolved in 0.05 M HCl and dried under vacuum in a hydrolysis tube. The tube was sealed under vacuum and heated for 3 h at 110 °C. It was then cooled and opened, and the peptide was dissolved in 200 μl of water. Samples were taken for amino acid analysis (base hydrolysis), HPLC comparison, and bioassay.

RESULTS

Staphylococcal Protease Digestion—Despite the peptide's highly acidic nature and high content of Gla, no fragmentation was evident after 18-h digestion at 37 °C. Almost all of the material eluted at the original position, with the remainder being present as a series of minor satellite peaks of lower mobility (data not shown). Similar satellite bands are produced by storage in dilute acid.

Trypsin Digestion—In our previous report, we digested for 4 h and obtained two components, one being the dipeptide Ser-Asn-X. In the present work, digestion was carried out for 18 h, and three fragments were obtained, as expected from the amino acid composition. Two of these were not retarded on HPLC and eluted together, while the third eluted somewhat later than undigested peptide. Amino acid analyses of material from the two HPLC peaks were carried out after acid hydrolysis (Table I).

Remaining material from peak A was subjected to electrophoresis at pH 6.5, with markers of Glu, Lys, Trp-Glu, Glu-NH2, Lys-Glu and Phe-Dap-Glu. One peak was eluted with Gln-Glu, the other with Lys-Glu, and the two were not retarded on HPLC and eluted together, while the third eluted somewhat later than undigested peptide. Amino acid analyses of material from the two HPLC peaks were carried out after acid hydrolysis (Table I).

4 The abbreviations used are: PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography; Dns, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Gla, γ-carboxyglutamate.
Lys, and Arg-Ala. Two ninhydrin-positive spots were found. One corresponded to the previously characterized carboxyl-terminal peptide, Ser-Asn-X, which stained yellow with ninhydrin and migrated close to Arg-Ala.² No free Glu or Lys were present, so the second component was presumed to be Glx-Lys. However, it migrated close to Trp-Glu, indicating a net charge of −1. Both Gln-Lys and pyroglutamyl-Lys carry a charge of ±1, while all isomers of Glu-Lys are neutral. A negative charge can arise only by some other modification. Both α- and ε-amino groups were free as shown by dansylation (X-

Standardization of Gla—Standard solutions of Gla were subjected to amino acid analysis, as were acid and base hydrolyzates. As had been found by other workers (9, 11), the ninhydrin color yield of Gla was low, only 38% of that of Glu. Quantitative recovery of Glu was obtained after acid hydrolysis, while Gla was recovered quantitatively after base hydrolysis.

Base Hydrolysis of Conotoxin GV—Amino acid analysis of a base hydrolyzate is given in Table I. The glutamic acid peak was greatly reduced, and a large peak was obtained that co-eluted with authentic Gla. Based on the color yield determined for Gla, a total of 5 mol/mol was found, which corresponds to the difference in Glu content between acid and base analyses (8 versus 3). Another way of expressing this equivalence is to calculate the relative color yields of the Gla produced in base and the extra Glu produced in acid.

\[ R = \frac{\text{peak area (Glu_{base})} - \text{peak area (Glu_{acid})}}{\text{peak area (Gla_{base})}} \]

With authentic Gla this value was 2.6, and it is clear from the values of R given in Table I that there is full equivalence between Gla destroyed and Glu produced.

Pepsin Digestion—A definitive set of fragments was obtained after purification of the pepsin digest by reverse-phase HPLC, with recoveries exceeding 90% at all parts of the molecule. Amino acid analyses after both base and acid hydrolysis are given in Table I. In addition to the fragments shown, fragment BC (5–14) was obtained in 5% yield.

Sequence Analysis—Manual degradation of whole toxin was carried out by Tarr’s procedure (6) before we were aware of Gla’s presence. No identifiable PTH-derivative was obtained at steps 3, 4, and 7, but otherwise the terminal sequence was shown to be Gly-Glu-X-Leu-Gln-Glu-Xn in Gln-Glu-X-Leu-Gln-Glu-Xn. Peptic fragments B and C were also analyzed by the manual method. Fragment B gave Asn-Glu-X-Leu, while C gave Ile-X-X-Lys-Ser-Asn.

Analysis of whole toxin by automated degradation gave completely concordant results (Table II). Positive identification of PTH-Arg was obtained at position 13. However, we obtained no trace of a derivative that might correspond to PTH-Gla in any of five steps that were otherwise unaccounted for; both organic and aqueous phases of the conversion medium were analyzed. Since the Polybrene carrier used is a positively charged polymer, it is likely that the anilinothiazolinone derivative of Gla does not extract from the sequenator cup. Similar negative results are consistently reported (10, 12). We have assigned Gla to the five positions (positions 3, 4, 7, 10, and 14) that gave no identifiable PTH-derivative on sequencing of whole peptide and fragments (Fig. 1). These assignments are in full accord with the distribution of Gla among fragments, as deduced from amino acid analysis (Table I).

Included in Table II is a report of yields of PTH-Glu at every step in the sequenator run. It is clear that the carboxylation is specific, with Glu2 being unaffected. Higher-than-background amounts of PTH-Glu were found at steps 3, 6, 7, 9, and 10, but the amounts are close to those expected from tailover and deamidation of PTH-Gln. Very little decarboxylation occurred during degradation.

Carboxyl-terminal Blocking Group—Our previous work had indicated that the carboxyl-terminal tryptic peptide was blocked, probably as Ser-Asn-NH₂. We obtained more direct confirmation of this as follows. Purified pepsin C fragment (residues 12–17) was digested with trypsin to produce a mix-
Decarboxylation—Analysis by HPLC of peptide treated according to Poser and Price (9) showed that all of the original material had been changed. Greater than 70% of the product ran as a single sharp peak, with several minor peaks accounting for the rest (Fig. 2). Base hydrolysis of crude decarboxylation product indicated a Gla content of approximately 0.35 nmol/mol instead of 5. Analysis of an acid hydrolysate of material from the major HPLC peak showed that it contained all the expected amino acids and was not a product of chain fragmentation. A mouse injected with 5 nmol of the crude decarboxylation product was unaffected, whereas 0.2 nmol of untreated toxin was sufficient to produce obvious symptoms.

**DISCUSSION**

The discovery of γ-carboxyglutamate (Gla) in a neuroactive peptide from a snail came as a great surprise. Initially suspected from an anomalous peptide mobility, this amino acid derivative was shown to be present in base hydrolysates of the intact peptide and of its pepsin fragments. The putative Gla, co-chromatographed with authentic Gla during amino acid analysis, was converted quantitatively to glutamic acid upon hydrolysis, gave the very low ninhydrin color yield that is characteristic of Gla, and was decarboxylated by heating of the dry protonated peptide. Decarboxylation resulted in loss of biological activity, as did trypsin digestion, indicating that the peptide is responsible for the observed sleeper symptoms and does not merely chromatograph with some other component.

During sequence analysis no identifiable PTH-derivative was obtained from Gla residues, but their positions could be inferred unambiguously by difference, and by amino acid analysis of fragments. Carboxylation occurs at only five of the six Gla residues, and not partially at all of them, as is clear from the sequenator results (Table II) and the almost quantitative recovery of discrete Gla-containing peptides (Ta-
ble I and Fig. 1). Overwhelmingly, the peptide exists in this form in the venom, and it is not merely one of several equivalent peptides. Typically, Gla-containing proteins are modified on all Glu residues close to the amino terminus (10), with an exception being human osteocalcin (12). Failure to carboxylate Glu2 of conotoxin GV might be attributed to its being very close to the amino terminus or to its being one of a series of three.

Apart from one report of its occurrence in bacterial and plant ribosomes (13), Gla has hitherto been found only in vertebrates. The carboxylation reaction is vitamin K-dependent, and the modification was discovered originally through analysis of the differences between normal prothrombin and the defective one produced during vitamin K deficiency (10). Gla occurs in several other proteins of the blood-clotting system and in some quite unrelated proteins involved in calcification (e.g. osteocalcin of bone (14), and a Ca²⁺-binding protein of chicken eggshell (15)). In all these instances Gla appears to function as a calcium ligand, the active form of the protein being the complexed one.

As yet we know nothing of the toxin’s mode of action, either naturally or in the mouse brain. It is inactivated by γ-decarboxylation, so presumably calcium-binding is important. The decarboxylation conditions have been shown to be specific for Gla (9), and it is most unlikely that the major inactive peak in Fig. 2 arises from deamidation. An effective dose in a 6-g mouse is approximately 0.2 nmol, perhaps representing an intracerebral concentration of 20 μM. This is well below the level of free Ca²⁺, so it seems more likely that the toxin acts not merely by sequestering needed Ca²⁺, but by forming a noxious complex directed towards a particular receptor. We do not suggest that receptors in mouse brain are the normal biological “target” of the peptide. In our original description of the peptide’s isolation, we speculated that its natural target might be peripheral synapses in predators such as crabs, and that its effects on the vertebrate central nervous system may reflect a purely incidental sharing of a common transmitter such as glutamate.

C. geographus venom has proven to be an extraordinary source of biologically active peptides, affecting various parts of the vertebrate neuromuscular and central nervous systems. A fascinating aspect of their chemistry is the degree to which they display post-translational modifications of various kinds. All toxins that have been adequately characterized, including members of four different series, are amidated at their carboxyl-terminal ends. Most have disulfide bridges, conotoxin GV being the only known exception. Two series contain hydroxyproline, and now we have shown that conotoxin GV contains γ-carboxyglutamate. It is perhaps of special significance that hydroxyproline and Gla are associated in vertebrates with calcification of bone. Molluscs, especially the Conus snails, have carried the art of building and decorating calcified tissues to extraordinary refinement. It may be that their system based on calcite has some fundamental biochemical links with the hydroxyapatite system exploited by the vertebrates.

Acknowledgments—Drs. Victoria de Santos and F. A. Luque purified some sleeper toxin preparations.

REFERENCES