Biochemistry

 Volume 23, Number 22 October 23, 1984

Accelerated Publications

Purification and Sequence of a Presynaptic Peptide Toxin from *Conus geographus* Venom†

Baldomero M. Olivera,* J. Michael McIntosh,‡ Lourdes J. Cruz, F. A. Luque,§ and William R. Gray

ABSTRACT: A novel toxin, ω-conotoxin (ω-CgTX), from the venom of the fish-eating marine mollusc *Conus geographus* has been purified and biochemically characterized. Recently, this ω-conotoxin has been shown to inhibit the voltage-activated entry of Ca^{2+}, thus providing a potentially powerful probe for exploring the vertebrate presynaptic terminal [Kerr, L. M., & Yoshikami, D. (1984) *Nature (London) 308*, 282–284]. The toxin is a basic 27 amino acid peptide amide with three disulfide bridges. An unusual feature is a remarkable preponderance of hydroxylated amino acids. The sequence of ω-CgTx GVIA is Cys-Lys-Ser-Hyp-Gly[5]-Ser-Ser-Cys-Ser-Hyp[10]-Thr-Ser-Tyr-Asn-Cys[15]-Cys-Arg-Ser-Cys-Asn[20]-Hyp-Tyr-Thr-Lys-Arg[25]-Cys-Tyr-NH_2.

In the last few years, there have been significant advances in understanding some key molecules in the neuromuscular system. Progress in characterizing the acetylcholine receptor (Conti-Tronconi & Raftery, 1982) and the voltage-sensitive sodium channel (Catterall, 1984) has been striking. These advances have been greatly aided by the availability of toxins with high affinity for the membrane-bound proteins—α-bungarotoxin for the acetylcholine receptor and tetrodotoxin and scorpion toxins for the sodium channel. Little success has been achieved with the presynaptic membrane, partly due to the lack of toxins specific for critical targets (Kelly et al., 1979). In this paper we report the purification and amino acid sequence of a peptide that irreversibly blocks the presynaptic terminus.

The toxin is from *Conus geographus*, a marine snail that captures fish by injecting a potent venom through a disposable harpoon-like tooth. We have been systematically isolating and characterizing the toxins from this venom and have described the structures and biological activities of several different types (Cruz et al., 1978; Clark et al., 1981; Gray et al., 1981; Stone & Gray, 1982; L. J. Cruz, B. M. Olivera, L. Kerr, D. Yoshikami, R. D. Zeikus, and W. R. Gray, unpublished results; B. M. Olivera, J. M. McIntosh, C. Clark, D. Middlemas, W. R. Gray, and L. J. Cruz, unpublished results). During routine bioassays of chromatographic fractions, we found some that caused a persistent shaking in mice that had been injected intracerebrally. Using this as an assay, we have been able to purify an active peptide to homogeneity. Kerr and Yoshikami showed that this homogeneous toxin preparation inhibits transmission at the frog neuromuscular junction. The action is certainly presynaptic, and the physiological evidence suggests that the toxin prevents the voltage-activated entry of calcium, which is needed to trigger acetylcholine release (Kerr & Yoshikami, 1984). The toxin, which we designate ω-conotoxin, is a 27 amino acid peptide amide containing three disulfide bridges. It is moderately basic in character, but its most striking feature is an extraordinary number of hydroxylated residues, including three of hydroxyproline. We report here the purification procedure and complete amino acid sequence.

Experimental Procedures

Assay for "Shaker" Activity. Crude venom is lethal upon intracerebral (ic) injection, so the assay can only be carried out on partially purified material, as has been described for a "sleeper" peptide (B. M. Olivera et al., unpublished results). Samples dissolved in normal saline are injected ic into young mice, which are then observed for unusual symptoms. With fractions containing the shaker peptide mice develop a characteristic tremor a few minutes after injection. The onset and persistence of the tremor is dose dependent; at high doses, symptoms may be observed for over 3 days. Normally, how-

† From the Department of Biology, University of Utah, Salt Lake City, Utah 84112. *Received July 12, 1984.* Supported by USPHS Grant GM22737.

‡ Present address: UCLA Medical School, University of California, Los Angeles, CA.

§ Present address: Department of Neurology, Washington University Medical School, St. Louis, MO.

0006-2960/84/0423-5087$01.50/0

ever, the mice are anesthetized and killed as soon as the assay result is clear. Although the assay is basically qualitative, a rough estimate of quantity can be obtained by serial 2-fold dilution until activity is no longer discernible. At low doses the shaker symptoms could be accentuated by briefly lifting the mice by their tails. To minimize the number of mice used, we did no end-point quantitation during the course of purification but measured specific activity only of highly purified materials.

Purification of ω-Conotoxins. Lyophilized venom was from snails collected around the island of Marinduque, Philippines, and was extracted as described previously (Cruz et al., 1978). In a typical experiment, 87 mg of protein was fractionated on Sephadex G-25 (2.7 × 160 cm, eluted with 1.1% acetic acid). Four major peaks of peptide material were obtained, detected by manual ninhydrin assay after acid hydrolysis of small samples. Shaker activity was found in the third peak. Material from this peak was then fractionated by reversed-phase HPLC[1] using a Supelco LC18 DB semipreparative column (1 × 25 cm, 5-μm particle size, not end capped). Peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 3 mL/min (Rivier, 1978). The effluent was monitored at 214 nm, and peaks were collected manually in polypropylene tubes. Fractions containing shaker activity were rechromatographed on a Vydac C18 column (4.6 × 250 mm, 5 μm, not end capped) under the same conditions, but with a shallower gradient and a flow rate of 1.5 mL/min.

For structural analysis samples of the major purified peptide were reduced with dithiothreitol and alkylated with iodoacetamide. Carboxamidomethylated peptide was purified by HPLC using the Vydac system. This material was used both for digestion with clostripain and for direct sequencer analysis.

Amino acid analysis was carried out on a Beckman Model 121 analyzer, after hydrolysis with redistilled 6 N HCl for 20 h at 105 °C.

Protease Digestion. Clostripain (Worthington Biochemicals) was activated by preincubation (5 mg/mL) in a solution containing 1 mM calcium acetate and 2.5 mM dithiothreitol for 2–4 h at 25 °C (Mitchell, 1977). Approximately 50 nmol of carboxamidomethylated peptide was digested in 50 μL of 0.025 M sodium phosphate, pH 7.5, containing 1 mg/mL activated clostripain, 0.2 mM calcium acetate, and 3 mM dithiothreitol. The mixture was incubated for 2.5 h at 37 °C; fragments were separated by HPLC using the system described above, but with a gradient of 0–40% acetonitrile.

Trypsin inactivation was carried out as described previously (Cruz et al., 1978).

Sequence analysis of peptides was carried out in a Beckman Model 890D sequencer (Edman & Begg, 1967) using a 0.1 M Quadrol program. Polybrene carrier (Tarr et al., 1978) was precycled by running four steps in the presence of 100 nmol of Ser-Gly. PTH-amino acids were analyzed by HPLC as described previously (Gray et al., 1981).

High-voltage electrophoresis was carried out at pH 6.5, for 2 h at 40 V/cm, with mobility markers of DNS-OH and DNS-NH_2. Peptides were detected by staining with ninhydrin and with the Pauly stain for His and Tyr.

Mass Spectroscopy. The molecular weight of the unmodified peptide (3 nmol) was determined by fast atom bombardment mass spectroscopy (Morris et al., 1981).

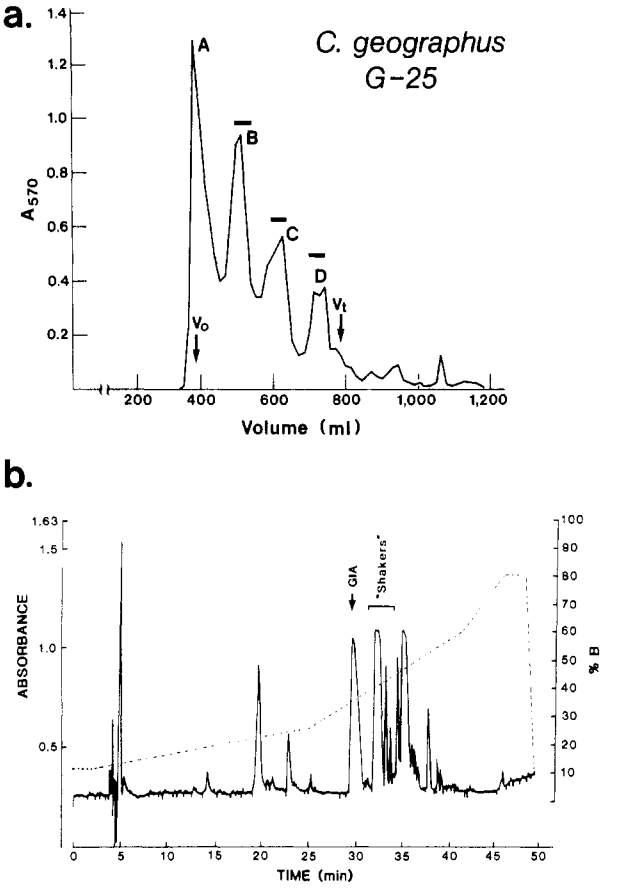

FIGURE 1: Purification of shaker peptide from *C. geographus* venom. (a) Chromatography on Sephadex G-25. Fractions of 5 mL were collected, and 5-μL samples were assayed by ninhydrin. (b) Rechromatography of material from peak C on a Supelco LC18 column, eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Buffer B consisted of 0.1% trifluoroacetic acid in 60% (v/v) acetonitrile/water. Major toxins located in the peaks from Sephadex G-25 were the following: A, conotoxin GIV (Clark et al., 1981); B, μ-conotoxin GIII (Stone & Gray, 1982; L. J. Cruz et al., unpublished results) and conotoxin GV (sleeper peptide; B. M. Olivera et al., unpublished results); C, α-conotoxin GIA (Gray et al., 1981) and ω-conotoxin GVI (this work); D, α-conotoxins GI and GII (Cruz et al., 1978; Gray et al., 1981).

Results

Purification of Toxin. The assay and purification of ω-conotoxin were carried out as detailed under Experimental Procedures. Four major peptide peaks were obtained from the Sephadex G-25 column, with shaker activity being found in the third (Figure 1a). Locations of other venom components in the Sephadex G-25 effluent are indicated in the legend to Figure 1. Fractionation by HPLC of material from peak C resolved several active components. The major active peak in Figure 1b is broad because of high loadings. Rechromatography showed that material from either edge of the peak was the same—a single very sharp peak accounting for about 95% of the absorbance, plus two or three minor shoulders and peaks. No other single chromatographic component is present at greater than 2%. Biological activity is found predominantly in the major sharp peak and is not preferentially with the minor components. The levels of contaminant amino acids (Table I) in the major peak are consistent with purity of approximately 95%. All structural work reported below, as well as the physiological experiments described by Kerr & Yoshikami (1984), were carried out on material from the main peak (see Figure 1b). With the purified peptide, shaker activity was just

[1] Abbreviations: Hyp, hydroxyproline; CAMCys, *S*-(carboxamidomethyl)cysteine; CMCys, *S*-(carboxymethyl)cysteine; DNS-NH_2, 5-(dimethylamino)-1-naphthalenesulfonamide; DNS-OH, 5-(dimethylamino)-1-naphthalenesulfonic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin. Standard abbreviations are used for amino acids.

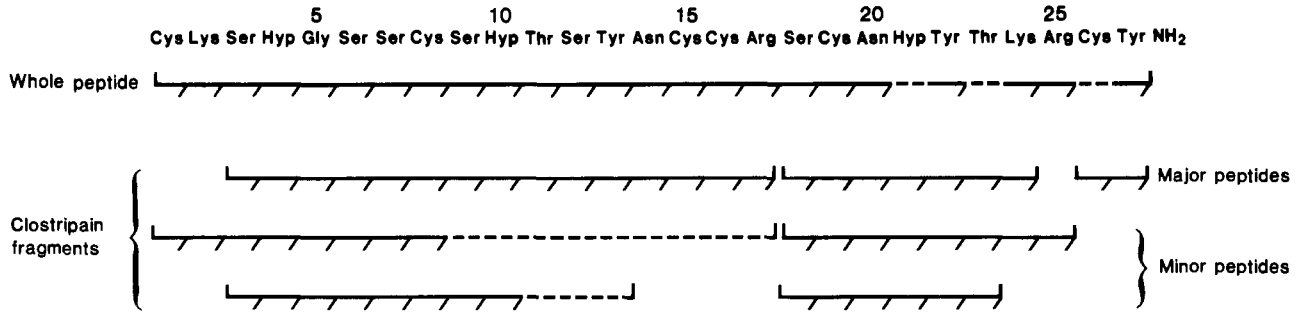

FIGURE 2: Amino acid sequence of ω-conotoxin GVIA. Reduced and carboxamidomethylated toxin was (a) subjected to direct sequencer analysis or (b) digested with clostripain, the fragments being separated by HPLC and analyzed on the sequencer. Amino acids marked with a barbed underline were identified unequivocally from sequencer analysis of the peptide; those marked with a dotted line are inferred from amino acid composition. Three different forms of the C-terminal dipeptide were obtained (see text). Peptides corresponding to residues 3–17, 3–13, 14–17, 18–22, 18–24, and 26–27 were also obtained by trypsin digestion but were not sequenced.

Table I: Amino Acid Analysis of ω-Conotoxin GVIA

	nmol	mol/mol		nmol	mol/mol
Lys	23.8	2.02 (2)	Gly	14.6	1.24 (1)
His		(0)	Ala	1.5	0.12 (0)
Arg	26.8	2.27 (2)	Val		(0)
Asx	23.2	1.97 (2)	Met	0.9	0.08 (0)
Thr	24.7	2.09 (2)	Ile		(0)
Ser[a]	62.8	5.41 (6)	Leu	0.7	0.06 (0)
Glx		(0)	Tyr	36.2	3.07 (3)
Pro	tr	(0)	Hyp	37.7	3.20 (3)
$^1/_2$-Cys[b]	68.9	5.85 (6)	total		27

[a] Corrected for hydrolysis losses: Thr, ×1.05; Ser, ×1.12. [b] Analyzed as cystine, uncorrected for losses. Values in parentheses indicate the number of residues found by sequence analysis (Figure 2).

discernible at a dose of 1 nmol (3 μg)/kg of body weight ic.

Characterization of the Major Active Component. The elution behavior on gel filtration suggested a molecular size of approximately M_r 2000–3000. Amino acid analysis gave the composition shown in Table I, which is noteworthy for the presence of hydroxyproline. This amino acid is also present in the μ-conotoxins (Stone & Gray, 1982; Sato et al., 1983; L. J. Cruz et al., unpublished results) and is the common *trans*-4-hydroxy isomer as judged by chromatography of the free amino acid and its PTH derivative. None of the other isomers was detected in ω-conotoxin. Included in Table I is the composition deduced from amino acid sequence studies, which is consistent with the direct analysis.

Sequencer analysis of the intact carboxamidomethylated peptide (5 nmol) was carried through 30 cycles and gave unequivocal identification of most residues up to position 27 (Figure 2). A series of fragments were isolated from the clostripain digest. All those present in significant amounts were analyzed on the sequencer, with results confirming and completing the sequence assignments (Figure 2). It is clear that clostripain was generating splits at Lys as well as Arg, although with lower efficiency.

The Cys residues appear to be present in the form of disulfides, as judged by the peptide's failure to give a positive reaction for sulfhydryl (<0.5 mol/mol). As with all other conotoxins that have been sequenced, the carboxyl terminus is amidated. Three forms of the dipeptide Cys-Tyr were isolated from the clostripain digest. The major component, which was also obtained from a tryptic digest, carried a net positive charge of 0.5 at pH 6.5, and therefore its α-carboxyl is blocked. It gave the expected CAMCys-Tyr on sequencing. Amino-terminal amides such as Asn have a low pK_a for the α-amino group, and this may explain the lower than expected charge. One of the minor components was present in too small an amount (15%) to be detected after electrophoresis but also gave CAMCys-Tyr on sequencing; we presume this is peptide that has been deamidated at the α-carboxyl. The other minor component gave CMCys-Tyr as its sequence, clearly being derived by deamidation of the carboxamidomethyl side chain. It was neutral at pH 6.5, again indicating that the α-carboxyl was blocked.

The identity of the blocking group was investigated by fast atom bombardment mass spectroscopy of unmodified toxin. This gave a strong molecular ion (MH^+ = 3035) and a doubly protonated ion ($MH^{2+}/2$ = 1518). This corresponds exactly to the expected molecular weight of the peptide amide having the sequence given in Figure 2, with all Cys residues present as disulfides. No other blocking group is compatible with this mass. The complete primary structure is thus accounted for by the sequence shown.

Discussion

We have purified to homogeneity and characterized a novel toxic peptide that causes shaker symptoms in mice and a presynaptic block at the frog neuromuscular junction. Several lines of evidence support the conclusion that the purified peptide is in fact the toxin. The homogeneous peptide and shaker activity were coincident in at least two different HPLC systems; in addition, treatment of the peptide with trypsin abolished shaker activity (data not shown). The same peptide was purified to homogeneity from two batches of venom by following the shaker activity; both homogeneous toxin preparations irreversibly blocked Ca^{2+} entry into the nerve terminal during the presynaptic action potential as previously described (Kerr & Yoshikami, 1984). The minor peaks of active material in Figure 1b have been partially characterized and are clearly related to the peptide whose sequence we have determined (data not shown). One of them appears likely to be the same, except for having Gly instead of amide at the carboxyl terminus. To maintain that biological activity is resident in a minor contaminant of the main peak, we should have to assert that similar active contaminants are present in the other minor peaks. Similarly, it is unlikely that the activity is due entirely to a small amount of deamidated peptide. We suspect that loss of 15% of the carboxyl-terminal amide occurred during clostripain digestion or the subsequent workup of fragments; some deamidation of the carboxamidomethyl side chain was also found. Typically, we are able to resolve amidated peptides from their carboxyl counterparts, and there was no evidence for the presence of a chromatographic contaminant at the level of 15%.

The peptide defines a new class of toxins from *Conus* venoms in both the chemical and physiological sense. Despite a general chemical similarity with some other types of conotoxins, there is no significant homology in their amino acid sequences. To distinguish the three classes of toxins known to act on the

vertebrate neuromuscular system, we use the Greek prefixes α for those that act on the acetylcholine receptor (by analogy with the well-studied snake toxins), μ for those that act on the muscle membrane sodium channels, and ω for these shaker toxins. In the expectation of characterizing other variants, we designate the peptide described here as ω-conotoxin GVIA.

As with most of the other toxins from *Conus* venoms, the peptide is basic and highly cross-linked by disulfide bridges. In 27 amino acids there are six half-cystines, four positively charged residues, and no acidic groups. Even the α-carboxyl is amidated, which is another feature shared with the other conotoxins. The molecule carries a net charge of 5+.

Perhaps the most striking aspect of the structure is the enormous number of side chains with hydroxyls or other hydrogen-bonding groups. Apart from the six Cys residues that are involved in disulfide bridges, the only amino acid lacking such a function is the solitary Gly. Three residues of hydroxyproline are present; as with the μ-conotoxins (Sato et al., 1983; L. J. Cruz et al., unpublished results) these occur in sequences that differ from those of typical collagen hydroxylation sites.

The physiological studies of Kerr & Yoshikami (1984) indicate that the ω-conotoxin GVIA acts presynaptically, preventing the voltage-activated release of acetylcholine. Release is normally mediated by an influx of calcium, and the evidence is consistent with the toxin's producing an irreversible block of this process. However, much work remains to be done at both the physiological and biochemical level to identify precisely the molecular target of the toxin. The toxin irreversibly blocks the neuromuscular junction, spinal cord, and sympathetic ganglion from frog and the Ca^{2+} component of the action potential in dorsal root ganglion cells in culture from embryonic chick. Clearly, it has some effect on the mammalian central nervous system, but it is without activity on the neuromuscular synapse of mouse. Overall, it appears that the toxin's target is a molecule that is widely present in presynaptic terminals; one exciting possibility is that the toxin irreversibly blocks voltage-activated Ca^{2+} channels.

Acknowledgments

We thank Drs. Doju Yoshikami and Lynne Kerr of the Biology Department, University of Utah, for helpful discussions on their physiological studies and Drs. Howard Morris and Graham Taylor of Imperial College, London, England, for carrying out fast atom bombardment mass spectroscopy.

Registry No. ω-CgTX GVIA, 92078-76-7.

References

Catterall, W. A. (1984) *Science (Washington, D.C.) 223*, 653–661.

Clark, C., Olivera, B. M., & Cruz, L. J. (1981) *Toxicon 19*, 691–699.

Conti-Tronconi, B. M., & Raftery, M. A. (1982) *Annu. Rev. Biochem. 51*, 491–530.

Cruz, L. J., Gray, W. R., & Olivera, B. M. (1978) *Arch. Biochem. Biophys. 190*, 539–548.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.

Gray, W. R., Luque, A. F., Olivera, B. M., Barrett, J., & Cruz, L. J. (1981) *J. Biol. Chem. 256*, 4734–4740.

Kelly, R. B., Deutsch, J. W., Carlson, S. S., & Wagner, J. A. (1979) *Annu. Rev. Neurosci. 2*, 399–446.

Kerr, L. M., & Yoshikami, D. (1984) *Nature (London) 308*, 282–284.

Mitchell, W. M. (1977) *Methods Enzymol. 17*, 165–170.

Morris, H. R., Panico, M., Barber, M., Bordoli, R. S., Sedgwick, R. D., & Tyler, A. (1981) *Biochem. Biophys. Res. Commun. 101*, 623–631.

Rivier, J. E. (1978) *J. Liq. Chromatogr. 1*, 343–366.

Sato, S., Nakamura, H., Ohizumi, Y., Kobayashi, J., & Yoshimasa, H. (1983) *FEBS Lett. 155*, 277–280.

Stone, B. L., & Gray, W. R. (1982) *Arch. Biochem. Biophys. 216*, 765–767.

Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622–627.

Amino-Terminal Arm of the λ Repressor: A ^{1}H NMR Study†

M. A. Weiss, R. T. Sauer,‡ D. J. Patel,§ and M. Karplus*

ABSTRACT: The N-terminal arm of the λ repressor is shown to be flexible in solution by one- and two-dimensional ^{1}H NMR methods. In particular, the relaxation of Thr-2 is largely independent of macromolecular tumbling. The conformation of the operator-binding domain is not affected by the removal of the first three residues nor by a point mutation, Lys-4 → Gln. These results support a proposed model of the λ repressor–operator complex in which the N-terminal arm of the repressor is assumed to be flexible and to wrap around the operator double helix.

The λ repressor consists of two structurally and functionally distinct domains that can be separated by proteolysis (Pabo et al., 1979; Sauer et al., 1979). The N-terminal domain mediates sequence-specific DNA recognition, whereas the C-terminal domain contains strong dimer (and higher order) contacts. The crystal structure of the N-terminal domain, as a fragment of 92 residues, has been determined (Pabo & Lewis, 1982). It consists of five α-helices; the first four fold into a globular structure and the fifth forms a dimer contact. Electron density was not observed for Ser_1-Thr_2-Lys_3, indicating that the first three residues are disordered in the crystal. Fragments lacking these residues bind to operator DNA 100-fold less tightly and give an altered pattern of chemical

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received June 13, 1984.* This work was supported by grants from the National Institutes of Health to M.K. (GM-30804) and R.T.S. (AI-16892). M.A.W. is a predoctoral fellow of the Medical Scientist Training Program at Harvard Medical School.

‡ Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

§ Present address: Division of Polymer Science, AT&T Bell, Murray Hill, NJ 07974.

0006-2960/84/0423-5090$01.50/0