Conotoxin GI: Disulfide Bridges, Synthesis, and Preparation of Iodinated Derivatives†

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ABSTRACT: The 13 amino acid toxic peptide from the marine snail *Conus geographus*, conotoxin GI, blocks the acetylcholine receptor at the neuromuscular junction. In this report, we describe a method for analyzing disulfide bonding in nanomole amounts of small cystine-rich peptides. The procedure involves partial reduction and a double-label alkylation of cysteine residues. Using this method, we show that the natural conotoxin GI has a (2–7, 3–13) disulfide configuration. The structure of conotoxin GI has been confirmed by chemical synthesis. The preparation and purification of molecularly homogeneous, iodinated derivatives of this toxin are also described. All derivatives, including the [diiodohistidine,diiodotyrosine]conotoxin GI, retained at least half of the biological activity of unmodified toxin. Since the tetraiodinated toxin, which is greater than 25% by weight iodine, retains considerable toxicity, unmodified histidine and tyrosine residues in conotoxin GI are not crucial for biological activity.

Molecules that possess extraordinarily high toxicity have long been a prime source of information about fundamental biochemical systems in higher organisms. From small molecules such as cyanide, malonate, and fluoroacetate, attention has swung to larger molecules that can be many orders of magnitude more potent on a molar basis. Foremost among these have been the alkaloids and small proteins from venomous animals. The sites of action of such toxins are frequently proteins that control the movement of ions through cell membranes and that are present in only minute quantities.

The best studied of these membrane proteins is the acetylcholine receptor of vertebrate skeletal muscle, particularly as it occurs in the electric organs of certain fish [for a recent review, see Conti-Tronconi & Raftery (1982)]. Much of the progress made in analyzing this system has come from the use of probes such as *d*-tubocurarine and α-bungarotoxin. We recently described a new series of such toxins, small peptides (13–15 amino acids) from the venoms of fish-eating snails of the genus *Conus* (Gray et al., 1981a,b; McIntosh et al., 1982). Their small size and modular construction make these peptides ideal for synthesis of a range of derivatives that should serve as versatile probes of the acetylcholine receptor. A prerequisite to this is a more complete understanding of their disulfide chemistry and a means of preparing radioactive derivatives.

This report describes experiments establishing the disulfide bond configuration of conotoxin GI from *Conus geographus*, the chemical synthesis of the peptide, and the preparation of several homogeneous, biologically active iodinated derivatives.

Materials and Methods

Materials. Conotoxin GI was purified from the venom of *C. geographus*, as described previously (Cruz et al., 1978). All radioactive isotopes were purchased from Amersham-Searle. Unless otherwise specified, enzymes were purchased from Sigma. Iodogen was bought from Pierce Chemical Co. Iodinated histidine and tyrosine standards were from Calbiochem and Cyclo Chemicals, respectively.

Methods. (*1*) *Bioassays* were carried out by intraperitoneal injection into mice, as described previously (Cruz et al., 1978).

(*2*) *High-Voltage Electrophoresis* (*HVPE*). HVPE was performed on a cooled flat-plate apparatus. In all analyses, Whatman 3 MM paper was used, and DNS-OH[1] and DNS-NH_2 were included in test samples as fluorescent markers for measuring peptide mobilities. From extensive records of known peptides in this system, we are able to predict the migration of most small peptides to within 1 cm using Offord's approach (Offord, 1966, 1977). Buffers used included 7% formic acid (pH 1.7) and 0.3 M ammonium acetate (pH 5.4).

(*3*) *High-Performance Liquid Chromatography* (*HPLC*). HPLC was carried out on a Hewlett-Packard Model 1084B, with a variable-wavelength detector. The column contained a reversed-phase VYDAC C18 support, 0.46 × 25 cm, 5-μm particle size, not end-capped. Elution of peptides was with a gradient of acetonitrile in 0.1% trifluoroacetic acid, the effluent being monitored at either 205 or 280 nm (Rivier, 1978), and peaks were collected manually in polypropylene tubes.

(*4*) *Amino Acid Analysis.* Peptide samples were hydrolyzed by 6 N HCl in sealed, evacuated tubes for 20 h at 105 °C. After removal of acid in vacuo, amino acids were analyzed on a Beckman Model 121C instrument.

(*5*) *Analysis of Disulfide Bridging.* Conotoxin GI (40 nmol) was partially reduced and alkylated with 0.04 M sodium borohydride, 0.1 M sodium phosphate, pH 8, and 0.1 M iodo-[^{14}C]acetamide (specific activity 9.4 × 10^7 cpm/μmol); the reaction was carried out for 12 min in a total volume of 20 μL. At the end of this time, excess unlabeled iodoacetamide was added (16 μL, 1.125 M) for 3 min at room temperature. The reaction was terminated by addition of 0.4 mL of 5% acetic acid, and the sample was desalted on Sephadex G-10. The void volume fractions were pooled and lyophilized. The carboxamidomethylated peptide was then redissolved in 30 μL of 0.1 M sodium phosphate, pH 7, and reduced with β-mer-

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[1] Abbreviations: DNS-OH, 5-(dimethylamino)-1-naphthalenesulfonic acid; DNS-NH_2, 5-(dimethylamino)-1-naphthalenesulfonamide; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; Acm, acetamidomethyl; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; TFA, trifluoroacetic acid.

0006-2960/84/0423-2796$01.50/0

captoethanol (1.5 μmol, 50 °C for 2 h). Iodo[^{3}H]acetic acid (9 μmol, specific activity 8.7×10^7 cpm/μmol) was added and allowed to react at room temperature for 1 h in a total volume of 60 μL. Unlabeled iodoacetic acid was added (20 μL, 2.5 M), and after 30 min at room temperature the reaction was stopped by the addition of 0.4 mL of 5% acetic acid. The modified peptide was desalted on Sephadex G-10 and lyophilized. All reactions were carried out under N_2 in the dark.

Trypsin digestion was carried out overnight at 37 °C in 80 μL of 0.05 M *N*-ethylmorpholine acetate, pH 8.5, containing 5 mM calcium chloride and trypsin (0.2 mg/mL). The peptide fragments were separated by using HVPE at pH 5.4. After electrophoresis for 4 h at 17.5 V/cm, sample strips were cut transversely into 1-cm segments, and each segment was counted in an LS233 scintillation counter. Labeled peptides were concentrated by ascending chromatography using 10% acetic acid; they were eluted by centrifugation at low speed.

Samples of the various nonapeptides isolated in this way (see Results) were digested further with dipeptidyl-aminopeptidase I (Callahan et al., 1972). The alkylated peptide was dissolved in 20 μL of 0.05 M pyridine acetate, pH 4.5, containing 5 mM EDTA, 15 mM β-mercaptoethanol, and 1% sodium chloride. A solution containing 0.4 unit of enzyme in 10 μL was added, and the reaction was carried out at 37 °C for 3 h. Digests were analyzed by HVPE at pH 5.4, as described above.

(*6*) *Peptide Synthesis.* Synthesis of conotoxin GI was carried out on a benzhydrylamine resin, by stepwise addition of amino acids from the C terminus. Cys-3 and Cys-13 were protected by acetamidomethyl (Acm) groups, while Cys-2 and Cys-7 were protected by *p*-methoxybenzyl. In this synthesis, treatment with HF resulted in deprotection of all residues but Cys-3 and Cys-13 and also caused release of the peptide from the resin as the C-terminal amide. The released peptide was converted to the Cys-2–Cys-7 monocyclic form by oxidation with potassium ferricyanide using the high dilution approach reported by Rivier et al. (1978) for somatostatin. The monocyclic intermediate was purified by reversed-phase HPLC, using 0.025 M triethylammonium phosphate–acetonitrile, and desalted by rechromatography in 0.1% trifluoroacetic acid–acetonitrile (Rivier, 1978). Oxidative closure of the second disulfide was carried out by a modification of the method of Kamber et al. (1980). The purified [Acm-Cys^{3,13}] peptide was dissolved in DMF–HOAc (8:2 v/v). With constant vortexing the peptide solution was added dropwise to a 0.067 M solution of iodine in the same solvent. The solution was briefly vortexed several more times and quenched after 8 min by the dropwise addition of a solution of $Na_2S_2O_3$. The clear, colorless solution was quickly dried on a rotary evaporator to a pale yellow oil and then dissolved in 0.025 M triethylammonium phosphate (TEAP), pH 2.25. The crude reaction mixture was repurified by HPLC on a Supelcosil C-18 (1 × 25 cm) column using the TEAP–acetonitrile system (Rivier, 1978) and then desalted on the same column using an increasing gradient of acetonitrile in 0.1% trifluoroacetic acid.

For the synthesis of [Cys^{12},Ser^{13}]conotoxin GI the polyamide support of Atherton and co-workers (Atherton et al., 1975, 1979; Arshady et al., 1981) having the *p*-(hydroxymethyl)-benzoic acid as linkage agent was used. Synthesis was from the carboxy terminus, coupling of the successive Boc amino acids being carried out by the symmetrical anhydride method. Cys-3 and Cys-12 were protected as the very acid stable acetamidomethyl derivatives (Veber et al., 1968); Cys-2 and Cys-7 were protected as the HF-labile 4-methylbenzyl derivatives. All other protecting groups were also removable by HF. After all amino acids were coupled, the resin-bound peptide was deprotected by treatment with anhydrous HF. This step left the peptide attached to the resin via its carboxy terminus and with exposed –SH groups on Cys-2 and Cys-7. The resin was suspended in 0.2 M ammonium acetate, pH 6, and exposed to air for 72 h. By carrying out this step with the peptide still attached to the resin it was hoped to maximize the yield of intramolecular disulfide bonding. The peptide intermediate was released by ammonolysis, thereby creating the carboxy-terminal amide. Chromatography on Sephadex G-25 showed that about 60% of the material eluted close to the V_t of the column, and this was assumed to be the monomer. A more rapidly eluting peak, but still included in the gel, was also obtained. After reduction with β-mercaptoethanol, and rechromatography, material from this peak moved to a position much closer to that of the monomer; presumably it represents dimers and small oligomers. The putative monomer was purified further by chromatography on carboxymethylcellulose, giving one major peak and two minor peaks. The peptide intermediate was lyophilized and dissolved in 50% acetic acid, and the solution was then added slowly, with vigorous stirring, to a 0.1 M solution of iodine in 70% acetic acid. This step oxidatively removes the Acm groups and creates the second disulfide bridge (Kamber et al., 1980). Excess iodine was destroyed after 10 min by addition of ascorbic acid, and the peptide was purified by chromatography on Sephadex G-25 and carboxymethylcellulose.

(*7*) *Reduction and Reoxidation.* Twenty nanomoles of synthetic conotoxin GI was dissolved in 25 μL of 0.2 M Bicine buffer, pH 8, and 2 μL of a 0.5 M dithiothreitol solution was added under argon; the solution was incubated at 55 °C for 2 h. The reaction mixture was fractionated by HPLC using 0.1% TFA–acetonitrile, as described above. The effluent was monitored at 205 nm, and the main peak (total volume, 0.7 mL) was collected.

A solution containing 120 μM potassium ferricyanide in 0.05 M ammonium bicarbonate–TFA, pH 7, was used as the oxidizing agent. The reduced conotoxin, as eluted directly from HPLC in the 0.1% TFA–acetonitrile, was gradually mixed with the ferricyanide solution over a period of 4 h with continuous stirring. A solution of 0.01 M ammonium hydroxide was simultaneously added over the 4-h period to maintain a neutral pH. These operations were carried out under argon at room temperature with a twin-syringe infusion pump. After oxidation, the reaction mixture was analyzed by HPLC and all peak fractions were collected.

(*8*) *Iodination.* Iodination of conotoxin GI was carried out by the method of Fraker & Speck (1978). Iodogen was dried in small polypropylene tubes from a solution in chloroform. Toxin samples were redissolved in 0.3 M ammonium acetate adjusted to either pH 5.3, pH 8.2, or pH 9.5. Sodium iodide was added, including where appropriate [^{125}I]iodide (Amersham/Searle) to label the peptides radioactively. The solutions were then added to the tubes containing iodogen, and reactions were carried out for 15 min at 25 °C. Unless otherwise specified, iodogen was always used in a 4-fold molar excess over the peptide, and the final conotoxin GI concentration was 0.125 mM. Reaction mixtures were diluted to 0.2 mL in 0.1% TFA, injected directly onto the HPLC column, and eluted with the 0.1% TFA–acetonitrile gradient.

Iodinated peptides purified in this manner were characterized by enzymatic degradation to free amino acids. Initial digestion was with trypsin (0.2 mg/mL) in 0.3 M ammonium acetate–5 mM calcium chloride, pH 8.5, for 30 min at 37 °C. Pronase dissolved in the same buffer was added to a final concentration of 1 mg/mL and digestion was continued for

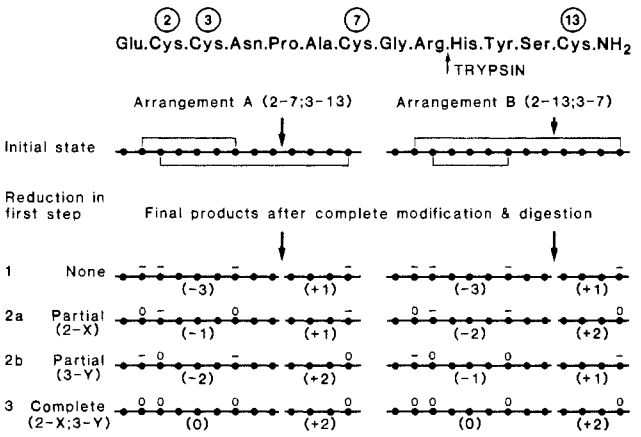


FIGURE 1: Scheme for analyzing disulfide bridging in conotoxin GI. Reduction of a disulfide in the first step results in ^{14}C labeling of its Cys residues with iodoacetamide but no change in net charge ("0" above residue); disulfides not reduced in this step are subsequently reduced and their Cys residues are ^{3}H labeled with iodoacetic acid, which creates a negative charge ("–" above residue). Net charges of the final nonapeptides are given in parentheses. The isotopic labeling of the nonapeptides resulting from *partial* reduction is indicative of the original disulfide arrangement.

an additional 4 h at 37 °C. The products of digestion were then separated by high-voltage paper electrophoresis at 1500 V for 2 h using commercial iodotyrosine and iodohistidine standards. The papers were autoradiographed with Kodak X-ray film for 12–24 h at −70 °C with an intensifier screen. Amino acid analysis was used to quantitate peptides prior to bioassay.

Results

Analysis of Disulfide Bridging in Conotoxin GI. Because of the limited amounts of natural material (<100 nmol), and the presence of adjacent Cys residues at positions 2 and 3 in the sequence, we adopted the novel strategy shown in Figure 1.

Conotoxin GI was partially reduced by sodium borohydride in the presence of iodo[^{14}C]acetamide, thus attempting to trap thiols as soon as they were formed. Suitable reaction conditions were explored with vasopressin (data not shown). The alkylation reaction was then pushed to completion by adding a high concentration of unlabeled iodoacetamide (0.5 M) for 3 min before stopping the reaction. The partially reduced and alkylated peptide was purified and then completely reduced and alkylated with iodoacetic acid. The population of molecules that had only one disulfide bond reduced in the borohydride treatment should therefore be alkylated with two (carboxymethyl)cysteine residues bearing a negative charge and two neutral (carboxamidomethyl)cysteine residues labeled with ^{14}C.

As shown in Figure 1, the product resulting from partial reduction should be a mixture of two isomeric forms, depending on which of the two disulfide bonds was reduced first. These forms are distinguishable after cleaving the peptides with trypsin. Digestion leads in all cases to a nonapeptide and a tetrapeptide. All tetrapeptides are positively charged at pH 5.4 and do not interfere with electrophoretic analysis of the nonapeptides, which carry charges of from 0 to 3–. The nonapeptides from the mixed alkylation product (containing both carboxymethyl and carboxamidomethyl groups) should have a charge of either 1– or 2–, depending on the isomer. The expected structures for the nonapeptides with charges of 1– and 2–, given the two alternative disulfide bond configurations, are diagramed in Figure 1; they differ in the location of the isotope.

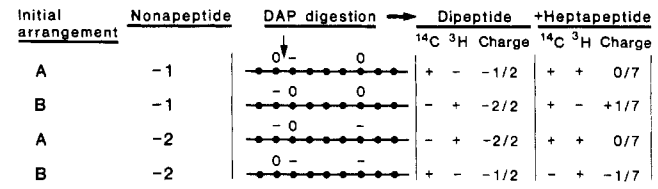


FIGURE 2: Expected disposition of isotopic labels after digestion of nonapeptides with dipeptidyl-aminopeptidase I (DAP).

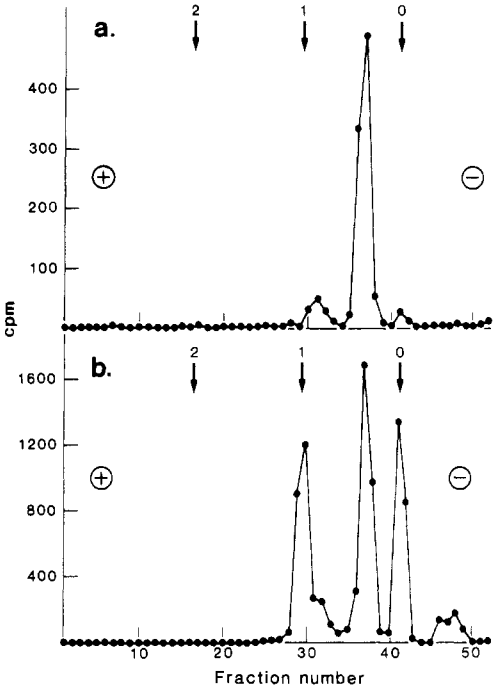


FIGURE 3: Analysis of dipeptidyl-aminopeptidase I digestion products by high-voltage electrophoresis. The purified alkylated nonapeptide having a charge of 1– produced by the scheme shown in Figure 1 was digested with DAP as described under Methods. The top panel shows the peptide before digestion; the lower panel shows the digestion products. Arrows 1 and 2 represent the mobility positions of the alkylated dipeptides Glu-(carboxamidomethyl)cysteine and Glu-(carboxymethyl)cysteine, respectively; these were prepared from toxin that had been fully reduced and alkylated with the appropriate derivatives. The anode is to the left in each case; the origin is at position 40, and neutral peptides migrate to position 41 (arrow 0) by electroendosmosis.

Electrophoresis of the trypsin digest gave an evenly spaced series of radioactive bands (not shown), having mobilities very close to those predicted from the empirical relationship between charge and molecular weight (Offord, 1966, 1977). Both isotopes were present in the 1– and 2– peptides; as expected, only ^{3}H was found in the 3– peptide. The neutral band, contrary to expectations, contained some ^{3}H, possibly due to contamination by tetrapeptide that had been alkylated in the imidazole of His-10. Samples of the nonapeptides were eluted from the paper, and the location of the ^{14}C label was analyzed for the 1– and 2– peptides, as outlined in Figure 2. The scheme uses the specificity of dipeptidyl-aminopeptidase I (DAP) to limit digestion to the bond between the modified Cys-2 and Cys-3; the enzyme cannot split peptide bonds on either side of a proline residue (MacDonald et al., 1972).

For disulfide arrangement A, digestion of the (1–) nonapeptide should give equimolar amounts of the dipeptide glutamyl([^{14}C]carboxamidomethyl)cysteine with a charge of 1– and a neutral ^{14}C-heptapeptide. For arrangement B, all ^{14}C label should appear as a 1+ heptapeptide. Results of the digestion are shown in Figure 3. Approximately 30% of undigested material remained, but the major digestion products

clearly indicate arrangement A. Digestion of the 2– nonapeptide should produce results opposite to those obtained with the 1– nonapeptide. Such was the case, although digestion was only about 40% complete (results not shown).

The extent of disulfide rearrangement was estimated in each case b analyzing the amount of incorrect peptides produced. In Figure 3b the small peak in fractions 46–48, corresponding in mobility to the 1+ heptapeptide, indicates approximately 6% scrambling. With the 2– nonapeptide there was found a small shoulder on the main peak. Repurification of material from this region gave the "scrambled" dipeptide in a yield of 19%. Some bias may exist in these estimates, as DAP digestion was incomplete, and the enzyme may not have been equally effective at removing the two forms of terminal dipeptide. Preferential digestion of the Glu–(carboxamidomethyl)cysteinyl peptide bond would lead to an underestimate of scrambling with the 1– nonapeptide and an overestimate with the 2– peptide. In neither case, though, is the amount of scrambling so great as to place the main analysis in serious doubt. We conclude, therefore, that the disulfide pairing is Cys-2–Cys-7 and Cys-3–Cys-13 in the natural toxin.

Analogous predictions can be made with respect to the distribution of ^3H in the digestion products (Figure 2). However, recoveries of this isotope were irregular and unsatisfactory, although the results (not shown) pointed in the same general direction as those with ^{14}C.

Chemical Synthesis of Conotoxin GI: Comparison of Synthetic and Natural Toxins. The chemical synthesis of conotoxin GI was carried out with the specific disulfide pairing (Cys-2–Cys-7; Cys-3–Cys-13) as described under Methods. Starting with 6 g of resin we obtained a final weight of 11.7 g after coupling, corresponding to 2.13 nmol of bound peptide. After HF deprotection and cleavage, ferricyanide oxidation, and desalting, the yield of crude peptide was 2.3 g (1.36 nmol; 64%). Highly purified monocyclic [Acm-Cys^{3,13}] peptide was recovered after HPLC (645 mg; overall yield 18%). Oxidative cyclization of the second disulfide proceeded smoothly to give pure synthetic conotoxin GI in 11% overall yield from crude resin-bound peptide.

Synthetic conotoxin GI and natural conotoxin GI were compared. In all tests carried out so far, the synthetic peptide (GI_s) has been indistinguishable from the natural material, GI_n. Fast atom bombardment mass spectroscopy showed the correct molecular weight (1436), and amino acid analysis gave the expected composition. Dansylation of 20 nmol of GI_s revealed no traces of DNS amino acids other than DNS-Glu and *O*-DNS-Tyr. On electrophoresis at pH 5.4 and 6.4 the two peptides had the same mobilities. Synthetic conotoxin GI was also subjected to disulfide bridging analysis as described for natural toxin, and very similar results were obtained.

Comparison of biological activities was made between GI_n and GI_s on samples that had been freshly purified by reverse-phase HPLC. Results of the ip bioassay on mice are shown in Table I. For each peptide the results were calculated in terms of activity units per nanomole (Cruz et al., 1978), on the basis of the amino acid analysis, the weight of the animals, and the death time. For GI_n and for GI_s, the values were 1.48 and 1.45 units/nmol, respectively; these are not significantly different.

Unmodified GI_n and GI_s were compared by reverse-phase HPLC, as were their tryptic and chymotryptic cleavage products. After enzyme digestion, a single new peak was generated, and 10–30% of undigested peptide was present. Excellent agreement was obtained between the elution times for the "nicked" products of GI_n and GI_s, in which the disulfide bonds were still present [Table I (d)]. The sensitivity of the chromatography to minor differences between peptides is illustrated by the clear separation of GI split by trypsin from that split by chymotrypsin.

Table I: Comparisons between Natural and Synthetic Conotoxin GI

	natural	synthetic
bioassay		
ip (units/nmol)	1.48	1.45
ic, LD_{50} (μg/kg)	21	28
HPLC retention times (min)[a]		
(a) conotoxin GI	16.51	16.49
(b) conotoxin GI	12.18	12.12
(c) conotoxin GI	10.78	10.84
(d) conotoxin GI	21.98	21.94
GI-trypsin	14.51	14.49
GI-chymotrypsin	18.69	18.58

[a] Chromatography was carried out on a Hewlett-Packard 1084-B instrument, with a variable-wavelength detector. All experiments were made at 23 °C, flow rate 1.5 mL/min, with effluents monitored at 205 nm. Systems used were as follows, gradients being expressed as linear segments giving % B reached at times given in parentheses. (a) Supelco, LC18, 0.46 × 15 cm; A, 0.1% TFA; B, 0.1% TFA in 60% acetonitrile; gradient 5 (0) → 5 (5) → 60 (25). (b) VYDAC, C18, 0.46 × 30 cm; A, TEAP, pH 3.3 (Rivier, 1978); B, TEAP, pH 3.3 in 60% acetonitrile; gradient 5 (0) → 5 (2) → 50 (25). (c) Column as in (b); buffers as in (a); gradient 15 (0) → 15 (2) → 50 (30). (d) Column and buffers as in (c); gradient 10 (0) → 10 (2) → 25 (25).

Initially conotoxin GI gave ambiguous results with respect to the carboxy-terminal sequence (Gray et al., 1981b), and we thought the more likely alternative was Cys-12–Ser-13. This structure with the homologous disulfide configuration (Cys-2–Cys-7; Cys-3–Cys-12) was shown to be incorrect by chemical synthesis. The final product showed the expected amino acid analysis and had the same electrophoretic mobility as the natural peptide at pH 6.5 but was inactive biologically in doses of up to 16 nmol, approximately 100 times the LD_{50} for natural peptide. Controls were run including dansylation (Gray, 1967) (to show that the amino-terminal glutamic acid had not cyclized) and electrophoresis (to indicate no loss of amide groups). The complete lack of activity is therefore a consequence of reversing the two C-terminal amino acids. In later experiments fully active conotoxin GI with the corrected Ser-12–Cys-13 amino acid sequence was readily synthesized by using the same polyamide support (P. Ward, E. Atherton, and R. C. Sheppard, unpublished experiments).

Reduction and Reoxidation of Conotoxin GI. In our preliminary work (Cruz et al., 1978) we found that reduction of the disulfides led to loss of biological activity. Attempts to reactivate by allowing the disulfides to re-form during prolonged exposure to air were unsuccessful. Sulfitolysis and reoxidation did not work either, despite the fact that apamine in the same reaction mixture was successfully inactivated and reactivated (our unpublished results). Availability of synthetic toxin enabled us to pursue the question further, using HPLC to analyze the products, rather than relying solely on regain of biological activity.

As shown in Figure 4b, reduction of conotoxin GI by dithiothreitol led to complete disappearance of the original material eluting at 15.89 min (Figure 4a) and appearance of a new peak at 14.30 min. When material from this latter peak was oxidized with ferricyanide, as described earlier, native toxin was regenerated, as evidenced by the peak at 15.98 min (Figure 4c). Peptide isolated from this peak was subjected to several tests and behaved in a manner identical with that of natural toxin: (1) it had the same specific activity on ip

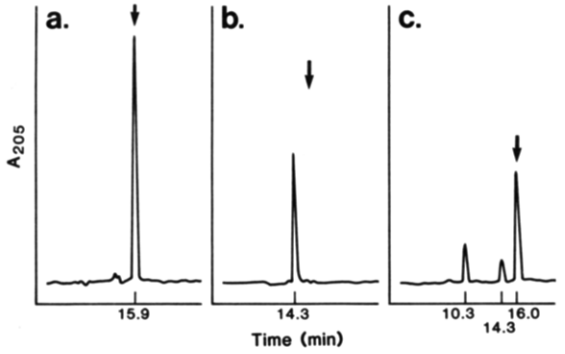


FIGURE 4: Reduction and reoxidation of conotoxin GI. Conotoxin GI was reduced with dithiothreitol and reoxidized with $K_2Fe(CN)_6$ as described under Methods. The original peptide (a), reduced conotoxin GI (b), and reoxidized conotoxin GI (c) were analyzed by reversed-phase HPLC using 0.1% TFA–acetonitrile; these analyses are shown from left to right, respectively. The arrow indicates the elution time for native conotoxin GI, 15.9 min under these conditions. The second peak in (c) corresponds in elution time to reduced GI.

assay in mice; (2) it cochromatographed with authentic toxin when the two were mixed; (3) trypsin digestion of a mixture of authentic and reoxidized toxins gave a single product with the previously observed HPLC behavior.

We conclude that conotoxin GI which has been completely reduced by dithiothreitol can be reoxidized in the correct disulfide conformation, by the action of ferricyanide under conditions of extreme peptide dilution. Failure to obtain reactivation by the other methods may be due to the much longer reaction times during which there was opportunity for intermolecular disulfides to form. In one such experiment the products were analyzed by HPLC; a complex series of peaks was obtained, with very little peptide running in the position of native toxin (results not shown). The material eluting at 10.27 min (Figure 4c) was biologically inert. It was formed also by exposure of native toxin to air at neutral pH.

Iodination of Conotoxin GI. Reaction products, as analyzed by HPLC, were found to vary in the expected fashion according to the pH of the reaction mixture and the ratio of iodide to peptide. Illustrative chromatograms are shown in Figure 5a,b. Identification of the individual peaks was made by electrophoretic analysis of the amino acids released by digestion with Pronase and trypsin (Figure 5c).

At pH 5.3, when the imidazole of His-10 is protonated, reaction is almost exclusively with Tyr-11 (Figure 5a), even in the presence of excess iodide. Under the latter conditions native conotoxin GI disappears completely, as does [monoiodo-Tyr[11]]conotoxin GI, but only trace iodination of His-10 occurs: the reaction product was almost exclusively [diiodo-Tyr[11]]conotoxin GI (data not shown).

Higher pHs led to iodination of His-10 also. At pH 8.2 and 0.25 mM iodide a complex mixture was obtained (Figure 5b). At pH 9.5 and 2.0 mM iodide the tetraiodinated derivative [diiodo-His[10],diiodo-Tyr[11]]conotoxin GI was by far the major product. In neither case did we find significant amounts of peptides that were iodinated on His-10 without Tyr-11 also being iodinated.

Evidence for the assignment of the various peaks is shown in Figure 5c. Extensive digestion with trypsin and Pronase was followed by electrophoresis at pH 1.7. Nonradioactive standards of various iodinated amino acids were included with each digestion mixture, and ninhydrin staining showed exact correspondence with the appropriate radioactive spots.

Peaks A and B (Figure 5a) are nonradioactive and represent unmodified conotoxin GI and a breakdown product of iodogen, respectively. Under conditions of iodogen excess relative to

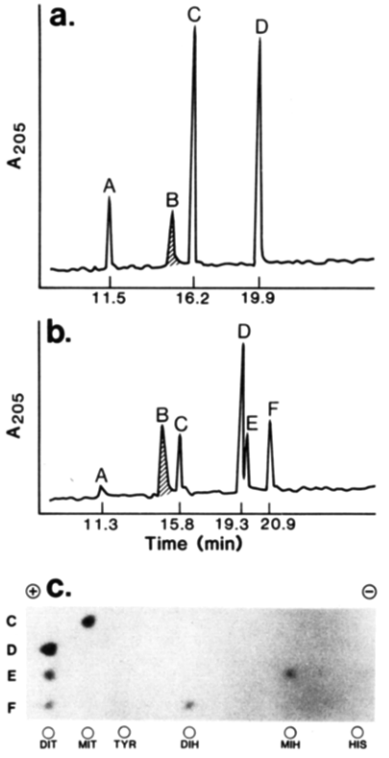


FIGURE 5: Chromatographic purification of iodinated derivatives of conotoxin GI. Iodination was carried out as described under Materials and Methods. Shown are HPLC separations of reactions carried out at (a) pH 5.3, 0.25 mM iodide, and (b) pH 8.2, 0.25 mM iodide. The peaks seen are as follows: A, unmodified conotoxin GI; B, an unidentified reaction byproduct (nonradioactive) that also appears in control reactions; C, [monoiodo-Tyr[11]]conotoxin GI; D, [diiodo-Tyr[11]]conotoxin GI; E, [diiodo-Tyr[11],monoiodo-His[10]]conotoxin GI; F, [diiodo-Tyr[11],diiodo-His[10]]conotoxin GI. (c) Autoradiography of enzymatic digests of peaks C–F, run on high-voltage electrophoresis at pH 1.7 as described under General Methods. The radioactive peaks were identified by inclusion of nonradioactive carriers of authentic iodotyrosine and iodohistidine standards. The markers were visualized by staining with ninhydrin, and their positions are indicated. When 1 mM iodide at pH 5.3 was used, greater than 90% of the peptide eluted in peak D with the remainder in peak E; When 2 mM iodide at pH 9.5 was used, approximately 80% of the peptide eluted in peak F, with about 10% in each of peaks D and E (data not shown).

iodide, chromatograms became very complex (not shown). Among the products were peptides that probably contained chlorine in place of iodine. In particular, proteolysis of some peptides gave radioactive materials with mobilities intermediate between those of monoiodotyrosine and diiodotyrosine and between those of monoiodohistidine and diiodohistidine. These almost certainly represent 3-chloro-5-iodotyrosine and 2-iodo-4-chlorohistidine, respectively. Apart from establishing that these materials were toxic, we did not investigate them further.

Biological Activity of Iodinated Conotoxin GI. Using the quantitative intraperitoneal assay that we have described previously, we obtained a linear relationship between death time and reciprocal dose. All tested iodinated derivatives were found to be biologically active (Table II): even that in which His-10 and Tyr-11 were fully substituted had about half the specific activity of unmodified toxin.

Discussion

In this report, we have described further characterization of conotoxin GI, a small peptide that blocks the acetylcholine receptor. We also report a successful chemical synthesis of the toxin and the preparation of labeled derivatives. A preliminary account of the synthesis was reported earlier (Gray et al., 1981a). We have postulated (W. R. Gray, D. Mid-

Table II

derivative	sp act. (units/nmol)	regression parameters[b] A	B
GI, natural	1.5	2.8	11.6
GI, synthetic	1.5	2.0	12.4
MIT-GI[c]	1.4	1.1	13.2
DIT-GI	1.0	3.3	16.7
DIT-MIH-GI	0.9	2.8	19.3
DIT-DIH-GI	0.7	2.9	25.4

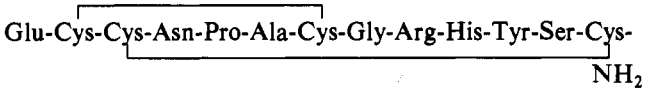
[a] A unit of activity is the quantity of toxin required to kill a 20-g mouse in 20 min (Cruz et al., 1978). [b] A and B are coefficients in the regression equation death time (min) = A (min) + B (min·nmol) × reciprocal dose ($nmol^{-1}$). Dose is standardized for a 20-g mouse. [c] MIT = monoiodotyrosine; DIT = diiodotyrosine; MIH = monoiodohistidine; DIH = diiodohistidine.

dlemas, A. Luque, L. J. Cruz, J. Rivier, and B. M. Olivera, unpublished results) that peptide toxins of this group have a conformation resembling that of the active tip of snake α-neurotoxins; as such, they are of exceptional interest as potential probes of the acetylcholine receptor. For the peptides to serve as useful probes, a more thorough understanding of their covalent chemistry and conformation is needed, as are larger amounts of synthetic toxin and radioactive derivatives.

Although the amino acid sequence of conotoxin GI has been established, along with those of some related peptides (Gray et al., 1981b; McIntosh et al., 1982), a direct analysis of the disulfide bridge configuration proved very difficult with the small amounts of material available. With two disulfides, there are formally three possible pairwise arrangements; however, since adjacent Cys residues (i.e., Cys-2–Cys-3) cannot pair without gross distortion, for conotoxin GI the only reasonable possibilities are (2–7, 3–13) and (2–13, 3–7). This simplification was more than offset by the necessity of splitting the peptide bond between adjacent Cys residues in order to deduce what other parts of the chain are linked to individual residues. Such cleavage must be done under conditions that do not result in disulfide rearrangement. The "classical" method is random acid hydrolysis in the presence of thiols, which inhibit interchange under acidic conditions (Ryle & Sanger, 1955). This approach requires large amounts of peptide—approximately 12 000 nmol was used to analyze the disulfides in apamine, a peptide of similar size (Callewaert et al., 1968). We were limited to less than 100 nmol.

We therefore designed a method that not only allowed us to use much smaller amounts of peptide but also gave a direct assessment of the level of disulfide scrambling taking place during the analysis.

The results of our analysis established that the disulfide bridges link Cys-2–Cys-7 and Cys-3–Cys-13:



Analysis of *both* complementary partial reduction and alkylation products indicated that rearrangement was occurring in about 10–20% of molecules. We regard this internal checking as an important feature of the method: it is not uncommon for disulfide bridge assignments to be made solely on the basis of identifying a particular bridged peptide, without establishing that alternative forms are absent. Small peptides with multiple bridges pose especially difficult analytical problems, which are exacerbated by adjacent Cys residues. We believe that our general approach should have wide applicability, although the details will necessarily depend on the specific sequences involved.

The correctness of our assignment is attested by the successful chemical synthesis of conotoxin GI having the appropriate pairing and the close similarity of natural and synthetic toxins. The synthesis also clearly resolves any ambiguity concerning the carboxy-terminal sequence and establishes the correctness of our published sequence (Gray et al., 1981b). Successful synthesis of a related molecule, conotoxin MI, with the analogous disulfide pairing adds further weight to the assignment of bridges (Gray et al., 1983).

Nishiuchi & Sakakibara (1982) have recently also determined the correct disulfide assignment of conotoxin GI by synthesizing all three possible disulfide configurations of the peptide and determining which had the highest biological activity. Contrary to our earlier findings with the natural conotoxin GI, they found that the correct disulfide pairing was the most stable. Availability of synthetic conotoxin GI enabled us to reexamine its reduction and reoxidation behavior. In contrast to our earlier experience, we found that reduced conotoxin can be successfully reoxidized to a form indistinguishable from the native material. Use of ferricyanide in excess, while reduced peptide was in extremely low concentration, was probably a key factor. Oxidation under these conditions is certainly very rapid relative to intermolecular disulfide reactions and probably is faster than the intramolecular process also. The converse is certainly true during the long slow reoxidation by exposure to air.

Smaller amounts of an inactive faster eluting material were also obtained during reoxidation. We cannot exclude the possibility that this represents another disulfide isomer such as (3–7, 2–13), which elutes earlier (Nishiuchi & Sakakibara, 1982). However, it was also produced simply by incubating natural toxin in air at neutral pH. Unless there was contamination by catalytic amounts of thiol, it is hard to see how disulfide interchange would be occurring. Such interchange could certainly occur in vivo and might be the source of the weak (<10%) activity observed by Nishiuchi and Sakakibara for the less stable isomers.

Iodination of the toxin, with retention of biological activity, was achieved by using iodogen. Preparation of the derivatives is very straightforward. At low pH only Tyr-11 was iodinated, while His-10 was also labeled at higher pHs. With this peptide we did not find conditions for selective iodination of His-10. Remarkably, all four iodinated products that were characterized were still highly toxic, so it is clear that bulky groups on the His and Tyr do not greatly interfere with binding to the receptor. The tetraiodinated molecule, which retains about 50% biological activity on a molar basis (Table II), actually contains greater than 25% by weight iodine!

The data presented here establish completely the covalent structure of conotoxin GI, provide a method for chemically synthesizing relatively large amounts of toxin, and describe methods for obtaining chemically defined labeled toxin. The availability of synthetic toxin and several homogeneous, chemically defined radioactive derivatives of conotoxin GI that are biologically active should permit measurement of kinetic constants and binding of the toxin to the acetylcholine receptor, as well as greatly facilitate the use of these toxins in neuroscience.

Acknowledgments

We thank Drs. Howard Morris and Graham Taylor of Imperial College, London, for carrying out the FAB mass spectroscopy.

Registry No. Conotoxin GI, 76862-65-2; [Cys^{12},Ser^{13}]conotoxin GI, 89556-48-9; reduced conotoxin GI, 78249-65-7; [diiodohistidine,-diiodotyrosine]conotoxin GI, 89556-49-0; [monoiodo-Tyr^{11}]conotoxin

GI, 89556-50-3; [diiodo-Tyr[11]]conotoxin GI, 89556-51-4; [diiodo-Tyr[11],monoiodo-His[10]]conotoxin GI, 89556-52-5.

References

Arshady, R., Atherton, E., Clive, D. L. J., & Sheppard, R. C. (1981) *J. Chem. Soc.*, 529–537.

Atherton, E., Clive, D. L. J., & Sheppard, R. C. (1975) *J. Am. Chem. Soc. 97*, 6584–6585.

Atherton, E., Gait, M. J., Sheppard, R. C., & Williams, B. J. (1979) *Bioorg. Chem. 8*, 351–370.

Callahan, P. X., McDonald, J., & Ellis, S. (1972) *Methods Enzymol. 25*, 282–298.

Callewaert, G. L., Shipolini, R., & Vernon, C. A. (1968) *FEBS Lett. 1*, 111–113.

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.

Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Common. 80*, 849–857.

Gray, W. R. (1967) *Methods Enzymol. 11*, 139–151.

Gray, W. R., Cruz, L. J., Olivera, B. M., & Rivier, J. (1981a) International Symposium on HPLC of Proteins and Peptides, Washington, DC, Abstr. 520.

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

Gray, W. R., Rivier, J. E., Galyean, R., Cruz, L. J., & Olivera, B. M. (1983) *J. Biol. Chem. 258*, 12247–12251.

Kamber, B., Hartman, A., Eisler, K., Riniker, B., Rink, H., Sieber, P., & Riltel, W. (1980) *Helv. Chim. Acta 63*, 899.

Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 159–212.

McDonald, J. K., Callahan, P. X., & Ellis, S. (1972) *Methods Enzymol. 25*, 272–281.

McIntosh, M., Cruz, L. J., Hunkapiller, M. W., Gray, W. R., & Olivera, B. M. (1982) *Arch. Biochem. Biophys. 218*, 329–334.

Nishiuchi, Y., & Sakakibara, S. (1982) *FEBS Lett. 148*, 260–262.

Offord, R. E. (1966) *Nature (London) 211*, 591.

Offord, R. E. (1977) *Methods Enzymol. 47*, 51–69.

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

Rivier, J., Kaiser, R., & Galyean, R. (1978) *Biopolymers 17*, 1927–1938.

Ryle, A. P., & Sanger, F. (1955) *Biochem. J. 60*, 535–540.

Veber, D. F., Milkowski, J. D., Denkewalter, R. G., & Hirschmann, R. (1968) *Tetrahedron Lett.*, 3057.

Kinetics of Appearance of Sulfhydryl Groups in α_2-Macroglobulin on Reaction of the Inhibitor with Amines†

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ABSTRACT: The mechanism of the appearance of sulfhydryl groups in α_2-macroglobulin in the reaction with amines was characterized by analyses of the kinetics with ammonia and methylamine. All reactions occurred under pseudo-first-order conditions in the range of pH (7.0–8.6) and amine concentration (10–600 mM) investigated. The logarithm of the pseudo-first-order rate constant increased linearly as a function of pH with a slope of unity, indicating that the unprotonated amine is the active species in the reaction. Plots of the observed pseudo-first-order rate constants vs. concentration of unprotonated amine at constant pH were also linear and gave second-order-rate constants of 0.32 and 13.8 M^{-1} s^{-1} for ammonia and methylamine, respectively, at pH 8.0; similar values were obtained at pH 8.6. Activation energies of 85 and 100 kJ mol^{-1} and activation entropies of 10 and 95 J K^{-1} mol^{-1} for ammonia and methylamine, respectively, were estimated from Arrhenius plots, suggesting that the higher reaction rate for methylamine is due primarily to a higher activation entropy. These results are consistent with the release of sulfhydryl groups being caused by a nucleophilic attack of the uncharged amine on a thio ester bond of α_2-macroglobulin in a bimolecular reaction occurring under pseudo-first-order conditions. The characteristics of the reaction suggest that the thio ester in each α_2-macroglobulin subunit reacts independently and equivalently with the amine and also that the thio ester bond cleavage initiates the reaction sequence leading to inactivation of the inhibitor. The α_2-macroglobulin thio ester was further characterized by a comparison of the rates of cleavage of this bond by a series of amines with the corresponding rates of cleavage of a small thio ester, ethyl thioacetate. The protein thio ester was cleaved more rapidly by small primary amines than the model thio ester. The thio ester bond in α_2-macroglobulin thus appears comparatively labile. The reactivity of the different amines with α_2-macroglobulin did not parallel the reactivity with the small thio ester but rather the inverse of the size of the amines, indicating that access of reagents to the protein thio ester is sterically hindered.

The protein α_2-macroglobulin is a plasma proteinase inhibitor with several unique properties. It has a high relative molecular mass (~725 000) and consists of four apparently identical polypeptide chains (Jones et al., 1972; Hall & Roberts, 1978; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1979). It inhibits a wide variety of proteinases of different classes and with different specificities (Barrett & Starkey, 1973; Harpel, 1976). Moreover, it binds the proteinase in a manner that abolishes or markedly decreases the activity of the enzyme against macromolecular substrates but largely preserves the activity against small substrates (Barrett & Starkey, 1973; Harpel, 1976). These properties have led to the suggestion that the inhibitor physically entraps the proteinase (Barrett

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0006-2960/84/0423-2802$01.50/0