Disulfide pairings in geographutoxin I, a peptide neurotoxin from *Conus geographus*

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The three intramolecular disulfide linkages of geographutoxin I, a peptidic neurotoxin isolated from the venom of the marine snail *Conus geographus*, were examined by a novel method for determination of the positions of disulfide linkages in peptides [(1989) Bull. Chem. Soc. Jp. 62, 1986–1994]. The disulfide bridges were found to be between Cys^3 and Cys^{15}, Cys^4 and Cys^{20}, and Cys^{10} and Cys^{21}, indicating that geographutoxin I has a rigid conformation consisting of three loops stabilized by these three disulfide linkages.

Geographotoxin; Synthesis; Disulfide pairing; Na^+ channel

1. INTRODUCTION

Geographutoxins (also called μ-conotoxins), peptidic neurotoxins, isolated from the venom of the marine snail *Conus geographicus*, block the Na channels of skeletal muscle and are distinct from α- and ω-conotoxins, which act on the acetylcholine receptor and voltage-sensitive Ca channels, respectively [1–3]. These neurotoxins are a family of peptides with highly homologous sequences consisting of 22 amido acid residues including six half-Cys and a C-terminal amide [4–6]. Recently, μ-conotoxin GIIIA (geographutoxin I) was synthesized and found to have the same biological action as the natural toxin [7,8]. This synthetic peptide should be useful in studies at a molecular level on the voltage-sensitive Na channels that regulate ion permeability through membranes, because its sequence can be modified by chemical synthesis with preservation or change of its biological activity [7,8]. Detailed information on the conformation of geographutoxin is necessary for defining its recognition site(s) for skeletal muscle Na channels, which is still unknown. A high content of disulfide bridges in small peptides such as geographutoxin constrains the orientation of their peptide chains. Therefore, in this study, we examined the arrangement of disulfide pairings in geographutoxin I (GTX-I) to obtain information on how the molecule folds in its tertiary structure. For this purpose we used a newly developed method for determining the positions of disulfide linkages in peptides [9]. The information obtained on the disulfide pairings in GTX-I will be useful in further studies on its three dimensional structure.

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2. MATERIALS AND METHODS

2.1. *Materials and apparatus*

Natural GTX-I was obtained as described [4]. β,β-Dideuterio (d_2)-DL-cysteine was synthesized as described [9]. All other compounds and solvents were reagent grade, unless otherwise stated. The apparatus for high-performance liquid chromatography (HPLC) consisted of a Waters M600 multi-solvent delivery system (Milford, MA) and Hitachi 655A variable wavelength UV monitor and a D-2000 chromato-integrator (Tokyo). A reversed-phase resin (Cosmosil $5C_{18}$) was obtained from Nacalai Tesque Inc. (Kyoto) and packed into a column (4 × 250 mm) in our laboratory.

2.2. *Principle of the procedure for determination of disulfide linkages in peptides*

The strategy of the method [9] is briefly as follows: a protected linear peptide with β,β-dideuterio(d_2)-Cys (abbreviated as Cys*, 2 atomic mass units (atm) larger than Cys) at two given residues of multiple Cys residues is first prepared by an appropriate method. After removal of the protecting groups, the resulting peptide is oxidized to form intramolecular disulfide linkages between the Cys residues and the fraction with the same retention time on a chromatogram as that of its corresponding natural or standard peptide is isolated. This purified peptide is then hydrolyzed to amino acids including cystine under conditions that cause the minimum disproportionation of disulfide linkages. In the case of a peptide with 6 Cys residues the hydrolyzate should contain Cys Cys and Cys* Cys* bonds in a ratio of 2:1 (4 atm difference) if the disulfide bond is linked between two Cys*, whereas the hydrolysate should contain Cys Cys and Cys Cys* in a ratio of 1:2 (2 atm difference) if the two Cys* are not linked. If the ratio of these cystines in the

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hydrolyzate can be determined exactly, the type of cystine present in the hydrolyzate, and so the disulfide pairing in the peptide, can be determined. In practice, the hydrolyzate is treated with 9-fluorenylmethyloxycarbonyl chloride (Fmoc-Cl) and the resulting Fmoc-amino acids are separated by HPLC. The fraction containing Fmoc-cystine is then analyzed by fast atom bombardment (FAB) mass spectrometry and the ratio of the signals of $(Fmoc\text{-}Cys)_2$ (m/z = 685) and $(Fmoc\text{-}Cys^*)_2$ (m/z = 689) or $(Fmoc\text{-}Cys)_2$ (m/z = 685) and (Fmoc-Cys, Fmoc-Cys*) (m/z = 687) is measured.

2.3. *Synthesis of peptides*

Peptide synthesis was carried out manually by the solid-phase method [10,11]. *p*-Methylbenzhydrylamine resin containing 0.5 mEq of amino groups per g of resin, used as starting material, was coupled with Boc-amino acids step by step from the C-terminus of the amino acid sequence of GTX-I. The following groups were used for protecting side-chains: 4-methylbenzyl for thiol groups of cysteine residues, *p*-toluenesulfonyl for guanidino groups of arginine residues, benzyl for hydroxyl groups of hydroxyproline and threonine residues, 2-chlorobenzyloxycarbonyl for ϵ-amino groups of lysine residues, and cyclohexyl for β-carboxyl groups of aspartic acid residues. The protected peptide resin was treated with anisol in anhydrous liquid HF [12] at 0°C for 60 min. After removal of HF, the resulting peptide was extracted with 10% acetic acid, washed three times with *n*-hexane, and air-oxidized in dilute solution (peptide concentration, 5×10^{-5} M) at pH 7.5. The solution was kept for 2 days at room temperature with occasional stirring, and then the air-oxidized peptide was purified as described below.

2.4. *Purification of synthetic peptides*

The air-oxidized peptide was purified by HPLC on a reversed phase column. The column was developed first with a linear gradient of 1–7% CH_3CN in 0.05% trifluoroacetic acid with increase in CH_3CN concentration of 0.25%/min, with an isocratic of 7% CH_3CN, and then with a linear gradient of 7–40% CH_3CN in the same solvent with increase in CH_3CN concentration of 1%/min at a flow rate of 1 ml/min at 30°C. The absorbance of the eluate at 220 nm was monitored.

2.5. *Analysis of purified peptides and* $(Fmoc\text{-}Cys)_2$

The amino acid compositions and molecular weights of purified peptides were examined by amino acid analysis and FAB mass spectrometry, respectively, as described [13]. The hydrolysis of peptides, the reaction of the hydrolyzates with Fmoc-Cl, and mass measurement of $(Fmoc\text{-}Cys)_2$ were carried out as described [9].

2.6. *Biological assay*

The biological activities of synthetic peptides were examined as described [14].

3. RESULTS AND DISCUSSION

Multiple disulfide linkages in small peptides are important for stabilizing their biologically active conformations. In this paper, we determined the positions of the three disulfide linkages in GTX-I that constrain the folding of the main peptide chain of this 22-residue peptide amide and conserve its bioactive conformation. For this purpose, we used the recently developed method for determining the arrangement of disulfide linkages in peptides [9] described in section 2.2.

First using the solid-phase method, we synthesized protected peptides with the sequence of GTX-I and with two β,β-d_2-Cys (Cys*) at the positions shown in Table I. These linear peptide amides were liberated from a polymer-support with HF, and air-oxidized in dilute aqueous solution to allow spontaneous formations of three intramolecular disulfide linkages. The HPLC profile of the synthetic peptide with normal Cys (peptide 1 in Table I) is shown in Fig. 1. The peak fraction with the same retention time as that of the natural peptide was isolated and examined by amino acid and mass spectrometric analyses ($Asp_{1.22}$, $Thr_{1.02}$, $Glu_{2.21}$, $Ala_{1.12}$, $Cys_{5.18}$, $Lys_{4.00}$, $Arg_{2.94}$, and $Hyp_{3.25}$ and $[M+H]^+$ 2608.3 (theoretical 2608.1), respectively). This fraction had the same inhibitory effect as the natural peptide on the contractile response of adult rat diaphragm. The synthetic peptide was eluted at a lower concentration of CH_3CN from a reversed-phase column, as predicted from its sequence [6], suggesting that synthetic intermediates are difficult to separate from excess reactants by HPLC and hence that the position of disulfide linkages in this peptide is not easy to determine by step-wise formation of disulfide linkages [11,15]. However, with the present method no problem was encountered in the synthesis and purification of this peptide, except that the yield of the desired product was low. The peptides (2–7), indicated by an arrow in Fig. 1, were hydrolyzed in 4 M methanesulfonic acid at 110°C for 24 h, under which conditions cystine can be recovered with little disproportionation of disulfide bonds [9]. The hydrolyzates were treated with Fmoc-Cl and subjected to HPLC, and the resulting $(Fmoc\text{-}Cys)_2$ fractions were isolated and analyzed by FAB mass spectrometry. The $(Fmoc\text{-}Cys)_2$ fractions from peptides 2, 3 and 5 gave the FAB mass spectrum shown in Fig. 2A, indicating that the ratio of the intensities of the signals of $(Fmoc\text{-}Cys)_2$ (m/z = 685) and (Fmoc-Cys) (Fmoc-

Table I

List of synthetic geographutoxin I peptides with β,β-dideuterio-Cys (Cys*) at two given positions

	Sequence (positions 1, 10, 20 marked)
1	Arg-Asp-Cys -Cys-Thr-Hyp-Hyp-Lys-Lys-Cys -Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
2	Arg-Asp-Cys*-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys*-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
3	Arg-Asp-Cys-Cys*-Thr-Hyp-Hyp-Lys-Lys-Cys*-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
4	Arg-Asp-Cys*-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys*-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
5	Arg-Asp-Cys-Cys*-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys*-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
6	Arg-Asp-Cys-Cys*-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys*-Cys-Ala-NH_2
7	Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys*-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys*-Ala-NH_2

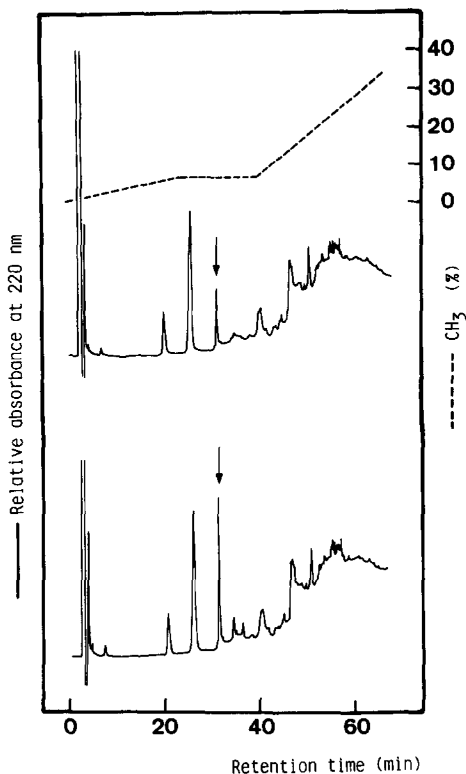

Fig. 1. HPLC profiles of (top) a deprotected and air-oxidized solution of a crude synthetic peptide and (bottom) coelution of a crude synthetic and natural GTX-I. The elution position of natural GTX-I is indicated by an arrow.

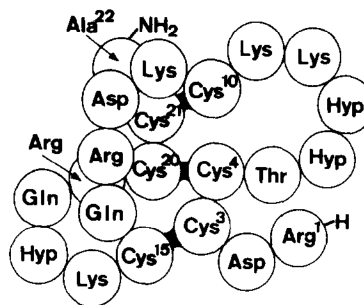

Fig. 3. Positions of disulfide linkages in geographutoxin I.

Cys*) (m/z = 687) was about 1:2. These results indicated that there was no disulfide bridge between Cys^3 and Cys^{10}, Cys^4 and Cys^{10} or Cys^4 and Cys^{15}. On the other hand, hydrolysates of peptides 4 and 6 gave the FAB mass spectrum shown in Fig. 2B in which the ratio of the signals of $(Fmoc\text{-}Cys)_2$ (m/z = 685) and $(Fmoc\text{-}Cys^*)_2$ (m/z = 689) was about 2:1, indicating that disulfide bridges were formed between Cys^3 and Cys^{15} and Cys^4 and Cys^{20}. Therefore, the remaining disulfide bridge should be between Cys^{10} and Cys^{21}, and this was confirmed by the synthesis of peptide 7. Thus, the disulfide pairings in GTX-I are between Cys^3 and Cys^{15}, Cys^4 and Cys^{20}, and Cys^{10} and Cys^{21}, as shown in Fig. 3.

The CD spectra of the synthetic and natural peptides were almost the same and were scarcely affected by

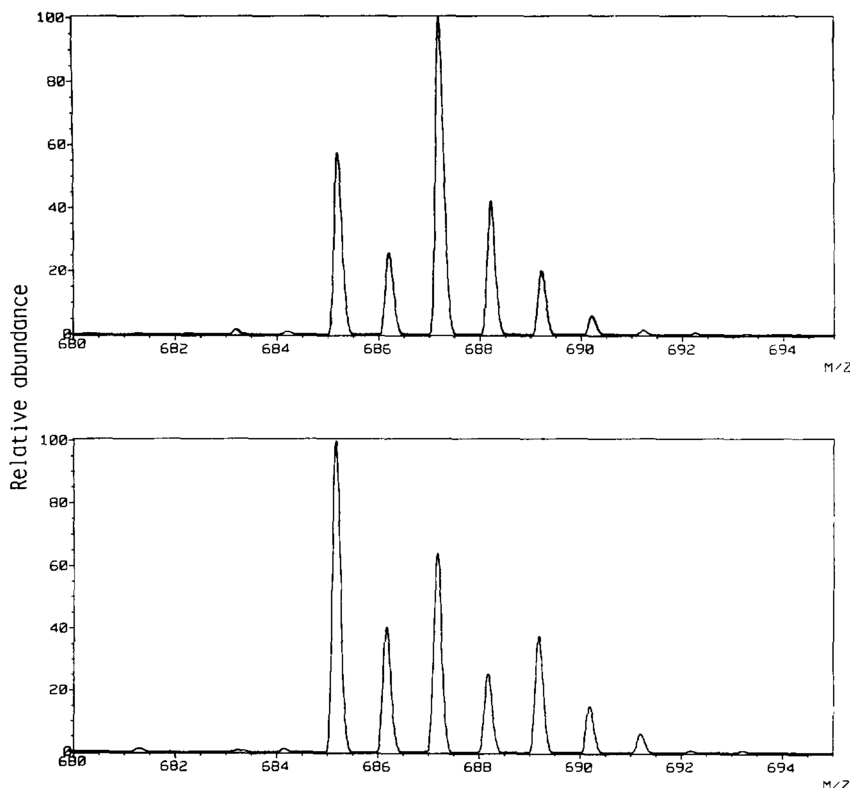

Fig. 2. FAB mass spectra of $(Fmoc\text{-}Cys)_2$ fractions isolated from the reaction products of the hydrolysates of synthetic GTX-I peptides with Fmoc-Cl. (top) From peptides 1, 2 and 4, (bottom) from peptides 3, 5 and 6.

change in the solvent [16], indicating that the conformation of the GTX-I molecule is fixed rigidly by three disulfide linkages, like those of apamin [17] and heat-stable enterotoxin [18]. The prediction of the secondary structure of GTX-I by Chou and Fasman [19] and CPK-model building with the three disulfide linkages (in Fig. 3) suggest that this peptide consists of three loops with β-turn structures from Hyp^6 to Lys^9, Cys^{10} to Arg^{13}, and Hyp^{17} to Cys^{20}, which are stabilized by the three intramolecular disulfide linkages, and hydrophilic side-chains on the outside of the molecule. These structural elements may be involved in the interaction of GTX-I with Na channels.

In this work we found that the disulfide bonds in GTX-I were as shown in Fig. 3. Judging from their close homology with GTX-I, other geographutoxins or μ-conotoxins probably have disulfide linkages in the same positions as those in GTX-I.

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