THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 20, Issue of October 25, pp. 12247–12251, 1983
Printed in U.S.A.

Conotoxin MI

DISULFIDE BONDING AND CONFORMATIONAL STATES*

(Received for publication, December 3, 1982)

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The toxic peptide from *Conus magus* venom (conotoxin MI) is a 14-amino acid peptide (McIntosh, M., Cruz, L. J., Hunkapiller, M. W., Gray, W. R., and Olivera, B. M. (1982) *Arch. Biochem. Biophys.* 218, 329–334) which inhibits the acetylcholine receptor. In this work we have confirmed the primary structure and established the disulfide bonding configuration (Cys 3-Cys 8; Cys 4-Cys 14) by direct chemical synthesis of the toxin with specific disulfide bridges. Natural and synthetic toxins were compared by several methods. Fast atom bombardment mass spectroscopy confirmed that the synthetic product had the expected molecular mass and number of exchangeable hydrogens. Ultraviolet CD spectra were closely comparable in shape and magnitude for the two materials, which were also identical in biological activity and chromatographic behavior. We have also established that, although the peptide is highly cross-linked with two disulfide bridges, it can slowly equilibrate between two conformations. A simulation analysis suggests that the conformers have half-lives of ~12 and ~72 min at 0 °C, decreasing approximately 2-fold for every 10 °C increase in temperature.

We have recently purified a number of toxins from the venoms of fish-eating marine snails of the genus *Conus* (1–4). All *Conus* species are venomous, and a group including *Conus magus* and *Conus geographus* specifically prey on fish. An elaborate venom apparatus is present in these snails, and the venom is injected into the prey by means of a disposable, harpoon-like tooth. One set of toxins (α-conotoxins) which have been identified specifically inhibits the vertebrate acetylcholine receptor at the neuromuscular junction. They are small basic peptides, 13–15 amino acids long, which are homologous in sequence. The four for which amino acid sequences have been assigned are shown in Table I.

The α-conotoxins are thus intermediate in size between the α-neurotoxins from snakes (typically small proteins of 60–75 amino acids) and alkaloids typified by curare which also inhibit this receptor. As with the α-neurotoxins from snakes, there is apparently a wide variety of homologous α-conotoxins in *Conus* species, which should provide a rich data set for analyzing structure-activity relationships in these small peptides. An advantage over the snake toxins is that the peptides are small enough to be amenable to direct chemical synthesis, and quite new structures can be created to test proposed relationships.

Bioassays showed that the peptide from *C. magus*, conotoxin MI, had significantly more activity than the three homologous α-conotoxins from *C. geographus* (4). *C. magus* is a rather small snail, yielding relatively small amounts of venom. In order to investigate the properties of this toxic peptide further, a more dependable source of the peptide and venoms had to be obtained. In this report, we describe the synthesis of biologically active conotoxin MI, thereby establishing the complete covalent structure of this α-conotoxin. In addition, we describe experiments showing that, unlike other conotoxins, conotoxin MI slowly equilibrates between two conformational states.

MATERIALS AND METHODS

Natural Conotoxin MI—Venom was collected from the snails, and bioassays were performed by intraperitoneal injection into mice, as has been described for *C. geographus* peptides (1). Crude venom was first fractionated on a column (2.5 × 92 cm) of Sephadex G-50 eluted with 1.1% acetic acid (v/v). Active fractions were pooled, lyophilized, and purified as described previously (4).

Chemical Synthesis of Conotoxin MI—The following protected peptide was built stepwise from the carboxyl terminus on a benzhydrylamine resin (4 g) (5): *t*-butoxycarbonyl-Gly-Arg(tosyl)-Cys(Sp-methoxybenzyl)-Cys(Acm[1])-His(tosyl)-Pro-Ala-Cys(Sp-methoxybenzyl)-Gly-Lys(2-chlorobenzyloxycarbonyl)-Asn(xanthyl)-Tyr(2,6-dichlorobenzyl)-Ser(OBzl)-Cys(Acm)-benzhydrylamine resin (1% cross-linked; 0.5 meq of amino group/g). Stepwise build-up of the peptide was done automatically on a Beckman 990B synthesizer using previously reported protocols (6) and 1 eq of protected amino acid/g of resin. N^{α}-*t*-butoxycarbonyl protection was used for all amino acid residues and was removed by treatment with trifluoroacetic acid. Since dicyclohexylcarbodiimide was used throughout as the coupling reagent, the following protecting groups were used for the side chain functional groups: benzyl for serine, 2,6-dichlorobenzyl for tyrosine, tosyl for arginine or histidine (liberated from the dicyclohexylamine salt immediately prior to coupling), 2-chlorobenzyloxy carbonyl for lysine, Acm for cysteine in positions 4 and 14, *p*-methoxybenzyl for cysteine in positions 3 and 8, and xanthyl for asparagine. All amino acids were purchased from Bachem and were checked for optical purity by determination of their optical rotation. Peptide was released from the peptide resin (7.9 g) as the COOH-terminal amide by treatment with anhydrous HF (100 ml) in the presence of distilled anisole (12 ml) at 0 °C for 40 min. After removal of the HF under reduced pressure, the peptide resin was washed with 300 ml of diethyl ether in portions. The peptide was extracted from the resin with 200 ml of 5% aqueous HOAc and quickly diluted to 6000 ml of distilled

* A preliminary account of this work was presented at the First International Symposium on HPLC of Proteins and Peptides, Washington, D. C., November 1981. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Grant GM22737 from the National Institutes of Health.

[1] The abbreviations used are: Acm, acetamidomethyl; HPLC, high performance liquid chromatography.

TABLE I
Primary structures of α-conotoxins
Disulfide bridges A and B connect the corresponding Cys residues within conotoxins MI and GI (Ref. 10 and this work). The homologous bridges are assumed to be present in GIA and GII, but have not been established experimentally.

Conotoxin	
	A B A B
MI	Gly-Arg- Cys-Cys-His-Pro-Ala- Cys-Gly- Lys- Asn-Tyr-Ser -Cys-NH_2
	A B A B
GI	Glu- Cys-Cys-Asn-Pro-Ala- Cys-Gly- Arg- His-Tyr-Ser -Cys-NH_2
GIA	Glu- Cys-Cys-Asn-Pro-Ala- Cys-Gly- Arg- His-Tyr-Ser -Cys-Gly-Lys-NH_2
GII	Glu- Cys-Cys-His-Pro-Ala- Cys-Gly- Lys- His-Phe-Ser -Cys-NH_2

H_2O. The acidic peptide solution was allowed to drip overnight into a stirred solution of 1 g of NH_4OAc and 1 g of $K_3Fe(CN)_6$ in 1 liter of H_2O; pH 8 was maintained using a pH-stat following the procedure of Rivier *et al.* (7). The yellow, turbid solution was acidified to pH 5 and treated with approximately 50 ml of Bio-Rad AG 3-X4A anion exchange resin for 30 min. After filtration through Celite, the clear, colorless peptide solution showed no evidence of free sulfhydryl groups after an Ellman test with 5,5′-dithiobis-(2-nitrobenzoic acid) reagent at pH 8. The solution was concentrated by passage through a column containing approximately 75 ml of Bio-Rad BioRex 70 cation exchange resin (H^+ form). After extensive washings with distilled H_2O (200 ml) and 5% aqueous HOAc (300 ml), the peptide was removed from the resin by the addition of 300 ml of 50% aqueous HOAc. The peptide solution was concentrated to a pale yellow oil on a rotary evaporator and immediately passed through a Sephadex G-25F column (2.8 × 200 cm) equilibrated with 5% HOAc and equipped with a flow-retarding peristaltic pump. A major product (700 mg) was isolated from the column effluent after lyophilization. Analysis by HPLC showed the monocyclic product to be contaminated with various impurities, a common observation for peptides obtained by solid phase methodology. Fifty-five mg of this product, crude [$Cys^{4,14}$(Acm)]monocyclic MI, was purified in five applications by passage through a semipreparative HPLC column (Supelco Sil C-18; 5-μm particle size, 1 × 25 cm) in the volatile buffer system 0.1% trifluoroacetic acid/acetonitrile. The flow rate was 5 ml/min with a gradient from 3% CH_3CN to 18% CH_3CN in 25 min. This procedure yielded highly purified [$Cys^{4,14}$(Acm)]monocyclic conotoxin MI (15.3 mg). This peptide gave a negative 5,5′-dithiobis-(2-nitrobenzoic acid) test and had the expected amino acid composition including a cystine molecule and two Cys(Acm) which co-eluted with proline.

Oxidative closure of the second disulfide was carried out by a modification of the method of Kamber *et al.* (8). [$Cys^{4,14}$(Acm)] Monocyclic MI (15.3 mg, 8.9 μmol) was dissolved in *N,N*-dimethylformamide/HOAc (1.5 ml; 8:2, v/v). This peptide solution was added dropwise over 2 min to a well stirred solution of I_2 (6.8 mg, 27 μmol) in the same solvent at 0 °C. Stirring was continued for 12 min at 0 °C and 12 min more at room temperature. The reaction was quenched by the dropwise addition of 0.1 M $Na_2S_2O_3$ solution until total disappearance of the deep amber I_2 color. H_2O (2 ml) was added, and the crude bicyclic peptide was purified by HPLC on a Supelco Sil C-18 column (5-μm particle size, 1 × 25 cm) in the 0.025 M triethylammonium phosphate, pH 2.25, acetonitrile (0–18% in 30 min) system (flow rate 4 ml/min) (9). The major product was desalted on the same column using an increasing gradient of acetonitrile (6–24% in 10 min; flow rate 4 ml/min) in 0.1% trifluoroacetic acid to yield synthetic conotoxin MI (5.0 mg).

Chromatography of the bicyclic peptide product in both triethylammonium phosphate/acetonitrile and 0.1% trifluoroacetic acid/acetonitrile gave highly asymmetrical peaks: a broad, gently sloping leading edge led to a relatively sharp peak at the trailing edge. Material from the sharp portion of the peak had the expected amino acid composition and was characterized further.

Reversed Phase HPLC of Natural and Synthetic Conotoxin MI—Except as noted, all experiments were carried out with a Hewlett-Packard 1084B instrument, using a VYDAC C18 column (5-μm particle size, 0.46 × 25 cm; not end-capped), at a flow rate of 1.5 ml/min. To facilitate bioassays we used only volatile buffers, either 0.1% trifluoroacetic acid/acetonitrile or 0.5% trifluoroacetic acid/acetonitrile. The column effluent was monitored by absorbance at 210 nm. Separations were carried out at several temperatures, in an attempt to improve peak characteristics. Specific conditions are given in the figure legends.

Enzymatic Digests of Natural and Synthetic Conotoxin MI—Trypsin and chymotrypsin were used to cleave the peptide chains, leaving disulfide bridges intact. Samples of 1–5 nmol of either natural or synthetic toxin were incubated in 5 μl of 0.1 M ammonium acetate, pH 8.5, containing 0.5 μg of the appropriate enzyme. After 4 h at 37 °C, the reactions were stopped by the addition of 180 μl of 0.1% trifluoroacetic acid. The digestion products were analyzed on the VYDAC C18 column, using 0.1% trifluoroacetic acid/acetonitrile at 25 °C.

Biological Activity of Conotoxins—Fifteen nmol of each peptide were re-purified by chromatography in 0.5% trifluoroacetic acid/acetonitrile, at 60 °C (see below). Samples of peptide were transferred to small polypropylene tubes and dried *in vacuo*. They were then redissolved in 150 μl of normal saline and injected intraperitoneally into young Swiss-Webster mice (14–16 g). Mice were observed for signs of paralysis; death times were recorded at the cessation of detectable heartbeat. Specific activities were calculated from the linear regression of death time upon reciprocal dose. A sample of each peptide solution was subjected to amino acid analysis to establish the quantitative standard.

Simulation Methods—A simplified simulation of the chromatography of conotoxin MI was done using a PDP-8 computer. The distribution of a peptide at equilibrium across a typical gaussian peak of the characteristic width obtained under our HPLC conditions (Fig. 2) was calculated in 81 slices representing a span of about seven standard deviations. Equilibrium between two conformers (A and B) such that there was a constant ratio of the two forms across the peak was also assumed. Eighty cycles of a calculation were then carried out in which 1) the A component in each of n slices of the peak was shifted by one place, simulating the chromatographic separation of A and B in a given time interval; 2) A and B were allowed to interconvert at fixed rates for the time interval considered in 1, keeping the equilibrium constant fixed; and 3) the new distribution profile of each component was calculated across the $n + 1$ slices created in 1 and used as an input for the next cycle. At the end of the series of 80 cycles, the profile was plotted graphically. The simulation was run repetitively, increasing both rate constants by a factor of 2 each time, thus obtaining a series of curves at each given equilibrium constant but with different rate constants relative to the rate of chromatographic separation of the two components. Complete series of curves were calculated for equilibrium constants of 4–8, each covering a range of 2^{15} in rates.

RESULTS

Chemical Synthesis of Conotoxin MI—We previously purified and determined the primary structure of conotoxin MI from the venom of the marine snail *C. magus*. Although the sequence, including the COOH-terminal amide group was established, the disulfide bonding was not. Unfortunately, the yield of purified toxin is small even under optimal conditions (~50 μg/100 snails). In addition, for unknown reasons, the content of conotoxin MI fluctuates greatly (over 20-fold) from one venom preparation to the next. To establish the disulfide bonding and to obtain enough material for further characterization of the toxin, we undertook a chemical synthesis of the

peptide. Since there are 2 neighboring Cys residues, only two possibilities are energetically feasible (Cys 3-Cys 8, Cys 4-Cys 14 or Cys 3-Cys 14, Cys 4-Cys 8). The peptide with the disulfide bonding pattern Cys 3-Cys 8, Cys 4-Cys 14 was first synthesized. Although no direct evidence for the disulfide bonding in conotoxin MI was available, we had preliminary evidence that the homologous native conotoxin GI had the indicated disulfide bonding pattern.[2]

Chromatographic purification of the peptide gave badly skewed peaks, as mentioned before. Material from the sharpest region was biologically active and gave a satisfactory amino acid analysis. We therefore made detailed comparisons with natural conotoxin MI. Both materials behaved identically, including the abnormal chromatography (see below), so we concluded that the correct covalent structure had been obtained.

Biological Assay of Natural and Synthetic Material—The chemically synthesized conotoxin MI was assayed for biological activity as a function of dose using intraperitoneal injection into mice. As previously described, there is a linear relationship between the observed time of death and the reciprocal of the dose. The results are shown in Table II. The chemically synthesized material shows the same specific activity as the natural conotoxin MI within experimental error. For comparison, the parameters for the homologous conotoxin GI are shown. Clearly MI has a specific toxicity 2.5-fold greater than that of conotoxin GI.

Chromatographic Comparison of Natural and Synthetic Conotoxin MI—As an additional criterion for the identity of the natural and synthetic material, chromatography on HPLC was used, coupled with enzymatic treatments. The natural conotoxin MI gave a broad skewed peak under our standard conditions, with an elution time of 13.7 min. Upon chymotrypsin digestion, the toxin eluted as a sharp peak at 11.1 min. With trypsin digestion, a sharp peak also was generated, but with a still shorter elution time (9.0 min). With synthetic toxin, we obtained a broad skewed peak with the intact toxin eluting at 13.6 min, a sharp peak eluting at 11.1 min after chymotrypsin digestion, and a sharp peak eluting at 8.8 min after trypsin digestion. The similar changes in elution times, sharpening of the peaks after digestion with protease, as well as the toxicity data above established the identity of natural and synthetic toxin.

Circular Dichroism—CD spectra of natural and synthetic toxins were measured using a Jarrell-Aske spectropolarimeter. Both toxins, in 0.01 M sodium phosphate, showed a negative Cotton effect in the peptide bond region, with mean residue ellipticities of -1.3×10^4 degrees $\times$ M $\times$ cm^{-1} (synthetic) and -1.5×10^4 degrees $\times$ M $\times$ cm^{-1} (natural). The total amount of natural material available from all snails obtained in 1982 afforded a concentration of only 10^{-5} M, precluding any more definitive analysis.

Mass Spectroscopy—Although the synthesis of conotoxin MI was designed to yield monomeric peptide, the possibility of dimeric or polymeric structures arising from interchain disulfide bonds needs to be eliminated. For this reason, synthetic toxin was analyzed by fast atom bombardment mass spectroscopy. The material gave an intense pseudomolecular ion at $(MH)^+ = 1493$, with lesser peaks corresponding to $(M + Na)^+$ and $(M + K)^+$. There was no evidence for the presence of materials having residual blocking groups or of shortened peptides due to incomplete coupling steps. After equilibration with glycerol/water containing 92% deuterium, the spectrum shifted to that calculated for a molecule having 27 exchangeable hydrogen atoms (10). Both the absolute mass and the number of exchangeable hydrogens are correct for a monomeric molecule having the proposed structure.

Conotoxin MI Equilibrates between Two Conformations—The broad, skewed peak obtained with both natural and synthetic conotoxin MI (see Fig. 1) contrasted with the sharp peaks obtained after trypsin or chymotrypsin treatment and with those obtained for conotoxin GI. A number of additional experiments strongly suggested that the broad peaks might be due to nonequilibrium behavior of the peptide during elution from the HPLC column.

We isolated material from the first third of a typical peak and reran it under the same conditions. The complete profile was regenerated (Fig. 1, *curve B*), eliminating explanations such as chemical breakdown or modification. In addition, a similar broad peak was seen on testing a different reversed phase column which had given sharp peaks with other conotoxins (Supelco LC18; data not shown). Finally, the basic

TABLE II

Bioassay of natural and synthetic conotoxins

Material	Regression parameters[a]		Specific activity[b]
	A	*B*	
			units/nmol
Conotoxin			
MI natural	4.3	4.1	3.8
MI synthetic	4.4	3.9	4.0
GI natural	2.8	11.6	1.5

[a] *A* and *B* are coefficients in the regression equation: death time (min) = $A + B \times$ (reciprocal dose (20 g/nmol)).

[b] The unit of activity is the quantity of material needed to cause death of a 20-g mouse in 20 min (Ref. 1).

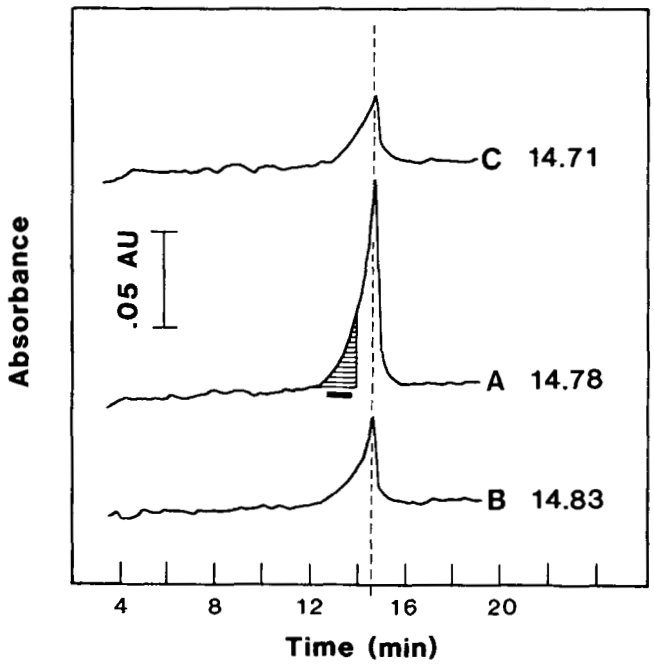

FIG. 1. **Chromatography of conotoxin MI on VYDAC C18 at 25 °C.** Solvent A, 0.1% trifluoroacetic acid in 60% (v/v) acetonitrile. Gradient was made up of linear segments, indicated as per cent B achieved at times given in parentheses (minutes): 10 (0)/10 (2)/35(25). Peak times are indicated at the *right*. *Curve A*, synthetic toxin, approximately 7.5 nmol. *Hatched area* indicates fraction that was rerun in *curve B*. *Curve B*, rerun of early portion of peak from *curve A*. *Curve C*, natural toxin, isolated by gel filtration and ion exchange chromatography, approximately 1 nmol.

[2] Gray, W. R., Luque, A. F., Stone, B. C., Alford, J., McIntosh, M., Olivera, B. M., Reyes, A., Cruz, L. J., and Rivier, J., submitted to *Biochemistry*.

features of peak shape were independent of loading in an 80-fold concentration range (0.5–40 nmol) of peptide.

It should be emphasized that the skewing behavior is so far unique to conotoxin MI. Several closely related conotoxins (GI, GIA, and GII) and various derivatives of GI give excellent symmetrical peaks on all HPLC systems tested.

The strong *forward* skew of conotoxin MI peaks suggested to us the occurrence of a slow interconversion between two or more forms of the peptide in solution, rather than nonequilibrium partitioning between free and bound phases. A 5-fold increase in concentration of the ion-pairing agent in the chromatographic elution buffer, to 0.5% trifluoroacetic acid, led to the expected increase in retention time (Fig. 2; 25 °C), but did not cause any obvious change in peak shape. Regardless of the cause of disequilibrium, we expected an increased temperature to improve peak shape by speeding up the attainment of equilibrium. A marked improvement was obtained at 50 and 60 °C (see Fig. 2).

The two modes of nonequilibrium behavior should give different results on *lowering* the temperature during chromatography. If disequilibrium is between sorption/desorption, one expects the problem to become worse at low temperature. By contrast, disequilibrium between two peptide conformations, when carried to an extreme, should lead to separation of two sharp peaks with an elevated base-line between them. Precisely this result was obtained by immersing the column in an ice-bath during chromatography of conotoxin MI (Fig. 3). This result strongly indicates that the anomalous behavior is due to retarded equilibrium between conformational states of a single peptide.

Simulation of Chromatographic Behavior—To obtain further insight into the peptide's behavior, we have carried out a simplified simulation of the chromatographic process. The assumptions involved in the simulation are based on the observation that in the linear parts of the gradients used for HPLC, well behaved peptides eluted with profiles that were close to gaussian distributions of constant width. We assume that a peptide was at equilibrium across a single peak of this shape and characteristic width. We then assume an equilibrium between two conformational forms (A and B conformers). In each simulation an equilibrium constant between the A and the B conformers was assumed, and A and B were then allowed to interconvert at fixed rates for a particular time interval. The details of the simulation are given under "Materials and Methods." Each simulation was plotted graphically, and the parameters for the simulation were chosen so that at low interconversion rates, the peak widths relative to the peak separation approximated those obtained for the real behavior of conotoxin MI at 0 °C.

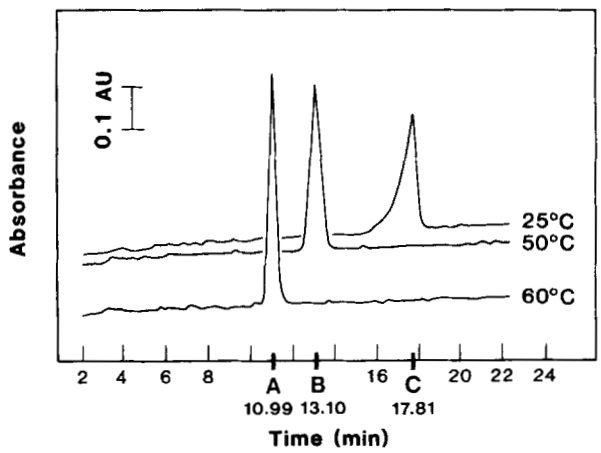

FIG. 2. **Effect of elevated temperature on chromatography of synthetic conotoxin MI.** Experimental details for HPLC are as described in the legend to Fig. 1, except that 0.5% trifluoroacetic acid was used, and chromatography was carried out at the indicated temperature.

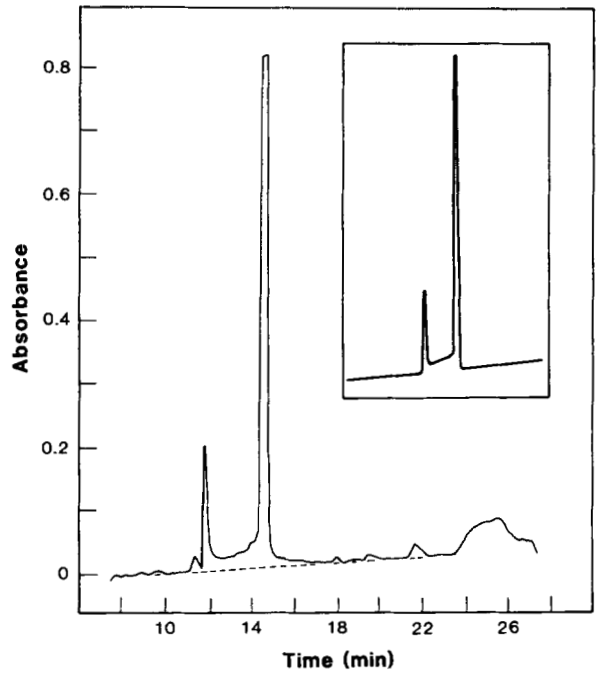

FIG. 3. **Chromatography of conotoxin MI at 0 °C on VYDAC C18 eluted with buffers described in the legend to Fig. 1.** Flow rate was 1.0 ml/min. Gradient: 10 (0)/10 (1)/50 (20). *Inset*, elution profile expected for slowly interconverting conformers with equilibrium ratio of 1:6. For both curves, the full height of the larger peaks is not drawn in.

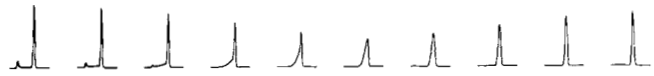

FIG. 4. **Simulation of chromatographic behavior of a peptide that exists as a pair of interconverting conformers.** See text for details of simulation. An equilibrium constant of 6 is assumed throughout the set, and the total area under the peak(s) is the same in each. Successive frames increase the individual rate constants by a factor of 2.

One such series of simulations which best fits the experimental data is shown in Fig. 4. In this series, it is assumed that the equilibrium constant for A → B is 6. The different curves are successive 2-fold increases in the interconversion rates of A and B, holding the equilibrium constant at a fixed value. It is seen that in this particular series, the simulated curves reproduce in a general way the changes observed in going from 0 to 60 °C in the chromatography of conotoxin MI. The *inset* in Fig. 3 shows a direct comparison between the observed chromatographic behavior at 0 °C and a simulation using the conditions of Fig. 4 (*second curve* from *left*). In this case the results were graphed on a sloping base-line such as was obtained in the actual chromatogram (the latter was recorded at a relatively high sensitivity to emphasize the elevated base-line between the two sharp peaks).

Although the simulation given is simplified and quite qualitative, the interconversion rates at 0 °C for conotoxin MI correspond to simulated half-lives of 12 and 72 min for conformers A and B, respectively. It is somewhat more difficult to match chromatograms at the higher temperatures and interconversion rates, but it appears that the rates increase

about 2-fold for every 10 °C rise in temperature, without any major effect on equilibrium constant.

DISCUSSION

In this report, we have confirmed our previous assignment of the primary structure of conotoxin MI by complete synthesis of the 14-amino acid peptide amide. Biologically active synthetic peptide has been obtained, which is indistinguishable from the natural conotoxin MI by all criteria used. The synthesis of conotoxin MI also establishes the disulfide-bonding pattern within the 14-amino acid peptide. The complete covalent structure of conotoxin MI is as follows.

Gly-Arg-Cys-Cys-His-Pro-Ala-Cys-Gly-Lys-Asn-Tyr-Ser-Cys-NH_2

In addition, we have quantitated our earlier observation (4) that conotoxin MI had significantly higher biological activity than the corresponding α-conotoxins from *C. geographus*. In this report we have presented evidence that the homogeneous conotoxin MI has a specific activity 2.5-fold higher than that of conotoxin GI. There is a strong sequence homology between conotoxins GI and MI (2); this report and recent work[2] with conotoxin GI establishes that the disulfide bonds are formed between homologous Cys residues in the two peptides. Thus, the difference in specific activity between conotoxins MI and GI must be due to the amino acid substitutions.

The various polymorphic forms of α-conotoxins from *C. geographus* have biological activities in a narrow range (2).[2] Two of the four differences between GI and MI occur also between GI and GII (Asn 4 → His and Arg 9 → Lys), so it is unlikely that they are major determinants of the increased biological activity of MI. An important difference is likely to be the increased positive charge at the NH_2 terminus, due to substitution of Glu 1 by Gly-Arg; potent antagonists of the acetylcholine receptor typically are highly basic molecules. The other substitution, His 10 → Asn, is also in the part of the molecule suspected of being involved in receptor binding, but no specific role is postulated for this residue.[3]

Our most remarkable finding with conotoxin MI is that it can apparently equilibrate slowly between two alternative conformational forms. This is in marked contrast to the other α-conotoxins isolated, for which there is no evidence for alternative conformations. It will be of interest to establish whether the same amino acid differences between GI and MI which are critical for higher biological activity are also important in the conformational transition in MI. The wide separation of the two forms during HPLC at low temperature suggests that the two conformations have different hydrophobic interactions with the HPLC column; we infer that their interactions with hydrophobic surfaces in the acetylcholine receptor would also be different. Our preliminary attempts to try to assay biological activities of the two forms have so far been unsuccessful, because under most physiological conditions, equilibration between the two forms is too rapid.

It is clear that although α-conotoxins as a group form a coherent set of toxins that strongly inhibit the vertebrate acetylcholine receptor, relatively subtle changes in amino acids at homologous positions can cause large effects in biological activity and in the biophysical properties of these small toxic peptides. We are optimistic that a knowledge of the solution conformations of these molecules will lead to a more thorough understanding of the processes occurring at the receptor.

[3] Gray, W. R., Middlemas, D., Luque, A. F., Cruz, L. J., Olivera, B. M., and Rivier, J., submitted to *Nature* (*Lond.*).

REFERENCES

1. Cruz, L. J., Gray, W. R. & Olivera, B. M. (1978) *Arch. Biochem. Biophys.* **190,** 539–548
2. Gray, W. R., Luque, A., Olivera, B. M., Barrett, J. & Cruz, L. J. (1981) *J. Biol. Chem.* **256,** 4734–4740
3. Clark, C., Olivera, B. M. & Cruz, L. J. (1981) *Toxicon* **19,** 691–699
4. McIntosh, M., Cruz, L. J., Hunkapiller, M. W., Gray, W. R. & Olivera, B. M. (1982) *Arch. Biochem. Biophys.* **218,** 329–334
5. Rivier, J., Amoss, M., Rivier, C. & Vale, W. (1974) *J. Med. Chem.* **17,** 230–233
6. Marki, W., Spiess, J., Tache, Y., Brown, M. & Rivier, J. E. (1981) *J. Am. Chem. Soc.* **103,** 3178–3185
7. Rivier, J., Kaiser, R. & Galyean, R. (1978) *Biopolymers* **17,** 1927–1938
8. Kamber, B., Hartmann, A., Eisler, K., Riniker, B., Rink, H., Sieber, P. & Rittel, W. (1980) *Helv. Chim. Acta* **63,** 899
9. Rivier, J. E. (1978) *J. Liq. Chromatogr.* **1,** 343–366
10. Sethi, S. K., Smith, D. L. & McCloskey, J. A. (1983) *Biochem. Biophys. Res. Commun.* **112,** 126–131