Contryphan Is a d-Tryptophan-containing Conus Peptide*

(Received for publication, August 19, 1996, and in revised form, September 18, 1996) Elsie C. Jimènez‡§, Baldomero M. Olivera§¶, William R. Gray§, and Lourdes J. Cruz‡¶

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In this report, we document for the first time the occurrence of d-tryptophan in a normally translated polypeptide, contryphan. The peptide, isolated from the venom of the fish-hunting marine snail Conus radiatus, produces the "stiff-tail" syndrome in mice. Characterization of the octapeptide gave the following sequence,

Gly-Cys-Hyp-d-Trp-Glu-Pro-Trp-Cys-NH₂

SEQUENCE 1

where Hyp = 4-trans-hydroxyproline.

The presence of d-tryptophan in position 4 of contryphan was confirmed by chemical synthesis. The post-translational epimerization in all other d-amino acid-containing small peptides characterized previously from vertebrates and molluscan systems is in position 2.

The standard amino acids in polypeptides translated from genes are exclusively in the L-configuration. In recent years it has been established that d-amino acids can be post-translationally introduced into such polypeptides (1). Several small peptides have been characterized, which contain a d-amino acid. The first of these was dermorphin, a potent heptapeptide containing small peptides characterized previously from vertebrates and molluscan systems (2). A number of other peptides have been characterized, which contain a d-amino acid described previously for all other small peptides. The cDNA encoding the precursor of fulicin was found to contain the usual L-Asn codon at the D-asn position (3, 4). The results demonstrated unequivocally the presence of mRNA encoding the peptide precursor, indicating that the d-amino acid was posttranslationally formed from the corresponding L-isomer.

In addition to these vertebrate systems, small peptides with d-amino acids have also been described in invertebrate systems, primarily molluscs. An FMRFamide analog from the bivalve, Mytilus edulis, which contains a d-leucine, has been characterized (5). Likewise, the land snail Achatina fulica has d-amino acid-containing small peptides, achatin-I and fulcin (6, 7). The cDNA encoding the precursor of fulcin was found to contain the usual L-Asn codon at the D-Asn position (8). Recently, the post-translational inversion of an amino acid was demonstrated in vitro for ω-agatoxin-Ivb (also termed ω-agatoxin-TK), a calcium channel inhibitor from funnel web spider (9). The peptide isomerase that preferentially acts on Ser⁴⁶ of the 48-amino acid peptide has been isolated and characterized.

The small peptides which appear to be post-translationally modified to convert an L- to a D-amino acid from a variety of phylogenetic systems are shown in Table I. Although there is no homology between vertebrate and invertebrate peptides (and the three molluscan peptides exhibit no sequence similarity), in every case the d-amino acid is found in the second position. This suggests that for small d-amino acid-containing peptides, the proteolytic event that generates the mature peptide and the post-translational enzymatic system that converts an L- to a D-amino acid work in combination to always generate the D-amino acid at position 2.

In this report, we describe the purification and characterization of a novel d-amino acid-containing peptide from the venom of Conus radiatus, which causes a "stiff-tail syndrome" in mice. This octapeptide, contryphan, has a D-tryptophan residue. This is the first report of d-tryptophan being formed through post-translational modification. Furthermore, in contrast to all of the small d-amino acid-containing peptides shown in Table I, contryphan does not have the d-amino acid in position 2. Like most peptides found in Conus venoms, contryphan is cross-linked by a disulfide bond; all other peptides in Table I are not disulfide-crosslinked. The discovery and characterization of contryphan indicates that the modification system for converting L- to D-amino acids evolved in Conus venom ducts differs significantly from the post-translational isomerization of an L- to a D-amino acid described previously for all other small peptides in both vertebrate and previously characterized molluscan systems.

MATERIALS AND METHODS

Preparation of Venom Extract—Specimens of C. radiatus were obtained from the Philippines. The venom ducts were dissected from the cone snails, and the venom was squeezed out of the ducts as described previously (10). The collected venom was lyophilized and stored in the freezer. Five-hundred milligrams of lyophilized venom was sequentially extracted with 10 ml each of water, 20% acetonitrile, 40% acetonitrile, and 60% acetonitrile. The venom suspension was sonicated in the extracting solvent for 30-s periods over ice water and centrifuged at 5000 × g for 5 min. The combined supernatants were stored at −20 °C for further purification.

Purification of Peptides—Crude venom extract was applied onto a Vydac C₁₈ semi-preparative column (10 × 250 mm) and eluted with a linear gradient of acetonitrile in 0.085% trifluoroacetic acid at 5 ml/min. Further purifications of bioactive peaks were done on a Vydac C₁₈ analytical column (4.6 × 250 mm) eluted with the acetonitrile/trifluoroacetic acid system at 1 ml/min. The effluents were monitored at 220 nm. Peaks were collected in polypropylene tubes and aliquots were assayed for biological activity.

Bioassay—Biological activity was assayed by intracranial injection in mice (9–21 days old). Aliquots (20–30 µl) of peptide in normal saline were injected using a 0.3 ml syringe with a 29-gauge needle. Each control mouse was injected with an equal volume of normal saline solution containing dissolved residue of lyophilized column buffer. After injection, the mice were placed in cages for observation.

Peptide Sequencing—The peptide was reduced and alkylated prior to sequencing. The pH of the peptide solution was raised to 7–8 by adding 0.5 M Tris base before dithiothreitol was added to a final concentration of 10 mM. The solution was flushed with nitrogen, incubated at 65 °C for 20 min, and cooled to room temperature. Three microliters of 4-vinylpyridine were added per 500 ml of solution. The mixture was left in

¶ This work was supported by National Institutes of Health Program Project GM48677 and a fellowship from Department of Science and Technology-Engineering and Science Education Program, Philippines (to E. C. J.). 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.

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the dark for 25 min at room temperature then diluted with 0.1% trifluoroacetic acid and applied onto a Vydac C18 analytical HPLC column.

The purified peptide was sequenced by automated Edman degradation on an Applied Biosystems 477A protein sequencer with a 120A analyzer (DNA/Peptide Facility, University of Utah). The 3-phenyl-2-thiophenylmethylamine derivatives were identified by HPLC. The predicted mass of the peptide was compared with the molecular masses obtained by Dr. Anthony Craig of the Salk Institute for Biological Studies using laser desorption mass spectrometry and liquid secondary ionization mass spectrometry (LSIMS). The LSIMS samples were measured at 3000 resolution from a glycerol matrix.

Peptide Synthesis—The protected peptide resin was synthesized using standard Fmoc (N-(9-fluorenylemethyl)carbonyl) chemistry and couplings using equimolar amounts of amino acid derivatives dicyclohexylcarbodiimide and hydroxybenzotriazole. Some amino acid side chains were protected as follows: Cys(trityl), OH-Pro(t-butyl), and Glu(t-buty1).

After synthesis, the terminal Fmoc group was removed by treatment with 1:4 piperidine:N-methylpyrrolidone (v/v). The peptide was cleaved from the resin by treatment with trifluoroacetic acid/H2O/ethanedithiol/phenol/thioanisole (80/5/2.5/7.5/5 by volume) for 1.5 h at room temperature. The mixture was filtered under vacuum into 3 ml of 0.1 M ascorbic acid. The reaction was quenched with a few drops of 0.1 M ascorbic acid and applied onto a Vydac C18 analytical column eluted with a very slow linear gradient of ACN in 0.085% trifluoroacetic acid (0.09% rise in ACN/min) in order to remove the trifluoroacetic acid residues.

The major peptide-containing fraction was oxidized with 30% hydrogen peroxide and eluted from the resin by treatment with trifluoroacetic acid/H2O/ethanedithiol/phenol/thioanisole (80/5/2.5/7.5/5 by volume) for 1.5 h at room temperature. The mixture was filtered under vacuum into 3 ml of 0.1 M ascorbic acid. The reaction was quenched with a few drops of 0.1 M ascorbic acid. The oxidized peptide was purified by HPLC.

### RESULTS

**Purification of Contryphans**—Crude venom was obtained from specimens of *C. radiatus* collected from Manila Bay and Marinduque. The crude venom extract prepared as described under “Materials and Methods” was applied to a C18 semi-preparative column, using a gradient of 0.45% CH3CN/min. A complicated HPLC profile, typical of *Conus* venoms was obtained (see Fig. 1A). When injected intracranially in mice, material from the peaks indicated by the arrows caused mild excitatory symptoms at low doses with tail raising and severe symptoms including “barrel rolling” and seizure observed with increasing dosages (see Table II).

The peak designated as *R* was resolved into several peaks on HPLC in a C18 analytical column eluted with a very slow gradient of acetonitrile. The first major peak, which showed the same activity as the *R* peak of *A* was rechromatographed to give a homogeneous peak shown in Fig. 2. Similar HPLC runs of the *R1* peak of *A* gave the profile in Fig. 2; rechromatography of this peak gave a homogeneous peptide.

**Biochemical Characterization and Biological Activity of Contryphan and Its Des-[Gly]
Derivative**—The purified peptides were reduced, alkylated, and sequenced as described under “Materials and Methods.” The amino acid sequence obtained for peak *R* is shown in Table III. Because of the high tryptophan content of this peptide, we designate it as “contryphan.” Peptide *R1* had an identical sequence except that it lacks the NH2-terminal Gly residue. Thus, the amino acid sequences of the two peptides are as follows.

| Peptide         | Sequence                  | Source     | Phylum     | Ref. |
|-----------------|---------------------------|------------|------------|------|
| Dermorphin      | YAGFYP*                   | *P. sauvagei* | Vertebrata | 2    |
| Met-deltorphin  | YMFHLMD*                  | *P. sauvagei* | Vertebrata | 20, 21 |
| Achatin-I       | GFAD                      | *A. fulica* | Mollusca   | 6    |
| Fulicin         | FNEFV*                    | *A. fulica* | Mollusca   | 7    |
| Mytilus-FFRFamide | ALAGDHFFRF*             | *M. edulis* | Mollusca   | 5    |

*The abbreviations used are: HPLC, high performance liquid chromatography; ACN, acetonitrile; LSIMS, liquid secondary ionization mass spectrometry.

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The calculated MH$^+$ value assumes that the Cys residues form a disulfide and an amidated COOH-terminal residue. The most striking feature of these peptides is the presence of D-tryptophan, the first occurrence of this unusual amino acid in a Conus peptide. Indeed, as far as we are aware, D-tryptophan has not been described previously in any mRNA-encoded polypeptidic structure. Analysis of a cDNA clone from a C. radiatus venom duct library encoding contryphan has shown that D-Trp is coded for by the standard codon for L-Trp. Previous reports of the occurrence of D-tryptophan are in peptide-like structures, which are almost certainly not translated through the normal ribosomal route, such as in the fungal aselacins, which are cyclic pentapeptolides (13).

In recent years it has been demonstrated that D-amino acids can be introduced into small peptides through post-translational modification (1). The sequences of D-amino acid-containing small peptides is shown in Table I. Contryphan is distinct from D-amino acid-containing peptides previously characterized in several respects. It appears to be the only small peptide (≤10 amino acids) containing a D-amino acid residue, which is also disulfide-crosslinked. Furthermore, in other small peptides, although a variety of different D-amino acids have been found (including alanine, asparagine, methionine, phenylalanine, and leucine), the D-amino acid is always the penultimate NH₂-terminal amino acid (Table I). It has been suggested that the isomerase for the Leu → Asp amino acid conversion may cue into the dibasic signal for proteolytic processing of peptide precursors (1), post-translationally converting the second amino acid into the D-configuration regardless of the identity of the amino acid at the designated locus. For contryphan the number 2 position rule could not apply, since the D-amino acid

### TABLE II

| Dose       | Activity                                                                 |
|------------|--------------------------------------------------------------------------|
| Low: 0.5–2.5 nmol/g | Grooming, licking and biting of paws, occasional tail-raising, occasional hyperactivity. |
| Medium: 4–6 nmol/g   | Stiffening and raising of tail to almost perpendicular to body; barrel-rolling, and subsequent passivity. |
| High: 8–20 nmol/g   | Paralysis of extremities, circular motion, barrel-rolling, seizure, and death. |

The calculated MH$^+$ = 933.3 (calculated MH$^+$ = 933.3) and a monoisotopic MH$^+$ of 933.3 (calculated MH$^+$ = 933.3) for des-[Gly¹]contryphan. These values are consistent with the amino acid sequences assigned with the Cys residues present as disulfides and the COOH termini as amide groups.

To independently verify the sequence, synthetic peptides containing standard L-amino acids were prepared as described under “Materials and Methods.” However, the synthetic peptides did not co-elute with native contryphan. The all-L analog of contryphan showed an unusually broad HPLC peak, and it appeared to be less stable than the native peptide; flushing the synthetic peptide solution with nitrogen seemed to retard its degradation to a purple derivative. Because of the identical mass of the native and synthetic L-amino acid-containing peptide, synthetic peptide homologs containing D-tryptophan at positions 4 and 7 of contryphan were synthesized. Both the fully reduced and the oxidized/folded forms of the synthetic D-Trp⁴ peptide co-eluted with the corresponding forms of the natural peptide (see Fig. 2). The D-Trp⁴ and D-Trp⁷ analogs did not co-elute with native contryphan (Fig. 3). The presence of D-tryptophan in position 3 of des-[Gly¹]contryphan was also confirmed by chemical synthesis and co-elution of native and synthetic material.

On intracranial injection into mice, synthetic contryphan elicited identical symptomaticity to the native material (tail raising and hyperactivity) at low doses. Surprisingly, the all-L analog, l-Trp⁴ contryphan shows a similar activity, whereas D-Trp⁴ contryphan elicited no detectable activity (>10 nmol/g body weight). Des-[Gly¹]contryphan elicited similar symptoms to contryphan but appears to be less potent; even at the highest doses (20 nmol/g), the peptide did not cause any lethality.

**DISCUSSION**

The data presented above document the purification, characterization, and chemical synthesis of two homologous peptides, contryphan and des-[Gly¹]contryphan from the venom of Conus radiatus. The most striking feature of these peptides is...
These systems, the D-amino acid is not found in position 2. Foraminal amino acid residues are post-translationally modified systems, very much larger polypeptides with D-amino acids positional cue. It is relevant to note that in two arthropod other peptides with amino acids that have been post-translational of Ser46 to the D-isomer has been found to enhance toxin Occurred, calculated from the elution time. The elution position of Peaks are labeled with the apparent percent ACN at which elution linear gradient from 13.5 to 40.5% ACN in 0.08% trifluoroacetic acid. Analytical column (Vydac, 218TP54, 4.6 x 250 mm) and eluted using a and analogs.

Mytilus, the modification system in Conus, contryphan; b, d-[Trp4]contryphan; c, L-[Trp7]d-[Try3]contryphan; d, L-[Trp4,D-Trp7]contryphan. Peptides were applied to a C18 Reverse-phase HPLC chromatograms of contryphan or present at much lower levels than the D-Trp isomer. Preliminary results suggest that contryphans are distributed more widely in Conus venoms, homologous peptides have been found in venoms collected from other Conus species. Thus, the ability to post-translationally modify an L- to a D-amino acid may be widespread in Conus, and additional D-amino acid-containing peptides unrelated to contryphan could well be present in Conus venoms.

When injected into the mouse central nervous system, contryphan elicits characteristic symptoms such as the stiff-tail syndrome (Table II). At higher doses, more generalized excitatory effects (i.e., barrel rolling and seizures) are induced. The mechanistic basis for the symptomatology observed is presently unknown. Recently, we suggested that at least some of the excitatory activities present in the venoms of fish-hunting cone snails may act together to cause an excitotoxic shock response in the prey (19), which leads to very rapid immobilization of the fish prey. Given the excitatory effects of contryphan in the mammalian central nervous system, a possible function for this peptide in the venom is to contribute to the excitotoxic shock response.

Acknowledgments—We thank Rick Jacobsen and Ki-Joon Shon for their contributions to this work.

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