Conantokin-G Precursor and Its Role in \(\gamma\)-Carboxylation by a Vitamin K-dependent Carboxylase from a Conus Snail*

(Received for publication, October 15, 1997, and in revised form, December 18, 1997)

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Conantokin-G isolated from the marine snail Conus geographus is a 17-amino acid \(\gamma\)-carboxyglutamate-(Gla)-containing peptide that inhibits the N-methyl-D-aspartate receptor. We describe the cloning and sequence of conantokin-G cDNA and the possible role of the propeptide sequence. The cDNA encodes a 100-amino acid peptide. The N-terminal 80 amino acids constitute the prepro-sequence, and the mature peptide is derived from the remaining C-terminal residues after proteolysis, C-terminal amidation, and a unique post-translational modification, \(\gamma\)-carboxylation of glutamate residues to Gla. Mature conantokin-G peptide containing Glu residues (E.Con-G) in place of Gla is a poor substrate for the vitamin K-dependent \(\gamma\)-glutamyl carboxylase (apparent \(K_m = 3.4\) mM). Using peptides corresponding to different segments of the propeptide we investigated a potential role for the propeptide sequences in \(\gamma\)-carboxylation. Prepropeptide segment –20 to –1 covalently linked to E.Con-G or the synthetic pentapeptide FLEEL increased their apparent affinities 2 orders of magnitude. These substrates are not efficiently carboxylated by the bovine microsomal \(\gamma\)-glutamyl carboxylase, suggesting differences in specificities between the Conus and the mammalian enzyme. However, the role of propeptide in enhancing the efficiency of carboxylation is maintained.

The vitamin K-dependent \(\gamma\)-carboxylation of glutamate residues was originally discovered as a novel post-translational modification in the blood coagulation cascade (1); some of the key clotting factors such as prothrombin must be \(\gamma\)-carboxylated in order for proper blood clotting to occur. Somewhat later, this post-translational modification was also found in certain bone proteins (2). This modification was restricted to these rather specialized mammalian systems until a very unusual peptide, conantokin-G, was described from the venom of the predatory marine snail, Conus geographus (3). Conantokin-G is a 17-amino acid peptide that inhibits the N-methyl-D-aspartate receptor (4). Unlike most Conus peptides, which are multiply disulfide-bonded, conantokin-G has no disulfide cross-links but has 5 residues of \(\gamma\)-carboxyglutamate residues; this remains the highest density of \(\gamma\)-carboxyglutamate found in any functional gene product characterized to date.

Most of the biologically active components of the Conus venom are multiply disulfide bonded peptides (the conotoxins). These have been shown to be initially translated as prepropeptide precursors, which are then post-translationally processed to yield the mature disulfide-cross-linked conotoxin. Conantokin-G differs strikingly from most conotoxins not only in having \(\gamma\)-carboxyglutamate residues, but also because it has no disulfide cross-links. We report below an analysis of a cDNA clone encoding the conantokin-G precursor. Furthermore, we establish the probable function of one region of the precursor that is excised during the maturation of the functional conantokin-G peptide.

The presence of \(\gamma\)-carboxyglutamate in a non-mammalian system was initially controversial because vitamin K-dependent carboxylation of glutamate residues had primarily been thought to be a highly specialized mammalian innovation. However, we have found that conantokin-G is only one member of a family of peptides; a variety of other conantokin peptides have been found including conantokin-T and conantokin-R from two other fish-hunting cone snails (5, 6). All three peptides have a high content of \(\gamma\)-carboxyglutamate (4–5 residues). \(\gamma\)-Glutamyl carboxylase has been purified from mammalian sources (7, 8) and has been expressed both in mammalian and insect cell lines (9, 10). Recently it was shown that, as is the case in the mammalian system, the carboxylation reaction in Conus venom ducts absolutely requires vitamin K, and the net carboxylation increases greatly in the presence of high concentrations of ammonium sulfate. In these respects, the mammalian and the Conus \(\gamma\)-carboxylation venom systems are very similar (11).

The propeptides of vitamin K-dependent blood coagulation proteins share extensive sequence similarity. This sequence is believed to interact with the carboxylase and constitutes the \(\gamma\)-carboxylation recognition sequence (\(\gamma\)-CRS). In this report, we analyze the conantokin-G precursor sequence for potential \(\gamma\)-CRS sequences. The results described below identify a sequence present in the –1 to –20 region of the conantokin-G propeptide, which when covalently linked to the N-terminal of the substrate stimulates carboxylation by the Conus enzyme.

MATERIALS AND METHODS

Conus radiatus venom ducts were obtained from Dr. L. J. Cruz (University of the Philippines). Vitamin K (phytonadione) was from Abbot Laboratories, and NaH\(^{14}\)CO\(_3\) 55 mCi/mmol from NEN Life Science Products. Bovine microsomes were a gift from Dr. D. W. Stafford (University of North Carolina, Chapel Hill, NC).

Conus microsome preparation from frozen venom ducts of C. radiatus was performed as described by Stanley et al. (11). Carboxylase assay using 1 \(\mu\)g of Conus microsomal protein per assay was performed as follows: Conus microsomes were solubilized in 0.7% CHAPS/0.5% phosphatidyl choline/1.5 mM NaCl for 20 min on ice. Final reactions were done in a total volume of 125 \(\mu\)l containing solubilized microsomes and a final concentration of reagents as follows: 25 mM MOPS, pH 7.4, 0.5 mM NaCl, 0.2% CHAPS, 0.2% phosphatidyl choline, 0.8 mM ammonium sulfate, 5 mM \(\gamma\)-carboxyglutamate. 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.

* This work was supported by National Institutes of Health Grant GM 48677 (to B. M. O.) and by Cognetix Inc. (to P. K. B. and C. S. W.).

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Characterization of the Conantokin-G Precursor—A cDNA clone encoding conantokin-G was identified as described under “Materials and Methods.” The clone was sequenced in both strands, and the nucleotide sequence obtained is shown in Fig. 1. The predicted amino acid sequence of the open reading frame encoded by this cDNA clone and the post-translational modifications that take place to yield the mature peptide are also indicated in the figure. The cDNA sequence predicts the presence of a valine residue at position 5 of the mature toxin sequence. Hybridization was done in 3× tetramethyl ammonium chloride (Aldrich), 0.1× NaHPO₄, 0.001× EDTA, 5× Denhardt’s solution, 0.6% SDS, 100 µg/ml sheared salmon sperm DNA for 24 h at 48 °C. Washes were done at room temperature in 3× tetramethyl ammonium chloride, 0.05× Tris-HCl, pH 8, 0.2% SDS for 15 min and 1 h at 50 °C in a solution of the same composition. The filters were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min each. The filters were then exposed to x-ray films. Double stranded DNA from purified clones were sequenced by dideoxynucleotide chain terminating method (13).

RESULTS

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μCi of NaH¹⁴CO₃, 6 mm dithiothreitol, 222 µm reduced vitamin K (prepared as described by Ref. 12). Substrate and inhibitor concentrations are indicated in the legends to the figures and tables. For experiments with bovine microsomes, 380 µg of microsomal protein was present in each reaction. Reaction mixtures were incubated at 30 °C for 30 min and were quenched by the addition of 75 µl of 1× NaOH. 160 µl of the quenched reaction mixture was transferred to 1 ml of 5% trichloroacetic acid and boiled to remove unincorporated ¹⁴CO₂. After cooling, 5 ml of Ecolite (NEN Life Science Products) was added, and the ¹⁴CO₂ incorporated was determined in a Beckman LS 8900 counter. The amount of microsomal proteins present in the various experiments is indicated under “Results.” All reported values are averages of three independent determinations.

Peptides were synthesized on a 357ACT peptide synthesizer (Advanced ChemTech) using Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry strategy. The peptides were cleaved from the solid support with treatment with trifluoroacetic acid/phenol/thioanisole (96/5/2.5/2.5 by volume) and purified by reverse-phase high pressure liquid chromatography. The integrity of the peptides were verified by electrospray mass spectroscopy. Experiments were done in triplicate, and the data were analyzed using Graph Pad Prism from GraphPad Software, Inc. (San Diego, CA).

Isolation of conantokin-G cDNA was as follows. A cDNA library from C. geographus, in a pUC plasmid derivative, was plated out in duplicate and screened with end-labeled degenerate oligonucleotides corresponding to the mature toxin sequences. Hybridization was done in 3× tetramethyl ammonium chloride (Aldrich), 0.1× NaHPO₄, 0.001× EDTA, 5× Denhardt’s solution, 0.6% SDS, 100 µg/ml sheared salmon sperm DNA for 24 h at 48 °C. Washes were done at room temperature in 3× tetramethyl ammonium chloride, 0.05× Tris-HCl, pH 8, 0.2% SDS for 15 min and 1 h at 50 °C in a solution of the same composition. The filters were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min each. The filters were then exposed to x-ray films. Double stranded DNA from purified clones were sequenced by dideoxynucleotide chain terminating method (13).

Identification of γ-CRS of Conantokin-G—In the case of mammalian proteins that undergo γ-carboxylation, the γ-CRS is contained in a 18-amino acid sequence (16-amino acid sequence in the case of bone peptides) immediately N-terminal to the mature peptide sequence (15–17). We investigated the ability of peptides shown in Table I to serve as substrates or affect the activity of the Conus carboxylase.

The concentration dependence for the carboxylation of peptides 1 and 3 (Table I) are shown in Fig. 2. The apparent K_m for E.Con-G is 3400 ± 215 µM, and that for -20.Pro-E.Con-G is 28 ± 3 µM. The results clearly indicate that covalent linkage of the propeptide sequence -20 to -1 makes E.Con-G into an efficient substrate (note that the -20 to -1 sequence has no Glu residues that would be substrates for carboxylation). Similar observations were made when the -20 to -1 peptide was covalently linked to FLEEL (Table I). The apparent K_m for peptide 3 is less than that of peptide 2, suggesting that propeptide sequences between -20 and -11 also interact with the carboxylase (Table I).

We then investigated the effect of the addition of pro(-20 to -1) in trans, on the carboxylation of FLEEL, E.Con-G, -10.Pro-E.Con-G, and -20.Pro-E.Con-G. We also determined the effect of addition of Pro(-20 to -11) and Pro(-11 to -1) on the carboxylation of -10.Pro-E.Con-G.

Inspection of the K_m values in Table I clearly indicates that substrates in which the propeptide sequences -20 to -1 are covalently linked to the N terminus are efficient substrates for carboxylation. In the case of both E.Con-G and FLEEL, the K_m decreases by 2 orders of magnitude. Pro(-20 to -1) stimulates the carboxylation of both FLEEL and E.Con-G when added in
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TABLE I
Primary sequence and apparent $K_m$/IC$_{50}$ of peptides used in this study

| Peptide | $K_m$ (μM) | IC$_{50}$ (μM) |
|---------|------------|---------------|
| FLEEL   | 2301 ± 6   |               |
| (E-Con-G) | 3400 ± 215 |               |
| (−10-Pro-E-Con-G) | 1836 ± 109 |               |
| (−20-Pro-E-Con-G) | 28 ± 3 |               |
| (−20-Pro-FLEEL) | 4 ± 0.2 |               |
| (−20-Pro−(−30 to 1)) | 490 ± 30 |               |
| (−20-Pro−(−40 to 21)) | 200 ± 50 |               |
| (−20-Pro−(−60 to 41)) | >1000 |               |

Fig. 2. Kinetic analysis of carboxylase activity using E.Con-G (A) and −20.Pro.E.Con-G as substrates (B). The data were fitted to a hyperbola in a single-site binding model. The apparent $K_m$ for E.Con-G was determined from a Lineweaver-Burk plot and for −20.Pro.E-Con-G from the best fit to a hyperbola.

trans, but the effects are quite small: ~25% enhancement in the case of E.Con-G and 40% for FLEEL. Pro(−40 to −21) and Pro(−60 to −41) have no effect on the carboxylation of FLEEL or E.Con-G (data not shown).

Propeptide sequence −20 to −1 is an inhibitor for the carboxylation of −10-Pro.E.Con-G (Fig. 3 and Table I) and −20-Pro.E-Con-G. The IC$_{50}$ of Pro(−20 to −1) is very similar to Pro(−30 to −1), suggesting that the interaction of the propeptide with the carboxylase in the region between −30 and −1 is probably limited to −20 to −1. Pro(−40 to −21) and Pro(−60 to −41) did not inhibit carboxylation of −10-Pro.E-Con-G even at concentrations 100-fold greater.

We also investigated the effect of alanine substitutions of the basic amino acids in the propeptide. $^{−20}$GKDR1TQMRIL-KQRGNKAR$^{+1}$GEEELY-NH$_2$, a 26-amino acid peptide containing propeptide sequences −20 to −1 of conantokin-G was used as the wild type substrate. Individual peptides in which KR at positions −12 and −11, KQR at positions −8, −7, and −6, NK at positions −4 and −3, and NK-R at positions −4, −3, and −1 were substituted by alanine were used as substrates in the carboxylation reaction. Carboxylation reactions were done at a substrate concentration of 40 μM, the apparent $K_m$ of carboxylation for the wild type substrate. Alanine substitutions in the context of the remaining propeptide sequences had little effect (<10%) on carboxylation.

We examined the ability of bovine microsomes to use −20.Pro.E-Con-G as substrate for γ-carboxylation. As shown in Table II, although this peptide is an excellent substrate for the Conus enzyme, it is extremely poor for the mammalian enzyme.

**DISCUSSION**

We have demonstrated that the γ-carboxylated 17-amino acid Conus peptide conantokin-G is initially translated as a propeptide of 100 amino acids. In general, the organization of the conantokin-G precursor is similar to that previously reported for disulfide-rich conotoxins from *Conus* venoms. The mature peptide is found in a single copy at the C-terminal end of the precursor. Before maturation of the peptide, a number of post-translational processing events have to take place (Fig. 1). These events include the γ-carboxylation of five glutamate residues, C-terminal amidation of asparagine-17 following excision of the C-terminal tripeptide, and a proteolytic event between Arg$^{-1}$ and Gly$^{1}$.

One notable feature of the conantokin-G prepropeptide is the length of the intervening region between the signal sequence and the mature peptide. The 59 amino acids in the intervening pro-region is the longest so far reported for any Conus venom peptide. We have demonstrated one potential function of this extended region: the presence of a γ-carboxylation recognition sequence in the propeptide and concentration of Pro(−20 to −1) was varied.

FIG. 3. Kinetic analysis of the inhibition of carboxylation of −20.Pro.E-Con-G by Pro(−20 to −1). The data were fitted to single-site competitive binding model. 50 μM −20.Pro.E-Con-G was used in the carboxylation reaction and concentration of Pro(−20 to −1) was varied.

In mammalian blood coagulation and bone Glα proteins, γ-carboxylation of glutamate residues is carried out by a vitamin K-dependent carboxylase. A conserved motif (16) γ-carboxylation recognition sequence in the propeptide sequence binds the γ-carboxylase and is required for a polypeptide substrate to be a high affinity target for the γ-carboxylase. In the experiments described above, we carried out an analysis using segments of the conantokin-G propeptide to identify potential sites that might serve as γ-carboxylase recognition signals for the Conus enzyme. The results reveal that a γ-carboxylation...
TABLE II

| Additions          | 14CO2 incorporated |
|--------------------|--------------------|
| Bovine microsomes  |                    |
| Endogenous         | 1                  |
| FLEEL (1.2 mM)     | 249                |
| −20.Pro-E.Con-G (0.28 mM) | 3                |
| Conus microsomes   |                    |
| Endogenous         | 11                 |
| FLEEL (1.2 mM)     | 818                |
| −20.Pro-E.Con-G (0.28 mM) | 2741             |

TABLE III

| Propeptide sequences of γ-carboxylated Conus and mammalian peptides |
|---------------------------------------------------------------|
| Pro-Con-G (−20 to −1) of conantokin-G; hFIX (21), hFX (22), and hPT (20) are the propeptide sequences of human Factors IX and X and prothrombin. |
| Pro-Con-G GKRDLQMKRKQRNGKAR                                     |
| hFIX T2VLDERNNK1LNPRPG                                          |
| hPT HVEAPQQRSSLQRVRR                                           |

troscopy. In all these structures, the Gla residues are on the same side of the conantokin structure; this would allow a membrane-bound enzyme to carry out efficient carboxylation of Glu residues oriented in the same direction with optimum stereochemistry.

In the discussion above, we have emphasized the differences in the γ-carboxylation recognition signal sequences in the mammalian and Conus systems. These differences may be due to the large evolutionary distance between the two species. However, there is an underlying general similarity between the two enzymes: the catalytic reaction they carry out, their cofactor requirements, and a recognition signal (albeit differing in sequence) in the −1 to −20 propeptide region. It will be important to purify the Conus enzyme and characterize it to determine the relationship between the two enzymes.

Acknowledgments—We thank Dr. Bob Schackmann of the DNA/PEPTID facility, Huntsman Cancer Center (supported by Grant NCI 42014) for synthesis of −20.Pro-E.Con-G and Dr. J. Rivier of Salk Institute for synthesis of Pro (−30 to −1). We thank Tom Stanley and D. Yoshikami for critical reading of the manuscript.

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The orientation in which a Glu presents itself to the active site of the carboxylase may determine whether it will be carboxylated. In the case of Con-G not all the Glu residues are γ-carboxylated (Glu2 is not carboxylated, whereas Glu3 and Glu4 are carboxylated). The solution structures of Con-G and Con-T as determined by CD and NMR spectroscopy (24, 25) are a mixture of a and 3_10 helices. Rigby et al. (26) also determined the structure of the metal-free conformer of conantokin-G by NMR spec-