Hydrophobic Amino Acids Define the Carboxylation Recognition Site in the Precursor of the γ-Carboxyglutamic-Acid-Containing Conotoxin ϵ-TxIX from the Marine Cone Snail *Conus textile*†

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Received July 16, 1999; Revised Manuscript Received September 1, 1999

ABSTRACT: To identify the amino acid sequence of the precursor of the Gla-containing peptide, ϵ-TxIX, from the venom of the marine snail *Conus textile*, the cDNA encoding this peptide was cloned from a *C. textile* venom duct library. The cDNA of the precursor form of ϵ-TxIX encodes a 67 amino acid precursor peptide, including an N-terminal prepro-region, the mature peptide, and four residues posttranslationally cleaved from the C-terminus. To determine the role of the propeptide in γ-carboxylation, peptides were designed and synthesized based on the propeptide sequence of the Gla-containing conotoxin ϵ-TxIX and used in assays with the vitamin K-dependent γ-glutamyl carboxylase from *C. textile* venom ducts. The mature acarboxy peptide ϵ-TxIX was a high K_M substrate for the γ-carboxylase. Synthetic peptides based on the precursor ϵ-TxIX were low K_M substrates (5 μM) if the peptides included at least 12 residues of propeptide sequence, from −12 to −1. Leucine-19, leucine-16, asparagine-13, leucine-12, leucine-8 and leucine-4 contribute to the interaction of the pro-conotoxin with carboxylase since their replacement by aspartic acid increased the K_M of the substrate peptide. Although the *Conus* propeptide and the propeptides of the mammalian vitamin K-dependent proteins show no obvious sequence homology, synthetic peptides based upon the structure of pro-ϵ-TxIX were intermediate K_M substrates for the bovine carboxylase. The propeptide of ϵ-TxIX contains significant α-helix, as estimated by measurement of the circular dichroism spectra, but the region of the propeptide that plays the dominant role in directing carboxylation does not contain evidence of helical structure. These results indicate that the γ-carboxylation recognition site is defined by hydrophobic residues in the propeptide of this conotoxin precursor.

The marine cone snail (*Conus*) is the sole invertebrate currently known to synthesize γ-carboxyglutamic acid. Cone snail venom is rich in γ-carboxyglutamic acid, where this amino acid represents 3−5% of the amino acid composition (*1*). Conantokin G, a 17 residue peptide that contains five γ-carboxyglutamic acid residues, was isolated from the venom of *Conus geographus* (*2*). Conantokin G binds calcium ions through a carboxylate network defined by five γ-carboxyglutamic acid residues and undergoes a calcium-induced conformational change upon binding to metal ions (*3*, *4*). Conantokin G inhibits the NMDA[1] receptor, an activity that requires γ-carboxyglutamic acid (*5*−*9*). Other γ-carboxyglutamic acid-containing neurotoxins from cone snail venom have been described, including conantokin T, TxVIIa, conotoxin GS, γPnVIIA, ϵ-TxIX, and the bromosleeper peptide (*10*−*15*). Some γ-carboxyglutamic acid residues in these peptides have been shown to be important for their biological activity (*11*, *12*).

In mammalian systems, specific glutamic acid residues in vitamin K-dependent protein precursors are modified to γ-carboxyglutamic acid by a vitamin K-dependent carboxylase. This enzyme, which requires molecular oxygen, CO_2, and vitamin K to catalyze the reaction, is a membrane protein that resides in the endoplasmic reticulum. The Gla-containing mammalian blood clotting and regulatory proteins are synthesized as prozymogens (for review, see ref *16*). The propeptide on the precursor form is cleaved as a late intracellular processing event. The propeptide of vitamin-K-dependent mammalian proteins, defined as residues −18 to −1 in Factor IX (*17*), contains a series of residues that are conserved (*18*). This propeptide contains a γ-carboxylation recognition site that is required and sufficient for γ-carboxylation (*19*, *20*). The propeptide binds to the γ-carboxylase (*21*), linking the carboxylase substrate to the carboxylase.

† This work was supported in part by grants from the National Institutes of Health (HL38216, HL42443, and HL18834) and the Swedish Medical Research Council.

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[1] Abbreviations: Gla, γ-carboxyglutamate; NMDA, *N*-methyl-D-aspartate; THAP, 2,4,6-trihydroxyacetophenone; MOPS, 4-morpholinepropane sulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; GSP, gene specific primer; HMP, hydroxymethylphenoxy; TFA, trifluoracetic acid; RACE, rapid amplification of cDNA ends.

10.1021/bi991640l CCC: $18.00
Published on Web 10/14/1999

Cone snail venom duct homogenate contains a carboxylase that is capable of in vitro carboxylation of certain glutamic acid-containing substrates, including FLEEL (*1*, *22*, *23*). Like the mammalian enzyme, the *Conus* carboxylase is vitamin K dependent and a membrane protein. Upon the basis of the recent cloning of the conantokin G precursor, the propeptide of conantokin G has been found to contain a region which directs γ-carboxylation by the *Conus* carboxylase activity (*24*). This is the first γ-carboxyglutamic acid-containing conotoxin precursor sequence to be reported, and the propeptide bears no sequence homology to the carboxylase recognition sites on known mammalian carboxylase substrates. To identify the residues that compose the carboxylation recognition site on the *Conus* vitamin-K-dependent peptide substrates, we have cloned the precursor of a γ-carboxyglutamic acid-containing conopeptide from *Conus textile*, ϵ-TxIX (*14*). In the current study, we demonstrate that the γ-carboxylation recognition site is defined by hydrophobic residues in the propeptide of the conotoxin precursor.

MATERIALS AND METHODS

Materials. The TA Cloning Kit and Fast Track Kit were from Invitrogen (Carlsbad, CA). Tip 20 Plasmid Purification Kits and Qiaquick Gel Extraction Kits were from Qiagen (Santa Clarita, CA). The Marathon cDNA Amplification Kit, polymerase, and PCR buffer were from Clontech (Palo Alto, CA). NuSieve GTG agarose was from FMC Bioproducts (Rockland, ME). Synthetic oligonucleotides were obtained from BRL Life Technologies (Gaithersburg, MD). Frozen cone snails were obtained from Vietnam. Live cone snails were obtained from Fiji and imported into the United States under the jurisdiction of the U.S. Fish and Wildlife Service. Vitamin K was from Abbott Laboratories (North Chicago, IL). ^{14}C-Labeled sodium bicarbonate was from Amersham Life Sciences (Arlington Heights, IL). Atomlight scintillation fluid was from Packard (Meriden, CT). All other reagents were of the highest grade available.

Construction of C. textile Venom Duct cDNA Library. Poly A+ RNA was isolated from 1 g of *C. textile* venom ducts using the Fast Track kit (Invitrogen). *C. texile* venom duct Poly(A)+ RNA (1 μg) was used as starting material for cDNA synthesis using a Marathon cDNA amplification kit (Clontech) to generate a library for RACE amplication of the full-length conotoxin cDNA.

Cloning of the Conopeptides. Degenerate oligonucleotides corresponding to the peptide sequence of ϵ-TxIX were synthesized. The degenerate sense oligonucleotide (5′-GARTGYTGYGARGAYGGNTG-3′) encoding for residues 1−7 of ϵ-TxIX along with the degenerate antisense oligonucleotide (5′-GGNGCNGCNGTRCARCA CCA-3′) encoding residues 13 to 7 were used in a PCR reaction with the *C. textile* RACE library as template in a Perkin-Elmer Cetus DNA thermal cycler. Amplification parameters were five cycles of 94 °C for 30 s and 72 °C for 2 min; five cycles of 94 °C for 30 s and 70 °C for 2 min; 25 cycles of 94 °C for 30 s and 68 °C for 2 min; and a 5 min extension at 68 °C. The PCR product was TA cloned into the pCR 2.1 vector (Invitrogen) and the resulting plasmid DNA sequenced on an Applied Biosystems model 373 DNA Sequencer using M13 reverse and forward primers. Gene-specific oligonucleotides corresponding to internal sequences, GSP1 and GSP2 (Figure 1), were used in 5′ and 3′ RACE PCR reactions, respectively. All RACE reactions were carried out using the designated gene-specific primer, the flanking primer AP1 (Clontech), and the *C. textile* RACE library as template. The resulting product of the 5′ RACE reaction was cloned and sequenced as described above. A gene-specific primer, GSP3, was used in an additional 3′ RACE reaction to confirm the full-length cDNA coding sequence.

Synthesis of Carboxylase Substrates. All peptides were synthesized using solid-phase Fmoc [*N*-(9-fluorenyl)methoxycarbonyl/*N*-methylpyrrolidone] chemistry on an Applied Biosystems model 430A peptide synthesizer as previously described (*25*). The deprotection chemistry was modified, however, and made use of 0.1 M *N*-hydroxybenzotriazole/piperidine in order to prevent modification of aspartate residues. The resin used was 4-hydroxymethylphenoxy (HMP). The shortest peptide, ϵ-TxIX-12, was cleaved from the resin in anisole/1,2-ethanedithiol (5:1) in trifluoroacetic acid (TFA) for 1.5 h at room temperature. The longer peptides were cleaved in water/thioanisole/phenol/1,2-ethanethiol (5:5:5:2.5) for 2.5 h at room temperature or 4.5 h for the longest peptides, ϵ-TxIX/37 and ϵ-TxIX/41. The peptide samples were purified by reversed-phase HPLC using a Vydac preparative C18 column (RPC18, 2.2 × 25 cm) and a Beckman System Gold HPLC system. The column was developed with a linear gradient from 15 to 50% acetonitrile in the presence of 0.1% TFA over 35 min and monitored at 214 and 280 nm. The peptides were sequenced using an ABI model 491 Procise Protein Sequencer. The purified peptides were analyzed by mass spectrometry on a Voyager Linear MALDI-TOF spectrometer (Perseptive Biosystems).

Preparation of Microsomal Vitamin K-Dependent Carboxylase. Live *C. textile* cone snails, maintained at the Marine Resources Center at the Marine Biological Laboratory, were placed on ice, the venom ducts removed by dissection, and the ducts immediately frozen in liquid N_2 and stored at −80 °C. The venom ducts were thawed and homogenized using a Tissue Tearor homogenizer in 250 mM sucrose, 500 mM KCl, 25 mM imidazole, pH 7.2, 0.1% CHAPS, and 0.1 mM PMSF. The resulting homogenates were centrifuged at 9000*g*, and the supernatants stored at −80 °C until gel filtration over a Superose 12 column. Fractions containing carboxylase activity were pooled. The fractions were centrifuged at 150000*g* at 4° C in a Beckman Ultracentrifuge. The pellets were solubilized in 25 mM MOPS, pH 7.0, 500 mM NaCl, 0.1% phosphatidylcholine, 0.1% CHAPS, 0.1 mM PMSF, and 20% glycerol by sonication using a model W220 F Sonicator (Heat Systems-Ultra Sonics, Inc.).

Carboxylase Assays and Kinetic Studies. The amount of $^{14}CO_2$ incorporated in the peptide substrates was measured in 125 μL reaction mixtures consisting of 222 μM vitamin K hydroquinone; 28 mM MOPS, pH 7.0; 500 mM NaCl; 0.16% (w/v) phosphatidylcholine and 0.16% CHAPS; 0.8 mM $(NH_4)_2SO_4$, and 0.72 mM $NaH^{14}CO_3$ (5 μCi) prepared as a master solution. Vitamin K was reduced with 8 mg sodium borohydride and 5 μL of β-mercaptoethanol for 30 min then diluted (1:4) with PBS and 0.5% CHAPS before use. Peptide substrate solutions were prepared in 8 mM DTT. Substrates (25 μL) of varying concentrations were added to the reaction tubes, followed by master solution (75 μL), and

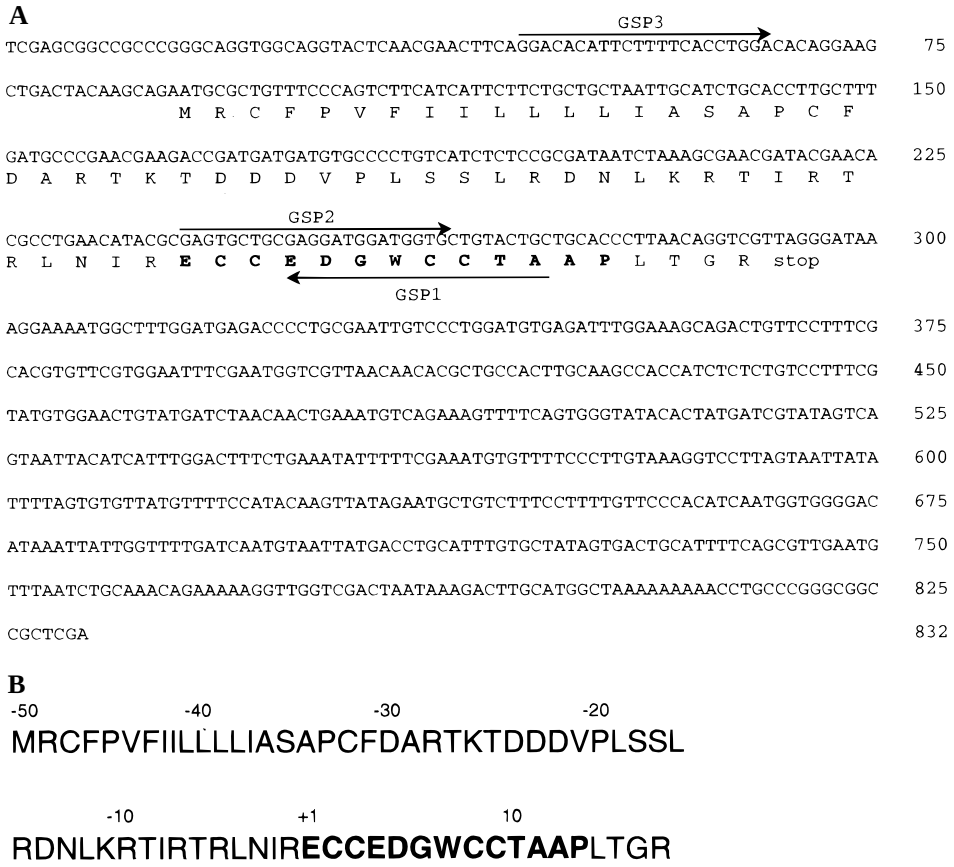

FIGURE 1: (A) Nucleotide sequence for cDNA encoding the precursor form of ϵ-TxIX and its predicted translation product, prepro-ϵ-TxIX. The mature conopeptide is shown in bold type. (B) Amino acid sequence of pre-pro-ϵ-TxIX.

the reactions initiated by the addition of a *C. textile* γ-glutamyl carboxylase preparation (25 μL). Reactions were incubated at 20 °C and stopped at 30 min by the addition of 1 mL of 10% trichlorolacetic acid. Residual unreacted $^{14}CO_2$ was removed by boiling for about 10 min, and the incorporation of $^{14}CO_2$ was measured in a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer.

Circular Dichroism Spectra. Circular dichroism spectra were obtained using an Aviv 62A DS spectropolarimeter. Peptides were dissolved in 10 mM sodium phosphate buffer, pH 7.0, 1.6 mM DTT, and 40% trifluoroethanol. Spectra were recorded between 250 and 200 nm.

RESULTS

Cloning of the Precursor Form of ϵ-TxIX. The complete amino acid sequence of the precursor form of ϵ-TxIX was predicted by cloning of the full-length cDNA. The cDNA encoding the mature peptide was cloned using degenerate oligonucleotide primers based on the amino acid sequence of the isolated peptide (*14*) and a *C. textile* RACE library as template. The partial nucleotide sequences determined for the mature conotoxin encoded the appropriate amino acid sequences for ϵ-TxIX. Gene-specific primers were designed based upon these nucleotide sequences and used for both 5′ RACE and 3′ RACE reactions. Bands of 650 and 300 bp were obtained from the 5′ RACE and 3′ RACE, respectively. The complete ϵ-TxIX precursor nucleotide sequence was obtained, and the precursor amino acid sequence predicted (Figure 1). To confirm this sequence, an additional gene-specific primer based upon the 5′ untranslated nucleotide sequence was used in a 3′ RACE reaction. This yielded a 900 bp product that was cloned and sequenced. These cDNA sequences predict a primary translation product of 67 amino acid residues that corresponds to the 13 residue conopeptide with a 50 residue signal peptide/propeptide that represents the prepro sequence and a 4-residue C-terminal extension.

Location of the γ-Carboxylation Recognition Site. To extrapolate from the presence of a γ-carboxylation recognition site on the propeptides of the mammalian vitamin K-dependent blood coagulation proteins (*19*, *26*), we prepared synthetic peptides based upon the structure of the precursor form of ϵ-TxIX as carboxylase substrates. Because of technical difficulties in preparing ϵ-TxIX peptides with a C-terminal proline by solid-phase peptide synthesis due to low coupling yields, all peptides based upon ϵ-TxIX were synthesized lacking proline 13.

The synthetic 12-residue acarboxy-ϵ-TxIX peptide lacking the C-terminal proline (ϵ-TxIX/12) was a substrate for the vitamin K-dependent carboxylase as determined in vitro by the incorporation of $^{14}CO_2$ into glutamic acids in this peptide. A K_M of 582 μM was obtained (Table 1). By comparison, pro-acarboxy-ϵ-TxIX containing residues -29 to -1 of the prepro sequence (ϵ-TxIX/41) had a K_M of 3.9 μM. These results indicate that the γ-carboxylation recognition site of this carboxylase substrate resides within the 29 amino acids of the conotoxin propeptide sequence.

A series of peptides was prepared to determine the minimal size of the propeptide consistent with the peptide being a low K_M substrate for the carboxylase. Pro-acarboxy-ϵ-TxIX peptides containing a 29 residue propeptide (ϵ-TxIX/41), a

Table 1: In Vitro Carboxylation of Synthetic Peptides Based upon the Sequence of Pro ϵ-TxIX

peptide	sequence	K_M (μM)	V_{max} cpm/30 min	V_{max}/K_M
ϵ-TxIX/12	**ECCEDGWCCTAA**	582	9945	17
pro ϵ-TxIX/21	TIRTRLNIR**ECCEDGWCCTAA**	19.	51 934	2733
pro ϵ-TxIX/24	LKRTIRTRLNIR**ECCEDGWCCTAA**	4.5	30 435	6763
pro ϵ-TxIX/37	TDDDVPLSSLRDNLKRTIRTRLNIR**ECCEDGWCCTAA**	4.0	14 631	3667
pro ϵ-TxIX/41	ARTKTDDDVPLSSLRDNLKRTIRTRLNIR**ECCEDGWCCTAA**	3.9	13 883	3560

Table 2: Effect of Substitution of Hydrophobic Residues with Aspartic Acid[a]

peptide	sequence	K_M (μM)
Pro ϵ-TxIX/24	LKRTIRTRLNIR**ECCEDGWCCTAA**	5.1
pro ϵ-TxIX/24(NI-3−2DD)	LKRTIRTRLDDR**ECCEDGWCCTAA**	17.5
pro ϵ-TxIX/24 (L-4D)	LKRTIRTRDNIR**ECCEDGWCCTAA**	15.3
pro ϵ-TxIX/24(I-8D)	LKRTDRTRLNIR**ECCEDGWCCTAA**	13.8
pro ϵ-TxIX/24(L-12D)	DKRTIRTRLNIR**ECCEDGWCCTAA**	9.9
pro ϵ-TxIX/37	TDDDVPLSSLRDNLKRTIRTRLNIR**ECCEDGWCCTAA**	4.8
pro ϵ-TxIX/37 (N-13D)	TDDDVPLSSLRDDLKRTIRTRLNIR**ECCEDGWCCTAA**	18.4
pro ϵ-TxIX/37(−13−12−8−4−3−2D)	TDDDVPLSSLRDDDKRTDRTRDDDR**ECCEDGWCCTAA**	370.
pro ϵ-TxIX/37(−19−16−12D)	TDDDVPDSSDRDNDKRTDRTRLNIR**ECCEDGWCCTAA**	178.
pro ϵ-TxIX/37 (L-16D)	TDDDVPLSSDRDNLKRTIRTRLNIR**ECCEDGWCCTAA**	51.5
pro ϵ-TxIX/37 (L-19D)	TDDDVPDSSLRDNLKRTIRTRLNIR**ECCEDGWCCTAA**	26.5

[a] In vitro carboxylation of synthetic peptides based upon the sequence of pro ϵ-TxIX. Amino acids altered from the native sequence are underlined. The mature sequence is presented in bold type.

25 residue propeptide (ϵ-TxIX/37), and a 12 residue propeptide (ϵ-TxIX/24) were characterized by K_M values of 3.9−4.5 μM (Table 1). These results indicate that the γ-carboxylation recognition site of ϵ-TxIX that directs carboxylation resides within residues −29 to −1 of the propeptide. Furthermore, −12 to −1 are sufficient to direct efficient γ-carboxylation. Elimination of residues −12, −11, and −10, as in the synthetic substrate pro ϵ-TxIX/21, is associated with an increased K_M of 19 μM.

Identification of Amino Acid Residues That Contribute to the γ-Carboxylation Recognition Site. To identify amino acid residues that are components of the γ-carboxylation recognition site, we prepared synthetic peptides based upon the structure of pro ϵ-TxIX/24, the low K_M substrate for the *Conus* carboxylase that contains 12 residues of the propeptide, from −12 to −1, and pro ϵ-TxIX/37, the low K_M substrate for the *Conus* carboxylase that contains 25 residues of the propeptide, from −25 to −1. We hypothesized that, as the mammalian carboxylation recognition sites are defined by hydrophobic amino acids (*27*), hydrophobic amino acids in this region of the conopeptide might define the interaction of this peptide with the γ-carboxylase. We prepared four variants of pro ϵ-TxIX/24: substitution of asparagine −3 and isoleucine −2 with aspartic acid, substitution of leucine −4 with aspartic acid, substitution of isoleucine −8 with aspartic acid, and substitution of leucine −12 with aspartic acid. Each of these synthetic peptides was assayed as a carboxylase substrate. Compared to pro ϵ-TxIX/24 (with a K_M of about 5 μM), each of these peptides was characterized by an increased K_M for the carboxylase. The K_M values obtained for these variants ranged from 10−17 μM (Table 2). These results indicate that substitution at leucine residues at −12 and −4, substitution at isoleucine at −8, or substitution of the asparagine/isoleucine pair at −3/-2 modestly perturb the interaction of these substrates with the carboxylase.

To evaluate the contributions of asparagine 13, leucine 16, and leucine 19 for γ-carboxylation, peptides based upon the sequence of ϵ-TxIX/37 were prepared, and the above residues were individually replaced with aspartic acid. These alterations were associated with changes in the K_M of these substrates from 4 to 18, 51, and 26 μM respectively (Table 2). Furthermore, significant disruption of the carboxylation recognition site was observed when individual peptides containing multiple substitutions of hydrophobic residues were evaluated. ϵ-TxIX/37/3D containing aspartic acid at −13, −16, and −19 was characterized by a K_M of 178 μM. ϵ-TxIX/37/6D containing aspartic acid residues at −2, −3, −4, −8, −12, and −13 was characterized by a K_M of 370 μM.

Table 3: In Vitro Carboxylation by the Bovine Carboxylase of Synthetic Peptides and Mutant Peptides Based upon the Sequence of Pro ϵ-TxIX

peptide	K_M (μM)
ϵ-TxIX/12	1500
pro ϵ-TxIX/21	202
pro ϵ-TxIX/24	69
Pro ϵ-TxIX/37	76
pro ϵ-TxIX/41	117
pro ϵ-TxIX/24(NI-3−2DD)	597
pro ϵ-TxIX/24 (L-4D)	324
pro ϵ-TxIX/24(I-8D)	225
pro ϵ-TxIX/37	76
pro ϵ-TxIX/37 (N-13D)	88
pro ϵ-TxIX/37 (L-19D)	584

Although the γ-carboxylation recognition sites of the ϵ-TxIX precursor and the precursors of the mammalian vitamin K-dependent proteins show no sequence similarity, we evaluated the analogues of the ϵ-TxIX precursor to establish whether these peptides are substrates for the bovine carboxylase. ϵ-TxIX/12 (which lacks the propeptide) is a high K_M substrate, with a K_M value of 1500 μM (Table 3). However, ϵ-TxIX/24, ϵ-TxIX/37, and ϵ-TxIX/41 are intermediate K_M substrates, with K_M values between 70 and 120 μM. These results indicate that the propeptide of ϵ-TxIX interacts with the bovine carboxylase albeit at reduced apparent affinity relative to the *Conus* carboxylase.

To determine whether these conopeptide precursors contain α-helix that might be important to substrate-carboxylase

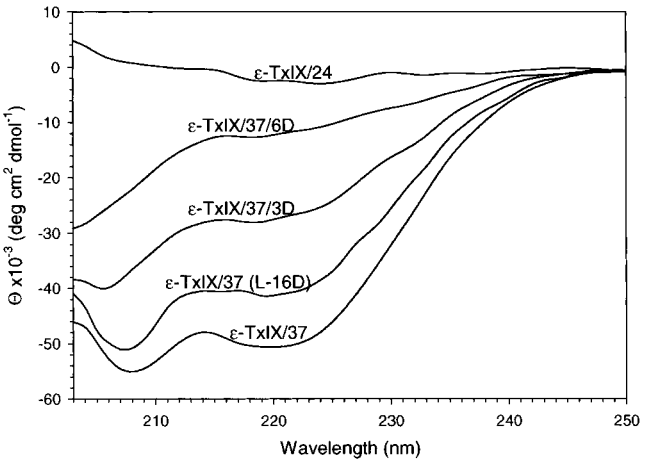

FIGURE 2: Helicity of propeptide. The circular dichroism spectra of the precursor forms of ϵ-TxIX were obtained in 10 mM sodium phosphate, pH 7.0, 1.6 mM DTT, and 40% trifluoroethanol. Peptide concentrations varied between 19 and 31 μM. The molar elipticity is presented as a function of the wavelength. ϵ-TxIX/24; ϵ-TxIX/37; ϵ-TxIX/37/3D includes mutations to aspartic acid at -19, -16, and -12; ϵ-TxIX/37/6D includes mutations to aspartic acid at -13, -12, -8, -4, -3, and -2; ϵ-TxIX/37(L-16D) includes a mutation at leucine -16 to aspartic acid.

recognition, the circular dichroism spectra of these peptides were obtained in 40% trifluoroethanol (Figure 2). The CD spectrum of ϵ-TxIX/37 is characterized by minima at 222 and 208 nm, consistent with a dominant α-helical secondary structure. ϵ-TxIX/37 peptides including single amino acid substitutions showed decreased helical structure, and the ϵ-TxIX/37/6D containing aspartic acid residues at -2, -3, -4, -8, -12, and -13 showed no helical structure. These results indicate the presence of α-helix in the propeptide of ϵ-TxIX. However, the major component of the carboxylation recognition site is not included in the α-helical region since ϵ-TxIX/24 is a low K_M carboxylase substrate but shows no evidence of α-helix even in a helix-stabilizing solvent.

DISCUSSION

The discovery of γ-carboxyglutamic acid in prothrombin in 1974 (*28*, *29*) led to identification of this amino acid in many mammalian proteins, including the vitamin-K-dependent proteins that regulate blood coagulation (for review, see refs *16*, *30*, and *31*), the proteins of mineralized tissue (*32*), and the growth arrest protein Gas6 (*33*). However, the sole example of γ-carboxyglutamic acid in invertebrates is in the conopeptides of the marine cone snail, *Conus*. To date, nine γ-carboxyglutamic-acid-containing conotoxins have been identified (*2*, *10*−*15*, *34*, *35*). To compare the biosynthesis of γ-carboxyglutamic acid in invertebrates to that in mammalian cells, we are examining the C*onus* vitamin-K-dependent carboxylase and identifying novel γ-carboxyglutamic acid-containing conopeptides from cone snail venom that are substrates of this enzyme. We have described a new γ-carboxyglutamic acid-containing conotoxin, ϵ-TxIX, that interacts reversibly with presynaptic voltage-gated Ca^{2+} channels (*14*). Like the α-conotoxins, this peptide also has four cysteine residues and two disulfide bonds, but the cysteines are arranged as two adjacent pairs (CC−CC). This unique cysteine pattern defines a new class of conotoxins, and its reversible presynaptic activity defines the new ϵ-conotoxins. Since γ-carboxyglutamic acid has been found

FIGURE 3: Propeptide alignment of *Conus* Gla-containing peptides, conantokin G and ϵ-TxIX. Residues -20 to -1 contain a major component of the carboxylation recognition site in conantokin G (ConG). Residues -12 to -1 contain a major component of the carboxylation recognition site in ϵ-TxIX. The homologous amino acids are highlighted.

in conotoxins without cysteine residues (e.g., conantokin G, conantokin T), conotoxins with six cysteines such as the γ-conotoxins and ω-like conotoxins, and peptides with four cysteines such as ϵ-TxIX, we see no suggestion that the presence of γ-carboxyglutamic acid relates to the disulfide bonding pattern.

To understand the molecular signals that define certain peptides as substrates for the vitamin-K-dependent γ-carboxylase, we have examined a precursor of a γ-carboxyglutamic acid-containing conotoxin as a substrate in an in vitro carboxylation system. Our goal has been to locate the γ-carboxylation recognition site that distinguishes conopeptide carboxylase substrates from conopeptides that do not get carboxylated. Preliminary results indicated that acarboxy forms of conantokin G were poor, high K_M substrates for the *Conus* carboxylase (*24*).[2] This led us to suspect that the carboxylation recognition site might reside on the propeptide in the precursor form of the conotoxin. We have previously shown that the γ-carboxylation recognition sites on Factor IX (*19*) and prothrombin (*26*, *36*, *37*) reside within the propeptides of the precursor forms of these proteins. This site is required for γ-carboxylation and is, for the model studied to date, sufficient to direct carboxylation of an otherwise unrelated glutamate-rich peptide adjacent to the γ-carboxylation recognition site (*20*).

Bandyopadhyay et al. have cloned the cDNA encoding the precursor form of conantokin G, a γ-carboxyglutamic acid-containing conotoxin from *Conus geographus* (*24*). These studies demonstrated that the propeptide sequence, specificially between residues -20 and -1, enhances carboxylase interaction with the peptide substrate, and strongly suggested that the recognition element of interest is located N-terminal of the mature conotoxin. Like the precursor of the Gla-containing conotoxin from *C. textile*, the propeptide of conantokin G shows no sequence similarity with the propeptides of the mammalian carboxylase substrates. Furthermore, this group established that a number of charged residues in the propeptide are not involved in directing carboxylation. In the current study, we have identified the propeptide sequence of a precursor of a γ-carboxyglutamic-acid-containing conotoxin from *C. textile*. This region does not show a sequence motif identical with the precursor of conantokin G. However, regions of homology between the C-terminal 12 propeptide residues of pro-ϵ-TxIX and the C-terminal 20 propeptide residues of conantokin G are apparent (Figure 3), with special reference to the pattern of hydrophobic residues. These propeptides are rich in hydrophobic amino acids that are spaced to suggest their participation as a hydrophobic face that interacts with the carboxylase. Upon the basis of the precedent of the importance of hydrophobic residues in the γ-carboxylation recognition site

[2] E.C., B.F., B.C.F., unpublished data.

of the mammalian carboxylase substrates, particularly phenylalanine-16 and alanine-10 in prothrombin and Factor IX, we have shown in the current study that hydrophobic residues are also important to substrate recognition by the *Conus* carboxylase.

Conotoxins have been previously demonstrated to undergo significant proteolytic processing following protein synthesis, and this appears to be the case for the Gla-containing conotoxin precursor described in this study. This peptide contains highly hydrophobic sequences in the N-terminus of the sequence, consistent with the role of a signal peptide. Furthermore, like the Gla-containing blood clotting proteins, there is an intervening sequence between the putative signal peptide and the mature conopeptide. This region, based upon our analysis and that of Bandyopandhyay et al. (*24*), contains the recognition element associated with designating the peptide as a carboxylase substrate.

The γ-carboxylation recognition site in the mammalian vitamin-K-dependent blood coagulation and regulatory proteins resides in the propeptide. The propeptide is cleaved as a late intracellular processing event, after the protein has undergone γ-carboxylation (*38*). In contrast, matrix Gla protein contains the carboxylation recognition site within the mature sequence (*39*). Three observations argue that the propeptide containing the carboxylation recognition site is sufficient to carboxylate vitamin-K-dependent protein precursors and that the glutamic acid targets and adjacent amino acids on the substrate do not define specificity: (a) the affinity of propeptide-containing substrates for the carboxylase is essentially the same, regardless of the length of the amino acid sequences C-terminal to the propeptide (*26*, *36*, *40*); (b) mutation of amino acid residues in the amino acid sequences around the target glutamic acid residues, including cysteine residues, does not interfere with γ-carboxylation (*20*); (c) the γ-carboxylation of a fusion construct expressing the prothrombin propeptide and a glutamic acid-rich region of thrombin argues that the propeptide is sufficient to direct γ-carboxylation (*20*). However, it remains to be shown whether *Conus* carboxylation occurs with the carboxylation recognition site solely on the precursor, whether some elements of the mature conopeptide are important for γ-carboxylation and whether the *Conus* carboxylase shows regiospecificity for the target glutamic acids. At present, our experiments only demonstrate that the hydrophobic precursor peptide contains a required component of the γ-carboxylation recognition site, a recognition element required for efficient γ-carboxylation in this single conotoxin, ϵ-TxIX. The propeptide of pro-ϵ-TxIX defines interaction with the *Conus* carboxylase, with a K_M of about 4 μM. This value is comparable to the K_M values for the mammalian blood clotting proteins for the bovine carboxylase, which have been found to be about 3 μM.

The carboxylation recognition sites for both the bovine carboxylase and the *Conus* carboxylase are defined by hydrophobic amino acids. The carboxylation recognition site of mammalian carboxylase substrates and the carboxylation recognition site of the *Conus* carboxylase substrate show no sequence similarity. However, we have shown that the pro-ϵTxIX carboxylation recognition site is a substrate for the bovine carboxylase, although the *Conus* carboxylation recognition site does not support optimal interaction. The propeptide of pro-ϵTxIX decreases the K_M of this peptide by about 150-fold when the *Conus* carboxylase is employed but only by about 20-fold when the bovine carboxylase is employed. The absence of sequence similarity but the presence of functional similarity reinforces our current view of the carboxylation recognition site: it is not defined by an amino acid sequence but rather a chemical surface with a unique topology that is complementary to the surface of the propeptide binding site of the carboxylase.

The molecular surface responsible for carboxylase recognition is apparently defined by this site, which may be stabilized in mammalian carboxylase substrates by an adjacent amphipathic α-helix (*41*). This appears to be the case for pro-ϵ-TxIX. The major component of the carboxylation recognition site appears to reside in the first 12 residues of the propeptide; this region showed no α-helical structure. However, residues −25 to −13 are dominated by helical structure, and this amphipathic α-helix is adjacent to the major component of the carboxylation recognition site and contributes to its interaction with the carboxylase as indicated by the effect of this region on V_{max} and of mutations in this region on K_M values. Given the common feature of regular spacing of hydrophobic amino acids in the propeptide and the prediction of an amphipathic α-helix adjacent to or involving part of the carboxylation recognition site, we believe that the carboxylation recognition site is enhanced by the hydrophobic patch formed by multiple hydrophobic residues. By contrast, conotoxin precursors that do not undergo γ-carboxylation do not have this pattern of hydrophobic patches with similar spacing. Comparison of carboxylation in vertebrates and invertebrates to identify both similarities and differences should provide insight into the role of vitamin K in the mechanism of γ-carboxyglutamic acid biosynthesis.

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BI991640L