Identification of a vitamin K-dependent carboxylase in the venom duct of a *Conus* snail

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Abstract Peptides from the venom ducts of cone snails (genus *Conus*) contain γ-carboxyglutamate residues. The γ-glutamyl carboxylase responsible for this post-translational modification is localized in the microsomal fraction, strictly dependent on vitamin K, activated by ammonium sulfate, and is associated with endogenous substrate. The K_m of the enzyme for vitamin K is comparable to that for the bovine carboxylase. However, a propeptide containing substrate related to the blood coagulation protein factor IX, a highly efficient substrate for the bovine enzyme, was poorly carboxylated by the *Conus* enzyme, suggesting differences in γ-carboxylase recognition signal sequences and/or structural requirements at the carboxylation site.

Key words: Vitamin K-dependent glutamyl carboxylase; Gamma-carboxyglutamate; Post-translational modification; *Conus radiatus*

1. Introduction

A large and diverse group of biologically active small peptides are found in the venom ducts of predatory cone snails (genus *Conus*). These peptides are the products of the normal transcription and translation machinery of the cell, but are notable for their very high frequency of post-translational modification (see for example [1]). One of the most unusual of these post-translational modifications is the carboxylation of glutamate (Glu) residues to γ-carboxyglutamate (Gla), first described in peptides belonging to the conantokin family [2–5]. The conantokins have been shown to be inhibitors of the NMDA (*N*-methyl-D-aspartate) receptors. The occurrence of γ-carboxyglutamate in other *Conus* peptides has also been established.

The discovery of γ-carboxylation of glutamate in the *Conus* venom duct system was completely unexpected because this modification had previously been described only in a small subset of mammalian proteins, which were either involved in the blood coagulation cascade, or in bone metabolism. More recently, another mammalian protein, *gas*6, related to the vitamin K-dependent negative coregulator in the blood coagulation cascade, has been identified and has been postulated to play a role in the control of cell growth [6] and tissue reorganization [7]. This post-translational modification had not been described in any invertebrate system. To date, the *Conus* venom duct carboxylase system remains the only nonvertebrate system where γ-carboxylation of glutamate has been firmly established.

While the mammalian carboxylase from bovine liver has been purified [8,9], cloned and sequenced [10,11], there is only one preliminary report on the *Conus* carboxylase in the literature [12], and the carboxylase activity in *Conus* venom ducts remains uncharacterized. The mammalian γ-glutamyl carboxylase is believed to be an integral membrane protein that is found in the rough endoplasmic reticulum [13] and has a strict requirement for the reduced form of vitamin K as a cofactor [13]. All the known substrates for the mammalian vitamin K-dependent carboxylase possess a highly conserved propeptide sequence. Except in the case of the bone matrix Gla protein, this sequence is removed from the mature protein. This sequence is believed to be the primary binding site for the carboxylase and has been designated as the γ-carboxylation recognition site (γ-CRS) [14].

The substrate routinely used for assaying the mammalian vitamin K-dependent carboxylase is the glutamate containing pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) [15]. The FLEEL peptide has a millimolar K_m for the carboxylase and its activity is stimulated by the addition of free propeptide [16–18]. It has been shown that Glu-containing peptides which have the propeptide sequence attached are high affinity substrates for the carboxylase ($K_D \approx 10^{-6}$ M) [19,20]. A peptide expressed in *E. coli* (factor IX (FIX)−18–41), which consists of the propeptide and all normally carboxylated residues of the vitamin K-dependent clotting protein factor IX, is an excellent substrate for the bovine enzyme in in vitro carboxylation assays with an apparent sub-micromolar K_m [21].

In this study, our aim has been to carry out an initial investigation of similarities and differences between the *Conus* venom duct carboxylase, and the mammalian enzyme system. For this purpose, we have used as the raw material the venom ducts of a fish-hunting *Conus* species, *Conus radiatus*. This species was chosen because its venom contains several peptides which have a high content of γ-carboxyglutamate. Included among these are a conantokin, conantokin-R which shows homology to previously characterized conantokins, and the recently characterized bromosleeper peptide [22]. The latter appears to be unrelated to the conantokin family but has a number of γ-carboxyglutamate residues. The presence of Gla-rich peptides in *Conus radiatus* venom made it likely that the venom duct of this species had substantial carboxylase activity, an expectation confirmed in the results presented below.

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2. Materials and methods

2.1. Materials

C. radiatus venom ducts were obtained from the Philippines. Aprotinin, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride (PMSF), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and the pentapeptide FLEEL were purchased from Sigma. Protease inhibitors FFRCK and FPRCK were from Bachem; vitamin K, (phytonadione), from Abbot Laboratories, and $NaH^{14}CO_3$ 55 mCi/mmol was from NEN.

2.2. Preparation of microsomes

C. radiatus ducts were frozen in liquid nitrogen and ground to a fine powder in a precooled mortar and pestle. This was then homogenized in buffer A, containing 25 mM MOPS pH 7.5, 500 mM NaCl and a mixture of protease inhibitors containing 2 mM DTT, 2 mM EDTA, 0.125 μg/ml FFRCK, 0.125 μg/ml FPRCK, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 34 μg/ml PMSF and 2 μg/ml aprotinin. The homogenate was centrifuged at 19000 rpm for 30 min at 4°C in a SS-34 rotor in a Sorvall RC-5B centrifuge. The supernatant, designated low-speed supernatant, was centrifuged in a TA 100.3 rotor at 75000 rpm for 60 min at 4°C in a Beckman TL 100 centrifuge. The pellet containing microsomes was resuspended in buffer A, distributed in small aliquots, quick-frozen in liquid nitrogen and stored at −84°C. The low-speed supernatant, microsomal fraction and post-microsomal supernatant were assayed for carboxylase activity essentially as described by Morris et al. [18].

2.3. Assay of carboxylase activity

Bovine microsomes prepared according to Girardot [23] and snail microsomes were solubilized in 0.7% CHAPS/0.7% phosphatidyl choline/1.5 M NaCl for 20 min on ice. Final reactions were done in a total volume of 125 μl containing solubilized microsomes and a final concentration of reagents as follows: 25 mM MOPS, pH 7.4, 0.5 M NaCl, 0.2% CHAPS, 0.2% phosphatidyl choline, 0.8 M ammonium sulfate, 1.2 mM FLEEL, 5 μCi $NaH^{14}CO_3$, 6 mM DTT, 222 μM reduced vitamin K (prepared as described by [24]). Reaction mixtures were incubated at 25°C for 30 min and were quenched by the addition of 75 μl of 1 N NaOH. 160 μl of the quenched reaction mixture was transferred to 1 ml of 5% trichloroacetic acid and boiled to remove unincorporated $^{14}CO_2$. After cooling, 5 ml Ecolite (NEN) was added and the $^{14}CO_2$ incorporated determined in a Beckman LS 9800 counter. The amount of microsomal proteins present in the various experiments are indicated in Results. All reported values are an average of three independent determinations.

2.4. Determination of apparent K_m for reduced vitamin K

Carboxylase activity was determined as described above except that reduced vitamin K concentration was varied to give a final concentration from 0 to 400 μM. A total of 7 mg of bovine microsomal protein and 0.3 mg of snail venom duct microsomal proteins were used in the reactions. Kinetic parameters were determined using non-linear least squares analysis and fitting the data to the Michaelis-Menten equation. All reported kinetic values are averages of at least three independent assays.

2.5. Assay for carboxylation of FIX−18–41

Carboxylase activity was determined essentially as described in Section 2.3 with the following changes. A total of 7 mg of bovine microsomal protein and 0.3 mg of snail venom duct microsomal proteins were used in the reactions, ammonium sulfate was omitted and the concentration of the substrate, FIX−18–41, was varied between 0 and 25 μM. Reactions were performed at 17°C for 9 h and processed as described above.

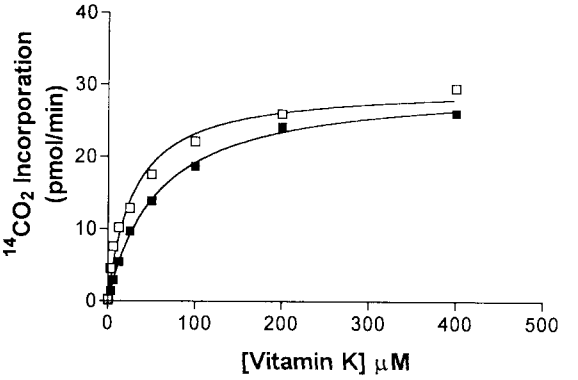

Fig. 1. Effect of reduced vitamin K on carboxylation with bovine (open squares) and *Conus* (closed squares) microsomes.

2.6. Protein concentration

Protein concentrations were determined by the micro assay using Bio-Rad protein assay reagents and bovine serum albumin as standard.

3. Results

3.1. Conus venom duct carboxylase, a microsomal fraction activity

Crude extractions of *C. radiatus* venom ducts were examined for carboxylase activity as described by Morris et al. [18]. Carboxylase activity in the crude extract was fractionated into a microsomal fraction and a post-microsomal supernatant. The post-microsomal supernatant had less than 4% of the specific activity of the microsomal fraction, and the *C. radiatus* venom duct microsomal fraction had a 6-fold greater specific activity; essentially all carboxylase activity found in the crude extract is recovered in the microsomal fraction (Table 1). This microsomal fraction was used for all subsequent experiments.

3.2. Conus carboxylase activity is vitamin K-dependent

The carboxylase activity found in the microsomal fraction was assayed using the standard reaction mixture for the mammalian enzyme. As shown in Table 2, no carboxylase activity was detected in the absence of either microsomes or reduced vitamin K. These results demonstrate that the *Conus* carboxylase is absolutely dependent on vitamin K, as previously established for the mammalian enzyme.

The K_m of the *Conus* enzyme for vitamin K is ~2-fold greater than that of the mammalian enzyme (28.9 ± 0.4 μM for the bovine enzyme and 57.2 ± 4.5 μM for the *Conus* en-

Table 1
Fractionation of *Conus radiatus* venom duct carboxylase

Fraction	Total activity (nmol of $^{14}CO_2$ incorporated in 30 min)	Specific activity (nmol of $^{14}CO_2$ incorporated in 30 min per mg protein)
Low-speed supernatant	32.8	4.2
Microsome	44.2	27.1
Post-microsomal supernatant	5.7	1.0

In three independent microsome preparations the specific activity of the low-speed supernatant was 3.3 ± 1.3, microsomes 36.1 ± 18.0, and post-microsomal supernatant 0.4 ± 0.06.

zyme), under conditions where the two activities are tested in parallel (see Fig. 1). Thus, the *Conus* carboxylase and the mammalian enzyme are very similar in their absolute requirement for vitamin K and in their affinity for the cofactor.

3.3. Endogenous substrate in Conus carboxylase preparation and the effect of ammonium sulfate on carboxylase activity

As shown in Table 2, the microsomal fraction apparently contains endogenous substrate. If the exogenous substrate peptide (the pentapeptide FLEEL) is omitted, a small but detectable carboxylase activity is observed. The *Conus* carboxylase is greatly stimulated (~18-fold under these conditions) by the addition of these high levels of ammonium sulfate (Table 2) an effect also observed for the mammalian enzyme. Thus, for all of the parameters examined in the experiments in Table 2, the *Conus* venom duct carboxylase and the mammalian enzyme appear to be similar.

3.4. Specificity differences between the mammalian and the Conus venom duct enzymes

In order to test whether the *Conus* enzyme recognizes the same signals that give the mammalian enzyme high affinity for certain substrates, a high affinity substrate for the mammalian enzyme was tested on both the mammalian enzyme and the *Conus* carboxylase. The propeptide-containing substrate for factor IX, comprised of amino acids −18–41, is a high affinity substrate for the bovine enzyme [8]. As shown in Fig. 2, although this peptide is an effective substrate for the mammalian enzyme ($K_m \sim 1.1 \pm 0.14$ μM), under these experimental conditions no activity (above that seen in the absence of exogenous substrate) is observed for the *Conus* enzyme.

4. Discussion

The venom duct of *Conus* is the only invertebrate system in which carboxylation of glutamate to γ-carboxyglutamate is known to occur. This modification is an unusual one and has only been characterized thus far in several proteins in the mammalian blood coagulation system, selected bone proteins, and in *Conus* venom ducts. The expectation of this modification in the mammalian cell growth regulator *gas6* suggests that γ-carboxylation may be more ubiquitous than previously thought. In the case of the *Conus* toxins, some of the Gla modifications are critical for biological activity. It was the goal of this study to characterize the γ-carboxylation activity of the *Conus* venom duct and compare its activity to the previously characterized mammalian vitamin K-dependent carboxylase. The comparison will also shed light on the evolution of this enzymatic activity in phylogenetically disparate systems.

Table 2
Carboxylase activity of *Conus radiatus* microsomes

	pmol of $^{14}CO_2$ incorporated in 30 min
Complete	455.16 ± 9.73
−microsomes	3.98 ± 0.26
−vitamin K	3.84 ± 0.26
−exogenous substrate	10.64 ± 0.33
$-(NH_4)_2SO_4$	25.63 ± 3.54

The complete reaction mixture contained microsomes (8 μg protein), CHAPS, phosphatidylcholine, $(NH_4)_2SO_4$, FLEEL, $NaH^{14}CO_3$ and reduced vitamin K at final concentrations described in Section 2. Experiments were done in triplicate.

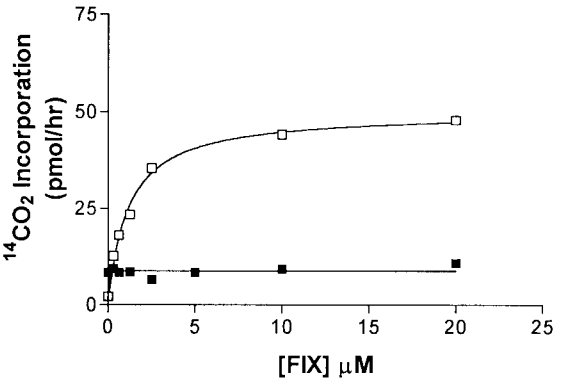

Fig. 2. Carboxylation of the factor IX−18–41 peptide with bovine (open squares) and *Conus* (closed squares) microsomes. Identical results were obtained for another independent experiment.

The enzyme activity characterized in the venom duct of the *Conus radiatus* exhibits striking similarities to its counterpart in mammals. The enzyme activity can be assayed by measuring the incorporation of $^{14}CO_2$ into the pentapeptide substrate FLEEL which is based on a carboxylated sequence in several of the vitamin K-dependent blood clotting proteins. The *Conus* carboxylase activity is found to be enriched in the microsomal fraction of fractionated venom ducts. In order to achieve the same level of carboxylation of FLEEL, 10–20 times as much bovine microsomal protein appears to be necessary compared to that from *Conus* suggesting that the specific activity of the *Conus* enzyme at this stage of purification is 10–20 times higher than for the bovine enzyme. The *Conus* carboxylase also requires the reduced form of vitamin K for activity as is seen for the mammalian enzyme and possesses a similar apparent affinity for this essential cofactor. Finally the *Conus* enzyme is dramatically stimulated by ammonium sulfate as is the mammalian enzyme.

A key feature of the mammalian carboxylase is its ability to recognize a highly conserved propeptide sequence present in all the known mammalian proteins which undergo γ-carboxylation. This γ-carboxylation recognition signal is found N-terminal to the glutamates that are carboxylated and then is removed to create the mature form of the protein. The only known exception to this motif is found in bone matrix Gla protein where the propeptide is present in the mature from of the protein [25,26]. The propeptide γ-CRS is believed to be the primary binding site for the mammalian carboxylase and confers a much higher affinity for the carboxylase to substrates which contain the CRS compared to glutamate containing substrates alone.

We tested whether the *Conus* enzyme could recognize and carboxylate a high affinity 59-amino acid substrate which contains the propeptide and carboxylated glutamates of the blood clotting protein factor IX. The *Conus* enzyme displays no activity above background for this substrate within the range of substrate concentrations tested. This suggests that this enzyme either does not recognize or poorly recognizes the mammalian γ-CRS or that the glutamates present in mammalian substrate are not positioned appropriately so they can be modified by the *Conus* enzyme. This suggests that the *Conus* enzyme may possess a totally different motif for recognizing its native substrate. In addition, the invariant unit Glu-Xaa-

Xaa-Xaa-Glu-Xaa-Cys, present in the Gla domains of all the mammalian proteins [26], is absent in the conantokins.

The pattern of γ-carboxylation of some of the *Conus* conantokin peptides may already provide a clue for the diverging substrate specificity of the two systems. In the 21-amino acid conantokin-T, only four of the six Glu residues are carboxylated [5]. The latter pattern of carboxylation seem to be more related to the vitamin K-dependent bone proteins where some glutamates are not carboxylated [25]. Although it is possible that even though the two enzymes are both vitamin K-dependent and are able to carboxylate small glutamate-containing substrates, at the level of substrate specificity the two enzymes may have diverged significantly. Comparison of the propeptide sequences of the conantokins and conotoxins will be necessary in order to identify the *Conus* γ-CRS. While a consensus amino acid sequence may emerge, the γ-CRS may be a multitude of sequences defining a tertiary structure, as in the case of the signal peptides involved in protein translocation into the endoplasmic reticulum [27]. Studies to identify the CRS for the *Conus* enzyme are in progress.

In conclusion, we have for the first time demonstrated that the γ-carboxylase activity in the *Conus* venom duct is vitamin K-dependent and appears to posses many of the characteristic of the mammalian vitamin K-dependent carboxylase, but is unable to carboxylate a native mammalian substrate. The purification, cloning, and sequencing of the *Conus* vitamin K-dependent carboxylase should permit a detailed comparison of its structural identity with the mammalian enzyme and suggest whether these enzymes are in fact related or whether unrelated proteins have converged upon a common function.

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