Mass spectrometric-based revision of the structure of a cysteine-rich peptide toxin with γ -carboxyglutamic acid, TxVIIA, from the sea snail, *Conus textile*

TAKEMICHI NAKAMURA,^{1,3} ZHONGHUA YU,¹ MICHAEL FAINZILBER,^{2,4}
AND ALMA L. BURLINGAME¹

¹ Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446

² Molecular Neurobiology, Free University of Amsterdam, The Netherlands

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Abstract

A mollusk-specific toxin, TxVIIA, having potent paralytic activity was isolated from the venom of sea snail *Conus textile* (Fainzilber M et al., 1991, *Eur J Biochem* 202:589–595). The structure reported above was based upon amino acid analysis and the Edman degradation. We have recently reinvestigated this toxin employing some of the most novel techniques in mass spectrometry. We now report a revised structure based primarily on high-energy collision-induced dissociation analysis of the two Asp¹⁷-N peptides of the reduced, pyridinylethyl derivative representing the entire sequence using matrix-assisted laser desorption ionization (MALDI) as CGGYSTYC γ VDS γ CCSDNCVRSYCTLF-NH₂ (γ , γ -carboxyglutamic acid or Gla).

The N-terminus of the previous sequence was incorrect, apparently due to a side reaction of reduction and alkylation, which led to the erroneous assignment of Trp for the N-terminal residue. In addition, the last two C-terminal amino acids and the C-terminal amidation had not been detected. Also, a combination of electrospray ionization mass spectrometry and positive and negative ion MALDI mass spectrometry provided information on the molecular weights of the native and derivatized toxin and presence of two Gla residues. Thus, TxVIIA does not have an "unusual" sequence as previously reported, but in fact belongs to the conserved Cys framework for ω - and δ -conotoxins. However, the four net negative charges with the cysteine-rich structure of this revised sequence is highly unusual for conopeptides.

Keywords: conotoxin; *Conus textile*; γ -carboxyglutamic acid; mass spectrometry; peptide structure; posttranslational modification

Conotoxins, a variety of peptide neurotoxins found in venom of *Conus* sea snails, are indispensable tools for neurophysiology and potential leads for structure-based drug design (Olivera et al., 1990; Myers et al., 1993). Precise structural analysis of diverse conotoxins is an important endeavor because the dis-

tinct biological activity of each toxin is directly related to its unique structure. Although conotoxins are relatively small peptide molecules (typically consisting of 10–30 amino acid residues), structural elucidation of these toxins can be challenging and complicated for several reasons, including: (1) the possibility of C-terminal amidation (Cruz et al., 1985; Ramilo et al., 1992); (2) the frequent observation of other posttranslational modifications, e.g., γ -carboxyglutamic acid (Gla) (McIntosh et al., 1984; Yanagawa et al., 1988), 4-hydroxyproline (Sato et al., 1983; Olivera et al., 1984), and phosphorylation or sulfation (Fainzilber et al., 1994a); and (3) the high content of cysteine residues, requiring reduction and alkylation prior to sequencing as well as sophisticated strategies for analyzing disulfide bridges (Gray et al., 1984; Hidaka et al., 1990; Shon et al., 1994). We have been tackling these difficulties by taking advantage of various techniques of advanced mass spectrometry.

Recently, we isolated and determined the primary structures of two mollusk-specific conotoxins, μ PnIVA and μ PnIVB, both of which have a new cysteine framework for conotoxins (Fain-

Reprint requests to: Alma L. Burlingame, Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446; e-mail: alb@itsa.ucsf.edu.

³ Present address: Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd., 2-58 Hiromachi 1-chome, Shinagawa-ku, Tokyo 140, Japan.

⁴ Present address: Department of Medical Biochemistry & Biophysics, Karolinska Institute, Stockholm, Sweden.

Abbreviations: CID, collision-induced dissociation; CPase-Y, carboxypeptidase-Y; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; Gla, γ -carboxyglutamic acid; LC/ESI MS, liquid chromatography electrospray ionization mass spectrometry; MALDI, matrix-assisted laser desorption ionization; PSD, post-source decay; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TOF, time-of-flight; oa-TOF, orthogonal acceleration TOF.

zilber et al., 1995). The new framework includes an N-terminal cysteine residue that displayed previously undescribed unusual behavior upon attempted reduction and alkylation. These cysteine residues gave an unidentified product as a result of treatment with dithiothreitol and 4-vinylpyridine. The unidentified cysteine derivative, generation of which may be attributable to some impurity in certain batches of reagent used for the reduction alkylation, gave a clear signal at the position of Trp in automated Edman sequencing and led us to assign erroneous N-terminal-Trp sequences in the initial stage of our structural studies. Subsequent measurement of the molecular weights of these two toxins revealed discrepancies with the sequences derived from the Edman data. As described elsewhere, mass spectrometry was essential in revealing the complete sequences of PnIVA and PnIVB, and establishing that the first two N-terminal residues of these toxins are Cys (Fainzilber et al., 1995). This experience led us to reinvestigate one of the previously reported sequences of conotoxins, which was deduced based on Edman sequencing and amino acid analysis, but without the involvement of mass spectrometry. This conotoxin, TxIIA, was first isolated from the venom of *Conus textile* and characterized as a structurally unique and physiologically potent mollusk-specific conotoxin by Fainzilber et al. (1991). The amino acid sequence of the toxin, published in 1991, included an N-terminal tryptophan residue and an uncommon odd number of cysteine residues (Fainzilber et al., 1991). Based upon our recent mass spectrometric-based investigations, we now report that the N-terminal tryptophan was an erroneous assignment, with cysteine being the correct assignment. In addition, these studies by tandem mass spectrometry have established unambiguously that the original Edman sequence was short two residues at the C-terminus. On the basis of the revised Cys framework reported herein, we proposed to rename this toxin TxVIIA, in accordance with a recent agreement to standardize conotoxin nomenclature (B.M. Olivera, in prep.). This article describes the importance of involving a variety of new techniques of mass spectrometry in establishing the revised structure of TxVIIA.

Results and discussion

The molecular weight of the main component in the TxVIIA sample, which was prepared in connection with the previous study (Fainzilber et al., 1991), was found to be 3,088.9 based on its measurement by ESI mass spectrometry, as shown in Figure 1. In addition, the electrospray spectrum reveals the presence of two other significant components whose molecular weights are 44 and 88 Da lower than the main component (Fig. 1). This finding is consistent with the originally reported amino acid analysis that included two residues of Glu. This observation suggests partial decarboxylation of the two Glu residues in the toxin (which gives a 44-unit decrease in mass per Glu residue) during the prolonged storage of the sample (McIntosh et al., 1984), because the amino acid analysis in the previous study gave nearly quantitative yield of Glu (Fainzilber et al., 1991). The electrospray molecular weight was supported by additional measurements using MALDI mass spectrometry, as well. The linear-mode positive ion MALDI mass spectrum is presented in Figure 2A, showing analogous ions corresponding to MH^+ , MH^+-CO_2 , and MH^+-2CO_2 , respectively. Interestingly, in the negative ion mode (Fig. 2B), virtually only a doubly decarboxylated species ($[M-H-2CO_2]^-$) was detected. Subsequently, this phenomenon was observed in the MALDI mass spectra of another Glu-containing conotoxin as well (T. Nakamura & A.L. Burlingame, unpubl. results). This suggested that the internal energy deposition in the positive ion mode should be relatively low, as is known to be the case for electrospray ionization, but significantly higher in the negative ion mode leading to facile, quantitative didecarboxylation. The molecular weight thus measured was in disagreement with the calculated average molecular mass (Carr & Burlingame, 1996) for the previously published sequence, 2,915.1 (assuming 2 X S-S) (Fainzilber et al., 1991; WGGYSTYC γ VDS γ CCSD NCVRSYCT, γ , Glu).

To reinvestigate the primary structure, the TxVIIA sample was reduced and alkylated with 4-vinylpyridine. The sample was used as the mixture of intact and partially decarboxylated ma-

Fig. 1. ESI mass spectrum of TxVIIA. Doubly and triply protonated peptide molecules were observed in the displayed mass range and are marked as X2 and X3, respectively, where X is A, B, or C. Observed average molecular mass (based on the mass of doubly and triply charged ions) for the neutral main component (designated as A) is shown in the upper right of the spectrum along with the masses for minor components, B and C. Ions designated with B and C are not attributable to a gas-phase fragmentation from A, but to minor components in the sample because an LC/ESI MS experiment gave a partial separation of these three components.

Fig. 2. Linear-mode positive ion (A) and negative ion (B) MALDI mass spectra of TxVIIA. A laser power of just above the desorption threshold was used for acquisition.

terial without further purification, to avoid loss of sample. A portion of this derivatized sample was analyzed by LC/ESI MS and gave the average molecular weight of 3,726.7 for the main component (data not shown). The mass increment of 637.8 units from the native peptide corresponds to reduction and alkylation of three pairs of half cysteine residues (212.3 unit by average mass per a pair of half cysteine residues, in theory) and strongly suggests the presence of a sixth Cys in the peptide, which was not found in the original Edman sequence. Further, the molecular weight difference between the calculated mass for the originally published sequence and observed value, 173.8, could not be explained by simply replacing any of the amino acid residues in the sequence with a Cys residue, or by simply adding a Cys residue to the sequence. Therefore, it appeared that residues other than just the Cys were missing from the published sequence as well.

To investigate the nature of this discrepancy, the reduced and pyridylethylated toxin was subjected to proteolytic digestion. Because there are two Asp residues in the reported sequence, the peptide was digested with Endoproteinase Asp-N, which is ex-

pected to cleave on the N-terminal side of Asp (Drapeau, 1980). The digestion mixture was analyzed by LC/ESI MS and revealed five major peaks designated Fragments A-E, as shown in Figure 3. It was found that the sum of the molecular weights of two of the peptide fragments (Fragment D, a peptide with an average molecular weight of 2,214.3, and Fragment E, a peptide with average molecular weight of 1,529.9) corresponded to the mass of the derivatized toxin prior to digestion. Hence, these peptides were purified with reversed-phase HPLC and re-analyzed by MALDI mass spectrometry in various ways. Although Fragment D showed an MH^+ peak corresponding to the mass observed by LC/ESI MS, its spectrum in negative ion mode showed virtually a single peak at roughly 90 Da (corresponding to loss of $2 \times CO_2$) lower, indicating again the presence of two Glu residues that have undergone facile didecarboxylation in the MALDI process as discussed above. Because the peptide fragment was generated by an Asp-N digestion, it is likely to correspond to the N-terminal hexadecapeptide terminating at Ser¹⁶. The calculated average molecular weight for the hexadecapeptide based on the published sequence was 2,192.4 (as pyridyl-

Fig. 3. Microbore reversed-phase LC/ESI MS trace (base-peak intensity) of the Asp-N digest of reduced and pyridylethylated TxVIIA. The average molecular masses of five major peptide fragments (A-E) were found to be 1,125.2, 1,063.2, 2,170.5, 2,214.3, and 1,529.9, respectively. The sum of the molecular weights of components D and E comprise the N- and C-terminal peptides of the derivatized toxin intact. The identity of the others are discussed in the text.

ethyl derivative) and 21.8 Da smaller than the observed value for Fragment D by ESI MS. The mass difference fits the presumed Trp/Cys misassignment due to a side reaction similar to the one observed for PnIVA and PnIVB (Fainzilber et al., 1995). To confirm this hypothesis, peptide D was analyzed by automated Edman degradation (Table 1) and MALDI CID analysis (Medzihradszky et al., 1996). The MALDI CID spectrum (Fig. 4) of Fragment D was determined by selecting only the ^{12}C isobar of the protonated molecular ion stable isotope cluster at 2,213.9 Da. The presence of sequence ions b_1 – b_8 establish the N-terminal octapeptidyl moiety as CGGYSTYC, whereas the b_9 falls at the residue mass expected for Glu, 44 Da lower than would be expected for intact Gla. In addition, the b_{10} – b_{12} fragments corresponding to the tripeptidyl sequence VDS are shifted similarly, whereas the b_{13} is lowered by an additional 44 Da as expected from the presence of the second decarboxylated Gla residue at that position. Thus, the additional carboxyl functions present in the intact molecular ion must be located at residues 9 and 13, consistent with the peaks due to facile loss of both one ($\text{MH}^+ - 44$ Da) and two ($\text{MH}^+ - 88$ Da) moles of CO_2 from MH^+ at 2,213.9. These results establish the revised N-terminal sequence, CGGYSTYC γ VDS γ CCS (as pyridylethyl derivative).

The MALDI reflectron mass spectrum of Fragment E (Fig. 5) clearly established the monoisotopic protonated molecule of the peptide at m/z 1,529.6, and thus the monoisotopic molecular mass is 1,528.6. This value is 259.1 units larger than the monoisotopic molecular mass of the C-terminal digest expected from the published sequence (DNCVRSYCT, 1,269.5, as pyridylethyl derivative). Because the Trp/Cys misassignment at the N-terminus has been established as described above, calculated average mo-

lecular mass for the revised sequence down to Thr²⁵ would now be 2,830.0 (assuming 3 X S-S) and is 258.9 units smaller than the observed value for the intact TxVIIA. This mass difference fits that observed for Fragment E and, therefore, the presence of two or three more residues at the C-terminus was strongly suggested.

In a similar manner, the sequence of peptide E was determined by MALDI CID analysis as shown in Figure 5. The ^{12}C isobar of the molecular stable isotopic profile was selected as the precursor species at $m/z = 1,529.5$ Da. Interpretation of this spectrum revealed that the missing C-terminal amino acids are Leu-Phe²⁷ and established that the C-terminus is amidated. Although these spectra are consistent with the new Edman data and are sufficient to establish the revised structure, it was nevertheless of interest to assign the remaining three components obtained in the Asp-N digest (Fig. 3).

Fragment C was identified as the γ -decarboxyl-Gla⁹ N-terminal Asp-N peptide by MALDI CID analysis (data not shown). Fragment B was assigned to an N-terminal portion of the peptide ending Cys⁸ (observed mass: 1,063.2; calculated average mass for CGGYSTYC: 1,063.2, as pyridylethyl derivative). The peptide was presumably formed by cleavage at Glu (Ingrosso et al., 1989), which is present in fragment C as the ninth residue instead of Gla⁹ as a result of spontaneous decarboxylation from Gla. The HPLC-purified sample of Fragment B was re-analyzed by MALDI reflectron mass spectrometry and the monoisotopic MH^+ of the peptide was confirmed at m/z 1,063.6 (calculated monoisotopic molecular mass: 1,062.4). The MALDI metastable ion (PSD) mass spectrum of this peptide supported the Cys¹ sequence. Fragment A was assigned to a fragment from partly decarboxylated material, E⁹VDS γ CCS¹⁶ (observed mass: 1,125.2; calculated mass: 1,125.2, as pyridylethyl derivative).

In conclusion, the revised primary structure of TxVIIA has been established as CGGYSTYC γ VDS γ CCSDNCVRSYCTLF-NH₂ (γ , Gla) by an integration of modern sequencing methods, which includes peptide mapping by LC/ESI MS, MALDI high-energy CID tandem mass spectrometry, automated Edman sequencing, and MALDI reflectron and metastable fragmentation (PSD) mass spectrometry. A combination of MALDI in both positive and negative ion modes, MALDI CID, and ESI mass spectrometry was essential in establishing the presence and location of Gla residues in this conotoxin. Although further investigation is necessary to establish the generality of this observation, MALDI mass spectrometry appears to be potentially useful for detecting Gla residues in peptides, i.e., the number of Gla residues in a peptide may be predicted by simply comparing positive ion and negative ion mode MALDI and ESI mass spectra of the peptide. However, only high-energy CID analysis using MALDI ionization was able to provide the required connectivity between the single intact molecular species (Fragment D), the presence of two Gla residues, and the amino acid sequence of the N-terminal Asp-N peptide, as well as the sequence and amidation of the its corresponding C-terminal counterpart (Fragment E).

Although the MALDI PSD mass spectra (Kaufmann et al., 1993) of various fractions supported certain features, this technique was not able to provide data that was sufficiently rigorous to establish the structure of the toxin. However, the presence of the immonium ions at m/z 120 and 86 suggested the presence of Phe and Leu or Ile residues, neither of which is present in the previously published sequence of TxVIIA. Because the sum

Table 1. Edman degradation of Asp-N digest of reduced and pyridylethylated TxVIIA

Cycle	Fragment D		Fragment E	
	Assigned residue	Yield (pmol)	Assigned residue	Yield (pmol)
1	Cys ^a	147.7	Asp	168.6
2	Gly	124.0	Asn	187.9
3	Gly	121.8	Cys ^a	314.3
4	Tyr	120.8	Val	212.8
5	Ser	21.2 ^b	Arg	65.4
6	Thr	29.1 ^b	Ser	30.8 ^b
7	Tyr	86.5	Tyr	107.4
8	Cys ^a	112.2	Cys ^a	111.4
9	— ^c	(Glu: 2.6)	Thr	12.1 ^b
10	Val	69.5	Leu	5.1
11	Asp	45.7	Phe	2.5
12	Ser	8.4 ^b		
13	— ^c	(Glu: 6.0)		
14	Cys ^a	52.9		
15	Cys ^a	57.1		
16	Ser	5.1 ^b		

^a Detected as pyridylethyl derivative.

^b Yield of these hydroxyamino acids was relatively low and degradation products were observed in the chromatogram, as expected.

^c Blank cycles corresponding to Gla position. No PTH-amino acids except weak PTH-Glu were observed at these cycles.

Fig. 4. MALDI high-energy CID mass spectrum of Fragment D ($m/z = 2,213.9$ Da), CGGTSTC γ VAS γ CCS.

of masses corresponding to residues of Phe and Leu or Ile is 260 and 1 unit larger than the observed mass difference, the C-terminal residue of the peptide was presumed to be amidated.

In summary, it was found that the previously reported sequence of TxVIIA, which was solely based on Edman sequencing and amino acid analysis without the involvement of mass spectrometry, was incorrect at both the N- and C-termini of this toxin. Erroneous identification of the N-terminal residue appears attributable to a side reaction of reduction and alkylation, whereas the Edman did not detect two residues at the C-terminus. The nature of the side reaction occurring at the N-terminal Cys in the previous study is still unclear because we were not able to reproduce the side reaction in the present study. Nevertheless, this study has shown the clear advantages of employing advanced methods of mass spectrometry in the investigation of posttranslationally modified Cys-rich peptides like conotoxins.

Taking into account these revisions, the TxVIIA is not "unusual" in terms of its Cys arrangement as reported in the previous publication, but displays the conserved Cys framework of δ -conotoxins (Hillyard et al., 1989; Fainzilber et al., 1994b; Shon et al., 1994) and ω -conotoxins (Olivera et al., 1994). Nevertheless, except for the Cys framework, there are few similarities between the amino acid sequence of TxVIIA, which contains two Gla residues and has a high negative net charge (-4), and the sequences of most other known δ - or ω -conotoxins. In addition, the paralytic activity of TxVIIA against mollusks is substantially

higher than that of the δ -conotoxin TxVIA, and may have a somewhat different mode of action (Fainzilber et al., 1991). These present results pave the way for chemical synthesis of this structurally and physiologically unique conotoxin for further investigations.

Materials and methods

Materials

Isolation of the conotoxin, TxVIIA (previously called TxIIA), from the venom of *C. textile* has been described elsewhere (Fainzilber et al., 1991). An additional aliquot of the purified peptide sample employed in the previous study, which had been dried and stored in a freezer at -20°C , was used without further purification.

The chemicals for reduction and alkylation were obtained from commercial sources (dithiothreitol: Pierce, lot 931214160, Rockford, Illinois; 4-vinylpyridine: Sigma, lot 73H1066, St. Louis, Missouri) and used without further purification.

Endoproteinase Asp-N and CPase-Y were purchased from Boehringer Mannheim (Indianapolis, Indiana).

Reduction and alkylation of TxVIIA

A dried peptide sample (ca. 5 nmol) was dissolved in 50 μL of 0.1 M NH_4HCO_3 , pH 8, containing 6 M guanidine-HCl and

Fig. 5. MALDI high-energy CID mass spectrum of Fragment E ($m/z = 1,529.5$), DNCVRSYCTLPNH₂.

10 μ M EDTA, and reduced with 300 μ g of dithiothreitol at 37 °C for 2 h under argon. To the solution 1.5 μ L of 4-vinylpyridine was added and the mixture was vortexed and kept at 37 °C for 2 h under argon. The alkylated peptide sample was purified on a reversed-phase HPLC immediately after the derivatization.

Digestion of the peptide by Endoproteinase Asp-N

An HPLC purified sample of reduced and pyridylethylated TxVIIA was dissolved in 40 μ L of 0.1 M NH₄HCO₃ and combined with 0.4 μ g of Asp-N in 10 μ L of a 10 mM Tris-HCl buffer, pH 7.5, solution. The peptide was digested for 18.5 h at 37 °C and the reaction was halted by freezing the incubation mixture at -80 °C. A portion of the digest was directly analyzed by LC/ESI MS, and the remainder was purified by reversed-phase HPLC. The pH of the digest was adjusted to approximately 3 just before injecting the sample on to the HPLC system to minimize the possibility of decomposition of Glu residues in an acidic solution (McIntosh et al., 1984).

Mass spectrometry

Microbore LC/ESI MS experiments were conducted on a VG/Fisons (Manchester, UK) Platform mass spectrometer using a C18 column (Macrosphere C18, 300-Å pore, 5- μ m particle, 1.0-mm i.d. $\times$ 250 mm, Alltech, Deerfield, Illinois) with a lin-

ear gradient elution (98% solvent A, 0.1% TFA in water, versus 2% solvent B, 0.08% TFA in acetonitrile, to 38% solvent A versus 62% solvent B in 60 min) generated by a Model 140B dual syringe pump (Applied Biosystems, Foster City, California). A post-column addition of a make-up solvent, 2-propanol/2-methoxyethanol (1:1), was used to optimize spraying and ionizing performance (Medzihradszky et al., 1994).

MALDI mass spectra were obtained with a VG/Fisons ToF-Spec (linear-type) or a ToFSpec SE (reflectron-type) MALDI TOF mass spectrometer using external calibrations. Estimated mass accuracy over the observed mass range was within $\pm 0.1\%$ for linear mode or $\pm 0.02\%$ for reflectron mode. The fragments resulting from metastable decay of MALDI molecular species were focused and recorded by stepping the voltage of an electrostatic ion mirror (reflectron) to obtain so-called PSD spectra. MALDI high-energy CID mass spectra were obtained using a VG Autospec 5000 oa-TOF mass spectrometer as described elsewhere (Medzihradszky et al., 1996).

A predissolved solution of α -cyano-4-hydroxycinnamic acid for MALDI mass spectrometry was purchased from Hewlett-Packard (Wilmington, Delaware).

Edman sequencing

A model 470A protein sequencer (Applied Biosystems) was used for Edman sequencing.

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