The Roles of Individual ϒ-Carboxyglutamate Residues in the Solution Structure and Cation-dependent Properties of Conantokin-T*

(Received for publication, November 19, 1997, and in revised form, January 22, 1998)

Scott E. Warder‡, Mary Prorok‡, Zhihang Chen‡, Leping Li‡, Yi Zhu‡, Lee G. Pedersen¶, Feng Ni§, and Francis J. Castellino‡‡

From the ‡Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556, the ¶Montreal Joint Center for Structural Biology, Biotechnology Research Institute, Biomolecular NMR Laboratory, National Research Council of Canada, Montreal, Quebec H4P 2R2, Canada, the §§National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709, and the ‡Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599

The solution structure of the Ca2+-loaded conantokin-T (con-T), a ϒ-carboxyglutamate (Gla)-containing 21-residue peptide (NH2-Gluγ2γEγ2γYγ2γKMLγ2γ2γNLRγ2γ15EVKKN20-A-CO(NH2)γ), has been elucidated by use of distance geometry calculations with experimental distances derived from two-dimensional 1H NMR spectroscopy. An end-to-end α-helix was the dominant conformation in solution, similar to that of apo-con-T, except that reorientation of several side chains occurred in the Ca2+-coordinated complex. The most notable examples of this were those of Gla10 and Gla14, which were more optimally positioned for complexation with Ca2+. In addition to the stabilization offered to the α-helix by Ca2+ binding, hydrophobic clustering of the side chains of Tyr3, Met8, Leu9, and Leu12, and ionic interactions between Lys7 and Gla9, Gla10 and between Arg13 and Gla14, along with hydrogen bonding between Gln8 and Gla10, were among the side chain interactions likely playing a significant role in maintenance of the α-helical conformation. Docking of Ca2+ in the con-T structure was accomplished using genetic algorithm-molecular dynamics simulation approaches. The results showed that one Ca2+ ion is most likely coordinated by four side chain oxygen atoms, two each from Gla10 and Gla14. Another bound Ca2+ ion has as its donor sites three oxygen atoms, two from Gla9 and one from Gln8. To examine the functional roles of the individual Gla residues, a series of variant peptides have been synthesized with Ala substituted for each Gla residue, and several properties of the resulting variants have been examined. The data obtained demonstrated the importance of Gla10 and Gla14 in stabilizing binding of the highest affinity Ca2+ site, and in governing the conformational change induced by Ca2+. The critical nature of Gla3 and Gla4 in inhibition of the spermine-induced potentiation of the binding of MK-801 to open ion channels of the N-methyl-D-aspartate receptor was established, as well as the role of Gla4 in stabilizing the apo-con-T α-helical conformation.

* This work was supported by Grants HL-19982 (to F. J. C.) and HL-06530 (to L. G. P.) from the National Institutes of Health, a grant from the National Research Council of Canada (Publication No. 41403), Grant MT-12566 from the Medical Research Council of Canada (to F. N.), the Kleiderer-Pezold family endowed professorship (to F. J. C.), and postdoctoral (to M. P.) and predoctoral (to S. E. W.) fellowships from the American Heart Association, Indiana Affiliate. 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.

‡‡ To whom correspondence should be addressed. Tel.: 219-631-6456; Fax: 219-631-8017; E-mail: castellino.1@nd.edu.

con-T3 is a neuroactive peptide found in the venom of Conus tulipa (1). It is among a vast number of small peptides, typically 10–30 amino acids in length, employed by these and other species of cone snails to immobilize their prey and their predators (2, 3). The targets of this array of peptides are neuroreceptors and skeletal muscle receptors, and the remarkable selectivity shown by this general class of peptides has also encouraged their use as laboratory reagents to block specific classes of these receptors (4, 5). Specifically, a general class of Conus peptides, the conotoxins, which contain a relatively high number of disulfide bonds, interact with nicotinic acetylcholine receptors (α-conotoxins), voltage-sensitive Na+ channels (ω-conotoxins), and voltage-sensitive Ca2+ channels (μ-conotoxins) (6). Conantokins, such as the 17-amino acid residue peptide con-G (7) and the homologous 21-residue con-T (1), are Gla-containing peptides without disulfide bonds. They have been shown to function as noncompetitive inhibitors of the enhancement by spermine and spermidine of Ca2+ flow into neurons, by targeted action on glutamate/glycine receptors of the NMDA subclass (8–11).

Certain structural features of these small peptides are relevant to their functions. con-G and con-T contain five and four residues of Gla/mol of peptide, respectively (1, 7). Because of this feature, both peptides interact with divalent cations that are important to the functions of neuronal cells, such as Ca2+ and Mg2+ (3, 12), as well as other metal ions (12, 13). The binding of Ca2+ and similar cations induces a large conformational change in con-G (12) from an essentially random structure to an α-helix. On the other hand, con-T, although it also interacts with these cations, does not undergo as dramatic a conformational change, because apo-con-T is already highly organized in an α-helical conformation (12, 14).

The conantokins possess the potential to serve as agents that inhibit the flow of Ca2+ into neurons via the NMDA receptor route and thus eliminate the harmful effects of entry of this cation into neuronal cells. However, these peptides cannot readily be directly employed as pharmaceutical agents for this purpose in humans because they do not cross the blood-brain barrier. Thus, as part of a rational drug design program, elucidation of the structure-function relationships of these peptides is essential. A recent emphasis on this topic is witnessed by publications on elaboration of the NMR-derived, three-dimensional structures of apo-con-G (13, 15), Ca2+-con-G (13), and apo-con-T (13, 14, 16). Most of these investigations have...
focused on backbone conformations, but one has rigorously
defined side chain orientations of apo con-T (14). In the present
phase of our efforts in this area, we have defined the three-
dimensional conformation of the Ca\textsuperscript{2+}-con-T complex and have employed mutant con-T peptides to attempt to understand the
role of individual Gla residues in defining its metal binding properties and its bioactivity. The results of this study are summarized in this report.

**EXPERIMENTAL PROCEDURES**

**Synthesis of N\textsuperscript{-}N-Fmoc-(γ',γ\textsuperscript{-}di-O-TBu)-L-Gla-OH**—The derivatized Gla was synthesized by previously described methodology (17), except for the resolution of the L- and D-isomers of N\textsuperscript{-}Z-(γ',γ\textsuperscript{-}di-O-TBu)-Gla-

**Peptide Synthesis, Purification, and Characterization**—The peptides were synthesized on a 0.1 mmol scale on a PAL resin support (PerSep-

tive Biosystems, Framingham, MA) using an Applied Biosystems (Fos-
ter City, CA) model 433A peptide synthesizer, as described previously (12). Purification of con-T and mutant con-T peptides was accomplished by fast protein liquid chromatography on a Biosep DEAE-20 column (Bio-Rad) equilibrated with 10 mM NaBO\textsubscript{3}, pH 8.0. A 500-ml linear gradient of NaCl (10 mM NaBO\textsubscript{3}, pH 8.0) (start solution), to 10 mM NaBO\textsubscript{3}, 500 mM NaCl, pH 8.0 (limiting solution), was employed. The target material was pooled, lyophilized, and then desalted on a Sephadex G-15 (Pharmacia Biotech Inc.) column that was equilibrated and eluted with 0.1% NH\textsubscript{4}OH. The peptides were characterized by reverse-phase high performance liquid chromatography and delayed extraction-matrix-assi-
sed laser desorption ionization-time of flight mass spectrometry, as described in an earlier communication (12).

**Calcium Binding**—The Ca\textsuperscript{2+} binding isotherms for each peptide were determined by potentiometry at 25 °C using a semi-micro Ca\textsuperscript{2+}-

**RESULTS**

**NMR of Ca\textsuperscript{2+}-con-T**—In a previous study, it was demonstrated that the CD spectrum of con-T underwent a small change to a more α-helical conformation consequent to the binding of Ca\textsuperscript{2+} to this peptide (12). In the present study, the nature of this conformational alteration was further explored, with the ultimate goal of elucidating the Ca\textsuperscript{2+}-bound structure of con-T. The one-dimensional 1H NMR spectrum of Ca\textsuperscript{2+}-con-T showed well resolved resonances, similar to those observed earlier for apo-con-T (14). This allowed ready assignment of all of the proton resonances using a combination of 2D (DQF-

**Identification of the Positions of the Metal Ions in con-T**—Docking of the metal ions in con-T and further energy minimization based refine-

**Peptide conformational freedoms, free from steric overlaps and consistent with the NMR data, were generated by DQF-COSY NOEs using the fixed bond lengths and angles provided in the ECEPP/3 data bank (24). All side chain-side chain or side chain-main chain medium range (i + 2 and i + 4) constraints were set to an upper bound of 5 Å and a lower bound of 2.0 Å. In the absence of metal coordination sites, we chose not to employ energy minimization as a structural refinement because of the potential bias that this could introduce in the side chain orientations. Because the major backbone NOE contacts in apo-con-T and in

Ca\textsuperscript{2+}-con-T were essentially the same, our approach to modeling the Ca\textsuperscript{2+}-con-T NMR-derived structure was to start with the 10 energy-

the metal ions in con-T and further energy minimization based refine-

ment of the metal-bound structure was accomplished by the genetic algorithm-molecular dynamics simulation approach described previ-

ously (25). The initial coordinates for con-T were those determined by NMR in this study for the Ca\textsuperscript{2+}-con-T complex. The genetic algorithm was employed to determine the initial positions of the Ca\textsuperscript{2+} ions by searching through the O-O midpoints that were within 6 Å of each other, using all oxygen atoms in the midpoint calculation. A total of 150 midpoints was found. The lowest Amber (26) energy structure, verified also by a systematic search, was subjected to a Particle Mesh Ewald (27) molecular dynamics simulation. For this simulation, the peptide was solvated in a 9.0-Å box of TIP3P water. The H\textsubscript{2}O, Na\textsuperscript{+}, and Ca\textsuperscript{2+} were then energy-minimized at constant volume as described (25). Next, a molecular dynamics simulation was performed on Na\textsuperscript{+}, Ca\textsuperscript{2+}, and H\textsubscript{2}O for 100 ps, followed by energy minimization. Another Particle Mesh Ewald molecular dynamics simulation was then performed for 150 ps on side chain residues, the ions, and H\textsubscript{2}O for 150 ps, with fixed backbone, followed by data collection over 300 ps.

A representative structure of the Ca\textsuperscript{2+}-con-T complex is illustrated in Fig. 1. Several of the proton resonance assignments of Ca\textsuperscript{2+}-con-T, obtained by use of this combination of methods, are listed in Table 1.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
con-T, and this is revealing in terms of possible differences in the structures of these peptides. A graphical summary of the chemical shift differences between Ca\(^{2+}\)-con-T and apo-con-T of the αNH and αCH backbone protons for each of the residues of this peptide are illustrated in Fig. 2. Also present in this figure are representative differences in the terminal (farthest sequentially removed from the β-carbons) nonexchangeable side chain protons (e.g. CH\(_2\) of Lys, δCH\(_3\) of Leu, δCH\(_2\) of Arg, and so forth) between these two peptides. The largest chemical shift changes observed in the backbone protons occur in the residue 10–18 region for the amide protons. Smaller differences are present in the αCH protons, except for that of Gla\(^{10}\), which undergoes a particularly large alteration. Of the terminal side chain protons, Gin\(^{9}\) and Arg\(^{23}\) show the most dramatic shifts.

Differences between the observed αCH chemical shifts and their random coil values provide valuable secondary structural information (28). Specifically, upfield shifts of these proton spins of ≈0.1 ppm from their random coil values indicate the presence of helical structure, if these values occur uninterrupted over four or more consecutive residues. The random coil values of each con-T residue were determined experimentally by obtaining the spectrum in 6 M urea. The differences between the αCH proton chemical shifts for Ca\(^{2+}\)-con-T from their same values in 6 M urea are illustrated in Fig. 3. Also plotted in Fig. 3, for comparison, are the values similarly obtained for apo-con-T (14). In the case of Ca\(^{2+}\)-con-T, as with apo-con-T, residues 2–21 possess relative chemical shifts >0.1 ppm, indicating a high content of α-helical structure. This is especially evident in the region of residues 5–17, where chemical shift differences >0.6 ppm are found, indicating the presence of a significant population of α-helices. The αCH proton chemical shift of Gla\(^{10}\), however, is opposite in sign from that expected for a residue in an α-helical conformation. This was also observed in a previous work (13), in which it was attributed to the unknown random coil chemical shift value of the αCH proton of Gla\(^{10}\). However, our data, which show that this chemical shift is not anomalous, allow this interpretation to be abandoned. Because neither CD nor NOESY data demonstrate any serious disruption in the continuum of α-helix of this peptide, we attribute the observation of the positive chemical shift of the αCH proton of Gla\(^{10}\) to the previously unknown consequence of direct Ca\(^{2+}\) coordination to this residue. This is also a very likely explanation for the slightly smaller relative αCH proton chemical shifts for several other Ca\(^{2+}\)-con-T residues as compared with those of apo-con-T (Fig. 3).

Structure of Ca\(^{2+}\)-con-T—The structure of con-T in complex with Ca\(^{2+}\) was primarily derived from DG calculations utilizing NOE connectivities present in this peptide. A summary of the NOE connectivities for intraresidue sequential (i + 1) and medium (i + 2, i + 3, and i + 4) NOEs used in the DG calculations are shown in Fig. 4. The high α-helical content of this figure is readily noted from the strong backbone αNH-αNH NOEs (nn(i + 1)), the stronger (nn(i + 1)) cross-peaks as compared with those of αCH-αNH (anti(i + 1)) connectivities, and the presence of many (an (i + 3)) and (an(i + 4)) NOEs. In addition, three-bond αCH-αNH coupling constants (\(^{3}\)J\(_{nn}\)) were approximately 5 Hz for all residues with well resolved αCH-αNH correlation peaks; these data are also strongly suggestive of the presence of an α-helical structure.

In addition to anti(i + 3) NOE contacts observed for most pairs of residues, backbone-side chain NOE connectivity were found between many proton pairs (Table II), such as those present between Lys\(^{18}\)-αCH and Ala\(^{21}\)-βCH\(_3\), between Gin\(^{-}\)αCH and both Leu\(^{-}\)γCH and Leu\(^{-}\)δCH\(_3\), and between Leu\(^{-}\)αCH and Leu\(^{12}\)-δCH\(_3\), among many others. Furthermore, side chain-side chain NOE connectivities were found between a number of residues, including Gin\(^{-}\)γCH\(_2\) and Leu\(^{-}\)δCH\(_3\), Arg\(^{-}\)-γNH and Val\(^{17}\)-γCH\(_3\), and Glu\(^{-}\)γCH\(_2\) and Asn\(^{20}\)-δCH\(_3\). All of this information strongly suggests a high structural order for con-T in complex with Ca\(^{2+}\), consistent with an α-helical nature.

The Ca\(^{2+}\)-loaded structure of con-T was generated from 160 distance constraints, including 4 intraresidue, 70 sequential, 86 of medium range, and 5 group NOEs (Table II). The convergent structures from the apo-con-T study (14) were used as starting templates for the DG calculations to generate the 10 metal-loaded structures with the lowest distance violations (Fig. 5) with their backbone (αNH-αCH) atoms superimposed. This cluster of structures possess RMSD values to the mean structure of 1.0 Å for backbone atoms and 1.5 Å for all heavy atoms. The average structure is provided in Fig. 6. Analysis of
this structure indicates that additional stabilization of the α-helix can originate from an extensive electrostatic network on one face of the helix, involving Gla3, Lys7, Gla10, Arg13, Gla14, and Lys18, and an appropriately spaced hydrophobic cluster of side chains, comprising Tyr5-Met8-Leu9-Leu12, on the opposite face. This situation also occurs in the apo-con-T structure (14).

The structure of Ca2+-con-T is compared with the apo-con-T (14) in Fig. 7. The structures are very similar in backbone conformations, and changes were observed in only a few of the backbone residues, viz, Gln6, Gla10, Arg13, and Gla14. These changes are noted in NOE data of Table II and from similar data for apo-con-T (14). For example, in apo-con-T, there are no side chain-side chain NOE connectivities between Gla10 and Gla14 (14), whereas in Ca2+-con-T, NOEs are seen between Gla10HG and Gla14HG and between Gla10HG and Gla14HN.

*Locating the Ca2+ Ions in con-T—With the establishment of the Ca2+-loaded con-T structure, it became of interest to attempt to locate Ca2+ ions in their binding site(s). For this purpose, a genetic algorithm-molecular dynamics simulation procedure was employed as described (25), using a

Table I

| Residue | αNH | αCH | βCH | Others |
|---------|-----|-----|-----|--------|
| Gly1    | 3.98 |     |     |        |
| Glu2    | 9.16 | 4.10| 1.98° | γCH2, 2.26° |
| Glu3    | 9.12 | 4.06| 2.11° | γCH, 3.10 |
| Gly4    | 8.08 | 3.98| 2.11/2.31| γCH, 3.22 |
| Tyr5    | 8.09 | 4.30| 3.01/3.12| δCH, 3.67°; εCH, 6.67° |
| Gln6    | 8.29 | 3.73| 2.05° | γCH2, 2.50°; εNH2, 7.98/6.95 |
| Lys7    | 7.87 | 4.03| 1.79° | γCH, 1.36/1.44; δCH2, 1.59°; εCH, 2.83° |
| Met8    | 7.79 | 4.03| 2.05/2.26| γCH2, 2.36/2.60; εCH, 1.89 |
| Leu9    | 8.62 | 3.80| 1.24° | γCH, 1.68; δCH2, 0.60° |
| Glu10   | 7.96 | 4.36| 2.05° | γCH, 3.38 |
| Asn11   | 8.32 | 4.30| 2.70/2.88| δNH2, 7.60/6.87 |
| Leu12   | 7.99 | 4.01| 1.44° | γCH, 1.80; δCH2, 0.76 |
| Arg13   | 8.09 | 3.89| 1.82° | γCH, 1.35/1.37; δCH2, 1.28/3.27; εNH, 8.30; εNH2, 6.91° |
| Gla14   | 8.50 | 3.91| 2.11/2.22| γCH, 3.50 |
| Ala15   | 7.72 | 4.07| 1.43 |        |
| Glu16   | 7.90 | 3.97| 2.05° | γCH2, 2.20° |
| Val17   | 8.05 | 3.65| 2.10 | γCH2, 0.85, 0.95 |
| Lys18   | 7.70 | 4.02| 1.73/1.80| γCH2, 1.33, 1.45; δCH2 ND; εCH2, 2.89° |
| Lys19   | 7.88 | 3.98| 1.79° | γCH2, 1.36/1.44; δCH2, 1.59° |
| Asn20   | 8.07 | 4.52| 2.67/2.76| εNH2, 7.60/7.02 |
| Ala21   | 7.77 | 4.12| 1.35 |        |
| Amide cap|       |     | 7.27/7.15|        |

*= Degenerate resonances.

Fig. 2. Proton chemical shift (αCH, αNH, and side chain) differences between apo-con-T and Ca2+-loaded con-T. The chemical shifts plotted were obtained by subtraction of the apo-con-T spectrum from that of Ca2+-con-T for each of the proton groups illustrated. The particular side chain protons chosen were those that were at terminal and nonexchangeable locations. The buffer was 10 mM NaBO3, 100 mM NaCl, pH 6.5, in the absence of Ca2+ or in the presence of 40 mM Ca2+ at 5 °C. The final con-T concentration was 2 mM.

Fig. 3. Chemical shift index for the αCH-proton resonances of Ca2+-con-T. The chemical shifts were calculated by subtraction of the values obtained in 6 M urea from those displayed in a buffer containing 10 mM NaBO3, 100 mM NaCl, 10% 2H2O, pH 6.5, ± 40 mM Ca2+ at 5 °C. The final con-T concentration was 2 mM.

Black bars, apo-con-T; white bars, Ca2+-con-T.
2Ca\textsuperscript{2+}-con-T binding model with 1 Na\textsuperscript{+} ion as the counterion to balance charge. An average structure was generated from the resulting coordinates and is illustrated in Fig. 8. This structure shows Ca\textsuperscript{2+} ions located at one site, containing the side chains of Gla\textsubscript{10} and Gla\textsubscript{14}, with another at Gla\textsubscript{3} and Gln\textsubscript{6}. Gla\textsubscript{4} is not suitably located to provide such a donor site for Ca\textsuperscript{2+}. In this model, two oxygen atoms from both Gla\textsubscript{10} and Gla\textsubscript{14} coordinate one Ca\textsuperscript{2+} ion, and two oxygen atoms from the \(\gamma\)-carboxylate of Gla\textsubscript{3} and one from the side chain carbonyl atom from Gln\textsubscript{6} are donor groups for binding of the other Ca\textsuperscript{2+}.

During the course of these calculations, energy minimization was conducted to derive the final structure with Ca\textsuperscript{2+} ions in their appropriate locations. In comparing the two models, viz, the NMR-derived structure (Fig. 7) and that further refined through Ca\textsuperscript{2+} docking and energy minimizations (Fig. 8), it is clear that no major differences in backbone or side chain conformations exit. The most notable changes occur in the ring orientation of Tyr\textsubscript{5} and the side chain positioning of Lys\textsubscript{7}, along with other minor differences. None of these, however, alter the face of the helix on which these residues reside, and none change the conclusions regarding the \(\alpha\)-helix stabilizing forces.

On the whole, the differences observed in the peptide structures revealed by the two models are surprisingly small. These are not further elaborated upon herein because the uncertain-
ties in deriving the models themselves from these disparate approaches are likely as large as the small differences in positions of flexible side chains between them. Instead, it is stressed that the two models are very similar and provide a very good set of models with which to explore structure-function relationships of this peptide.

**con-T Variants**—To test the importance of side chains found to be critical in stabilization of Ca$^{2+}$ binding to con-T, several variant peptides were constructed, and their properties were assessed. This group consisted of changes of all Gla residues and one other residue, Gln6, that was identified as a possible contributor to stabilization of Ca$^{2+}$ in the molecular dynamics simulation. In all cases, Ala was the amino acid that was substituted, because Ala would favor a-helix stability (29) but would not provide side chain atoms that could coordinate Ca$^{2+}$.

The effects of these substitutions on the stability of the apo-con-T helix and that of the Ca$^{2+}$-loaded peptide, as well as the quantitative ability of Ca$^{2+}$ (C$_{50}$ values) to induce the a-helical conformation in the variant peptides, are summarized in Table III. Representative CD titrations with Ca$^{2+}$ of con-T, con-T[Gla3-Ala] and con-T[Gla14-Ala] are provided in Fig. 9. The only variation in this series that resulted in significant destabilization of the apo-con-T a-helix was that of Gla4-Ala, where approximately one-half of the a-helical content was observed as compared with the wild-type peptide (Table III). However, the C$_{50}$ values characterizing the Ca$^{2+}$-induced conformational transition in this peptide were similar for all peptides, except for those of con-T[Gla10-Ala], con-T[Gla14-Ala], and con-T[Gla10-Ala/Gla14-Ala], where substantially higher concentrations of Ca$^{2+}$ were required to induce their Ca$^{2+}$-dependent conformations. Furthermore, in these latter cases, the conformational change appeared to be very small and of marginal significance, and it was opposite in character to that

| Group NOEs | Group NOEs |
|------------|------------|
| X1 X2 5.0, 2.0 | X7 X8 5.0, 2.0 |
| X3 X4 5.0, 2.0 | X9 X10 5.0, 2.0 |
| X5 X6 5.0, 2.0 |          |

**Group definitions**

| Group definitions | Group definitions |
|-------------------|-------------------|
| X1 2HG 8HB       | X6 3HG 5HB       |
| X2 5HD 6HB       | X7 11HD          |
| X3 5HN           | X8 5HA           |
| X4 3HB 4HB       | X9 15HM          |
| X5 4HN           | X10 14HG         |

* Backbone-backbone, backbone-side chain, and side chain-side chain.
induced in the other mutant peptides, the final extents of which were similar to those of con-T and con-T[Gla3-Ala] (Fig. 9).

The observation that the α-helicity of con-T[Gla10-Ala], con-T[Gla14-Ala], and con-T[Gla10-Ala/Gla14-Ala] decreases as the peptide is titrated with Ca²⁺ can be due to two possible factors: 1) electrostatic screening of favorable side chain-side chain or backbone-side chain interactions, and/or 2) the coordination of metal to individual Gla residues that leads to a specific disruption of critical charge-charge interactions. These possibilities were examined by investigating the ionic strength dependence on α-helicity for the peptides con-T[Gla10-Ala], con-T[Gla14-Ala], and con-T[Gla10-Ala/Gla14-Ala] using NaCl. The results revealed that nonspecific ionic strength changes alone cannot account for the decrease in α-helicity for these peptides. Therefore, the Ca²⁺-induced decreases are primarily due to specific coordination with the remaining Gla residues.

The binding isotherms of Ca²⁺ to each of the con-T mutant peptides were established by Ca²⁺-specific electrode titrations. Examples of the titration data obtained are illustrated in Fig. 10. In most cases, the binding data were fit to a model with two independent sites, one strong site with a $K_d$ within the range of 0.2–0.5 mM and one weaker site of $K_d$ approximately 10-fold higher. Exceptions have been observed in the cases of con-T[Gla10-Ala], con-T[Gla14-Ala], and con-T[Gla10-Ala/Gla14-Ala], wherein the data could be fit several different models of very weak Ca²⁺ binding. For these peptides, the binding characteristics were determined through Michaelis-Menten fits, which showed that the amount of free Ca²⁺ required to reduce the binding to 50% was approximately 1–4 mM. These results indicate that only the strong Ca²⁺ sites have been eliminated by mutations at Gla10 and Gla14 and/or that these two mutations led to peptides that required different Ca²⁺ binding models to satisfy the experimental data.

Lastly, the effects of the mutations in con-T on the ability
of the resulting peptides to inhibit MK-801 binding to open rat brain NMDA receptor channels in the presence of exogenous spermine has been examined. The neuronal membranes were washed and thus contained only low levels of glutamate and glycine, but these ligands were present at sufficient concentrations to lead to significantly increased ion channel opening upon addition of spermine. The amount of $[^3H]$MK-801 binding to the membranes, as a function of the concentration of con-T-derived peptides, is shown for con-T and for con-T[Gla10-Ala/Gla14-Ala] in Fig. 11. Values of the IC$_{50}$ for peptide inhibition have been calculated from the concentration midpoints of the differences between the basal level of MK-801 binding to the peptide and that induced by spermine at a peptide concentration of zero. The values obtained are summarized in Table III. Very large increases in the IC$_{50}$ value were seen for con-T[Gla3-Ala] and con-T[Gla4-Ala], and a more modest increase was observed for con-T[Gla10-Ala] and con-T[Gla10-Ala/Gla14-Ala]. Thus, major binding determinants for the peptide to its site on the membrane are provided by Gla3 and Gla4 of con-T.

**DISCUSSION**

A variety of divalent cations, including Ca$^{2+}$ and Mg$^{2+}$, alter the CD properties of con-T (12) in a manner suggesting that the relative $\alpha$-helical content of the peptide is increased. The Gla residues of this peptide, which serve as Ca$^{2+}$ binding loci when present in blood coagulation proteins and in proteins and peptides present in bone, are the strongest candidate residues for metal ion coordination in con-T. Titrations by ion-specific electrode methods reveal that con-T has one strong Ca$^{2+}$ binding site, and possibly a second weaker site of interaction (12). A previous study has detailed the backbone.
and side chain conformations of con-T and concluded that a significant population of molecules were end-to-end a-helices (14). Despite this, changes occurred in both the NMR and in the CD spectra of con-T as a result of addition of Ca$^{2+}$ that indicated that a higher content of a-helix resulted from this interaction (Figs. 2–4 and Table II). As shown in Fig. 2, these changes did not appear to be in backbone conformations but rather in side chain orientations, and such types of changes should not have such dramatic influence on the a-helical content. It is most likely that the population of molecules in the a-helical conformation increases in the presence of Ca$^{2+}$, and the side chain reorientations reflect the optimization of Ca$^{2+}$ binding sites.

The NMR-derived solution conformation of Ca$^{2+}$-loaded con-T has been determined by methods similar to those published for the apo-con-T structure (14). However, in this case, only DG calculations of backbone-backbone NOEs and of backbone-side chain and side chain-side chain NOE constraints were employed to calculate the structure at this stage. Because the exact position of the bound Ca$^{2+}$ ion(s) was unknown, full energy minimization was not performed in these structure calculations. This is because the exclusion of the bound ions from the energy minimizations would have resulted in nonoptimal definition of metal-induced conformation. However, the major differences in side chain conformation that result from Ca$^{2+}$ binding to con-T are centered at Gln6, Gla10, Arg13, and Gla14. Such changes are consistent with the binding of Ca$^{2+}$ to Gla10 and Gla14. In apo-con-T, the guanidino group of Arg13 appears to structurally mimic the role of Ca$^{2+}$ in the metal-loaded form, reducing electrostatic repulsion between the Gla10 and Gla14 side chains. This model is predicted from our previous structural work (14) and is consistent with the observation that con-T[Arg13-Ala] reduces the apo-a-helicity by >50% (data not shown). Therefore, binding of a metal ion to the tight site concomitant with displacement of the Arg13 side chain is consistent with a significant chemical shift in this residue.

Gla3 and that of Gln 6 are in spatial proximity in the metal-peptide complex, either as the result of hydrogen bonding or because they are directly coordinated with the metal. The chemical shift changes of the Gla6-$\epsilon$NH$_2$ side chain protons in the Ca$^{2+}$-con-T complex can be perhaps explained by the amide

![FIG. 9. Effects of Ca$^{2+}$ on the a-helical contents of apo-con-T, con-T[Gla3Ala], and con-T[Gla4Ala]. The molar ellipticities at 222 nm are plotted against the free Ca$^{2+}$ concentrations. The buffer was 10 mM NaBO$_3$, 100 mM NaCl, pH 6.5, at 25 °C.](image)

![FIG. 10. Binding isotherms of Ca$^{2+}$ to con-T mutants. Modified Scatchard plots are used to display the data. ■, wild-type con-T; ○, con-T[Gla3-Ala]. The experimental line is fit to wild-type con-T using an independent two-site model with $K_d$ values 0.25 mM and 2.5 mM.](image)

![FIG. 11. Effects of con-T and con-T[Gla14Ala] on the binding of $[^3H]$MK-801 to washed rat neuronal cells. The concentration of spermine used was held constant at 50 μM under conditions of endogenous levels of glutamate and glycine in washed membranes. $[^3H]$MK-801 binding was then measured as a function of the concentrations of con-T (●) or con-T[Gla10-Ala/Gla14-Ala] (○). The basal binding level of $[^3H]$MK-801 under these conditions in the absence of spermine is approximately 500 cpm.](image)
The Ca$^{2+}$-Conantokin-T Complex

upon addition of Ca$^{2+}$ disrupts to the con-T have been examined through the effects of these peptides. The Ca$^{2+}$ binding isotherm displays one clear, tight binding site of approximately 0.25 mM (12), and additional evidence suggests that at least one additional, weaker site is present, with a $K_d$ > 10-fold higher than the strong site (Table III). Alteration of Gla3 or Gla4 to Ala residues preserves the binding at the tight Ca$^{2+}$ site, but diminishes Ca$^{2+}$ binding at the weaker site (Table III). This result is in concert with the model of the Ca$^{2+}$-docked con-T (Fig. 8), which shows that these two residues are capable of coordinating Ca$^{2+}$. Alteration of Gla3 to Ala did not affect Ca$^{2+}$ binding, again in agreement with this model, which predicts that Gla4 would not coordinate Ca$^{2+}$. Both this model and that derived from NMR measurements (Fig. 7) place Gla4 on the opposite side of the face of the $\alpha$-helix that appears to house the Ca$^{2+}$ binding loci. On the other hand, alteration of Gla4 and/or Gla14 to an Ala residue resulted in Ca$^{2+}$ binding that was so weak that a unique binding model could not be fit. Therefore, for these peptides, an average binding constant was estimated from a Michaelis-Menten fit of the binding data (Table III). The value obtained was at least 15-fold larger than that displayed for the strong Ca$^{2+}$ binding site of native con-T. Thus, we propose that Gla10 and Gla14 serve as coordination sites for the tightly bound cation.

The only mutant that affected the stability of the apo-con-T $\alpha$-helix was con-T[Gla3-Ala], which reduced the population of $\alpha$-helical molecules to one-half of their values in wild-type con-T and the other mutants tested (Table III). Thus, despite the change of Gla4 to a residue that in itself should not be disruptive to the $\alpha$-helical character of this peptide, destabilization of the $\alpha$-helix nonetheless occurred. This conclusion is in agreement with our analysis of the apo-con-T structure, in which Gla4 was predicted to be an important capping residue at the amino terminus and to interact favorably with the $\alpha$-helix.

Toxin Rev.

REFERENCES

1. Haack, J. A., Rivier, J., Parks, T. N., Mena, E. E., Cruz, L. J., and Oliveira, B. M. (1996) J. Biol. Chem. 265, 6025–6029
2. Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. F., Woodward, S. R., Hillyard, D. R., and Cruz, L. J. (1996) Science 274, 253–263
3. Myers, R. A., McIntosh, M., Macechini, M.-L., and Skolnick, P. (1996) J. Biol. Chem. 271, 17173–17178
4. Skolnick, P., Boje, K., Miller, Pennington, M., Macechini, M.-L., Skolnick, P., and Macechini, M.-L. (1992) J. Neurochem. 59, 1516–1522
5. Skolnick, P., Zhou, L.-M., Chandra, T., Pennington, N., Skolnick, P., and Macechini, M.-L. (1994) in Pharmacology and Toxicology, Basic and Clinical Aspects: Direct and Allosteric Control of Glutamate Receptors (Palmer, M. G. Reynolds, I. J., and Skolnick, P., eds) pp. 155–165, CRC Press, Boca Raton, FL
6. Zhou, L.-M., Szendrei, G. I., Fossom, L. H., Macechini, M.-L., Skolnick, P., and Oros, L. (1996) J. Neurochem. 66, 620–628
7. Prorok, M., Warder, S. E., Blandt, T., and Castellino, F. J. (1996) Biochemistry 35, 16528–16534
8. Skjærbaek, N., Nielsen, K. J., Lewis, R. J., Alwood, P., and Craik, D. J. (1997) J. Biol. Chem. 272, 22967–22970
9. Warder, S. E., Chen, Z., Zhu, Y., Prorok, M., Castellino, F. J., and Ni, F. (1997) FEBS Lett. 411, 19–26
10. Skolnick, P., Zhou, L.-M., Chandra, T., Pennington, N., Skolnick, P., and Macechini, M.-L. (1994) in Pharmacology and Toxicology, Basic and Clinical Aspects: Direct and Allosteric Control of Glutamate Receptors (Palmer, M. G. Reynolds, I. J., and Skolnick, P., eds) pp. 155–165, CRC Press, Boca Raton, FL
11. Zhou, L.-M., Szendrei, G. I., Fossom, L. H., Macechini, M.-L., Skolnick, P., and Oros, L. (1996) J. Neurochem. 66, 620–628
12. Prorok, M., Warder, S. E., Blandt, T., and Castellino, F. J. (1996) Biochemistry 35, 16528–16534
13. Skjærbaek, N., Nielsen, K. J., Lewis, R. J., Alwood, P., and Craik, D. J. (1997) J. Biol. Chem. 272, 22967–22970
14. Warder, S. E., Chen, Z., Zhu, Y., Prorok, M., Castellino, F. J., and Ni, F. (1997) FEBS Lett. 411, 19–26
15. Badeja, J. D., Furse, B. C., and Furse, B. (1997) Biochemistry 36, 6906–6914
16. Lin, C. H., Chen, C. S., Hsu, K. S., King, D. S., and Lyu, P. C. (1997) FEBS Lett. 407, 242–248
17. Colpitts, T. L., and Castellino, F. J. (1993) Int. J. Pept. Protein Res. 41, 567–575
18. Clapes, P., Valverde, I., and Torres, J. L. (1996) Tetrahedron Lett. 1993, 417–418
19. Colpitts, T. L., and Castellino, F. J. (1994) Biochemistry 33, 3501–3508
20. Chen, Y.-H., Yang, J. T., and Martinez, H. M. (1972) Science 6906–6914
21. De Lisi, C., and Castellino, F. J. (1993) Int. J. Pept. Protein Res. 41, 567–575
22. Levy, C., and Brothers, T. (1971) J. Biol. Chem. 249, 257–263
23. Levy, G. C., Jeong, G. N., and Zhu, Y. (1992) in NMR of Proteins and Nucleic Acids, John Wiley & Sons, Inc., New York
24. Zhu, Y. (1992) Technical Report, Department of Electrical Engineering, Syracuse University, Syracuse, NY
25. Levy, C., and Brothers, T. (1971) J. Biol. Chem. 249, 257–263
26. Levy, G. C., Jeong, G. N., and Zhu, Y. (1992) in Proceedings of the 33rd Experimental NMR Conference, p. 152
27. Némethy, G., Gibson, K. D., Palmer, K. A., Yoon, C. N., Paterlini, G., Zagari, A. P., and Castellino, F. J. (1993) Biochemistry 32, 12566–12570
28. Colpitts, T. L., and Castellino, F. J. (1994) Toxin Rev. 9, 197–202
29. Skolnick, P., Zhou, L.-M., Chandra, T., Pennington, N., Skolnick, P., and Macechini, M.-L. (1994) in Pharmacology and Toxicology, Basic and Clinical Aspects: Direct and Allosteric Control of Glutamate Receptors (Palmer, M. G. Reynolds, I. J., and Skolnick, P., eds) pp. 155–165, CRC Press, Boca Raton, FL
30. Zhou, L.-M., Szendrei, G. I., Fossom, L. H., Macechini, M.-L., Skolnick, P., and Oros, L. (1996) J. Neurochem. 66, 620–628
31. Prorok, M., Warder, S. E., Blandt, T., and Castellino, F. J. (1996) Biochemistry 35, 16528–16534
32. Skjærbaek, N., Nielsen, K. J., Lewis, R. J., Alwood, P., and Craik, D. J. (1997) J. Biol. Chem. 272, 22967–22970
33. Warder, S. E., Chen, Z., Zhu, Y., Prorok, M., Castellino, F. J., and Ni, F. (1997) FEBS Lett. 411, 19–26
34. Badeja, J. D., Furse, B. C., and Furse, B. (1997) Biochemistry 36, 6906–6914
35. Lin, C. H., Chen, C. S., Hsu, K. S., King, D. S., and Lyu, P. C. (1997) FEBS Lett. 407, 242–248
36. Colpitts, T. L., and Castellino, F. J. (1993) Int. J. Pept. Protein Res. 41, 567–575
37. Clapes, P., Valverde, I., and Torres, J. L. (1996) Tetrahedron Lett. 1993, 417–418
38. Colpitts, T. L., and Castellino, F. J. (1994) Biochemistry 33, 3501–3508
39. Chen, Y.-H., Yang, J. T., and Martinez, H. M. (1972) Biochemistry 11, 4320–4329
40. Wüthrich, R. (1986) NMR of Proteins and Nucleic Acids, John Wiley & Sons, Inc., New York
A., Rumsey, S., and Scheraga, H. A. (1992) *J. Phys. Chem.* **96**, 6472–6484

25. Li, L., Darden, T. A., Freedman, S. J., Furie, B. C., Furie, B., Baleja, J. D., Smith, H., Hiskey, R. G., and Pedersen, L. G. (1997) *Biochemistry* **36**, 2132–2138

26. Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. S., Cheatham, T. E. I., Ferguson, D. M., Seibel, G. L., Singh, U. C., Weiner, P. K., and Kollman, P. A. (1995) *Amber*, Version 4.1, University of California, San Francisco

27. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) *J. Chem. Phys.* **103**, 8577–8593

28. Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) *Biochemistry* **31**, 1647–1651

29. Argos, P., Hanei, M., and Garavito, R. M. (1978) *FEBS. Lett.* **93**, 19–24
The Roles of Individual \(\gamma\)-Carboxyglutamate Residues in the Solution Structure and Cation-dependent Properties of Conantokin-T

Scott E. Warder, Mary Prorok, Zhigang Chen, Leping Li, Yi Zhu, Lee G. Pedersen, Feng Ni and Francis J. Castellino

*J. Biol. Chem.* 1998, 273:7512-7522.
doi: 10.1074/jbc.273.13.7512

Access the most updated version of this article at [http://www.jbc.org/content/273/13/7512](http://www.jbc.org/content/273/13/7512)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 24 references, 6 of which can be accessed free at [http://www.jbc.org/content/273/13/7512.full.html#ref-list-1](http://www.jbc.org/content/273/13/7512.full.html#ref-list-1)