Role of modified glutamic acid in the helical structure of conantokin-T

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Abstract Circular dichroism (CD) and 2-dimensional NMR were used to study the solution conformation of conantokin-T (Con-T), a small peptide toxin found in the venom of fish-hunting cone snails, and its Glu-substituted analog. Con-T lacks disulfide bonds but contains many γ-carboxyglutamic acids (Gla), a post-translationally modified residue. Our results show that Con-T adopts an α-helical conformation in aqueous solution even in the absence of calcium. Glu replacements diminish both helicity and function of Con-T. The helical content of Con-T is higher than most natural helical peptides of this length in aqueous solution. The sequence of this small toxin incorporates several known elements that stabilize α-helical structure in peptides. Gla residues form several salt bridges that stabilize helical conformation of Con-T.

Key words: Conantokin-T; NMDA receptor; γ-Carboxyglutamic acid; Helix; Circular dichroism; NMR

1. Introduction

The venom of the cone snails (*Conus*) contains an extraordinary variety of small pharmacologically active peptides [1]. The targets of these toxins include voltage-sensitive calcium channels, sodium channels, acetylcholine receptors, vasopressin receptors, and *N*-methyl-D-aspartate (NMDA) receptors in the neuromuscular system [2]. Conantokins are peptide toxins found in the venom of fish-hunting *Conus*. Unlike other toxins from *Conus* venoms, conantokins lack cysteine residues [3]; they incorporate, instead, numerous γ-carboxyglutamic acid (Gla), a post-translationally modified residue. Conantokin-G (Con-G) is a 17-amino-acid peptide, containing five Gla residues, found in the venom of *C. geographus*. Conantokin-T (Con-T), which contains four Gla residues, is a 21-residue peptide isolated from *C. tulipa* venoms, sharing about 40% sequence homology with Con-G. Both Con-G and Con-T induce a sleeping effect in young mice but hyperactive behaviour in old mice when administered by intracranial injection [2]. It was suggested that conantokins form helical structure upon chelation of calcium ion by the Gla residues [4]. Calcium binding has been the primary function attributed by γ-carboxyglutamic acid residues in proteins. One possible role is Ca^{2+}-mediated intrachain or interchain interactions with other Gla residues. Gla residues can also mediate binding to membrane surface through Ca^{2+} ion bridges with the anionic phosphate head groups [5]. Although several studies on Gla residues, mostly concentrating on the blood-clotting proteins, have emerged recently [6,7], little is known about the role of Gla residues in conantokins. In order to understand the importance of Gla residues in these molecules, we replaced all Gla residues in Con-T by Glu (for sequence see Fig. 1), and investigated the effect on the structure and function. We report here the circular dichroism (CD) and NMR studies of the Con-T and its Glu analog. We also examined the effect of Con-T and its Glu analog on the NMDA receptor in rat hippocampal CA1 neurons by electrophysiological techniques. The substitution of all Gla residues by Glu not only reduces the helicity, but also diminishes the NMDA antagonism of Con-T.

2. Materials and methods

2.1. Sample preparation

Con-T and Con-ET were synthesized using an Applied Biosystems 430A peptide synthesizer, using standard F-moc Chemistry. Crude peptides were purified by reverse-phase HPLC on a Vydac C_{18} semi-preparative column (300 Å, 10 mm × 25 cm) with a gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid. The molecular weight of purified peptides was confirmed by FAB-Mass spectroscopy.

2.2. Circular dichroism measurements

CD spectra were run on an Aviv model 62A DS spectropolarimeter. The wavelength was checked by a two-point calibration with D-10-camphorsulfonic acid [8]. The concentration of stock solutions was determined by using the absorption of Tyr at 275.5 nm in 6 M guanidine hydrochloride for Con-T and Con-ET [9]. For Ca^{2+}-free CD measurements, peptides were treated with chelex-100 (Sigma) for 1 h and passed through a 0.45 mm filter (Lida, WI) to remove Ca^{2+} ions. The fraction of helix was obtained from the relationship $f = [\theta]_{obs}/[\theta]_{max}$, $[\theta]_{obs}$ is the mean residue ellipticity observed at 222 nm, and $[\theta]_{max} = (n-4/n)[\theta]\infty$ = the maximal mean residue ellipticity value for chain length n, where $[\theta]\infty = -40\,000$ deg·cm^2/dmol [10].

2.3. Sedimentation equilibrium

Sedimentation equilibrium analysis was performed using a Beckman XL-A analytical ultracentrifuge operating between 40 000 and 55 000 rpm. The partial specific volume (vbar) was calculated as suggested by Laue et al. [11], the value was 0.71 ml/g for Con-T peptide. The solvent density was taken as 1.006 g/ml. Data were fit to a single exponential using NONLIN [12]. Peptide concentrations of 150, 75, and 30 μM were analyzed at 22°C. Buffer consisted of 10 mM potassium phosphate, 100 mM NaCl, pH 7.0.

2.4. ^1H NMR spectroscopy

NMR spectra were recorded on a Bruker DMX-600 spectrometer equipped with an X32 computer at 25°C. Time-proportional phase incrementation (TPPI) [13] was used to acquire phase-sensitive NOESY [14], DQF-COSY [15], and TOCSY [16] spectra. Typical 2D data sets contained 512 FIDs with 2K data points each, requiring 32–64 scans/FID, a spectral width of 6204 Hz, and a recycle delay of at least 1.5 s. The acquired data were processed using UXNMR (Bruker). The peptide samples were prepared at a concentration of approximately 5 mM in 90% H_2O/10% D_2O.

2.5. Slice preparation and intracellular recordings

Hippocampal slices (500 mm thick) were isolated from Sprague-Dawley rats (120–150 g) and used for intracellular recording as reported [17]. A submerged recording chamber was used and the superfusing artificial cerebral spinal fluid (ACSF) solution was maintained

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at 32 ± 1°C. The composition of the ACSF solution was (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11 (pH 7.4), bubbled with a mixture of 95% O_2 and 5% CO_2. The chamber consisted of a circular well of a low volume (1–2 ml) and was constantly perfused at a rate of 2–3 ml/min. Intracellular recordings were made from CA1 pyramidal neurons using glass microelectrode filled with 3 M potassium acetate (80–100 MW) as described previously [18]. Electrical signals were recorded using an Axoclamp-2B amplifier (Axon Co.), and a IBM-486-based microcomputer with pCLAMP software (version 6.0.2, Axon Co.) was used to acquire and analyze the data.

3. Results

3.1. Con-T adopts helical conformation in the absence of calcium ions

The peptide absorption bands in the far UV region become optically active in the asymmetric environment of α-helix, producing a characteristic double minima at 222 and 208 nm and a maximum at 195 nm [19]. Fig. 1 shows the CD spectra of conantokins and their analogs at 25°C in aqueous solution. Con-T exhibits a typical helix spectrum with about 75% helicity. Although the helix content is not as high as its wild type, Con-ET still shows considerable helical structure in aqueous solution. It should be noted that all measurements were carried out in the absence of calcium ion. Chelex 100 was used to remove trace metal ions. However, addition of calcium to Con-T and Con-ET only affects their CD spectra slightly. Recent studies by Castellino and co-workers [20] found that addition of Ca^{2+} increases the helical content of Con-G substantially whereas the existing helical structure of Con-T increases only by a small amount. This is different from the observation by Chandler and co-workers [21], where they found that Ca^{2+} titration did not affect the solution conformation of Con-G at all. We think this apparent discrepancy may be due to the pH employed. The ionization state of γ-carboxyl groups of Gla greatly affects its calcium binding ability.

There is a known tendency of α-helices to associate intermolecularly, especially for those peptides with amphipathic sequences. Although the sequences of conantokins do not reveal a strong tendency to form a hydrophobic face in helical wheel projections, they show a clustering of Gla residues on one side. Conantokins may form stable α-helices as a consequence of intermolecular association of this highly charged Gla face, mediated by calcium ion [4]. Thus, it is essential to verify the molecularity of these peptides. The dependence of ellipticity on peptide concentration (up to 400 μM) in the

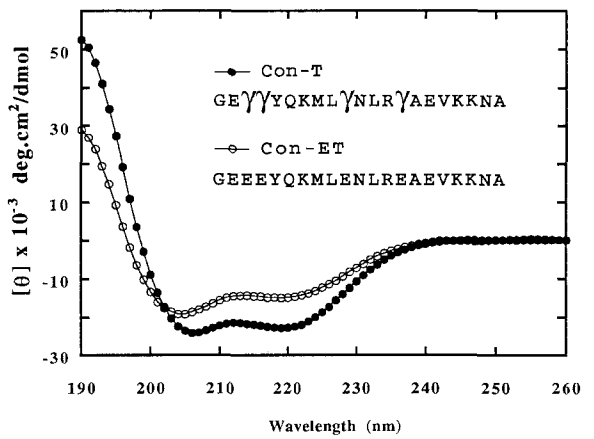

Fig. 1. The CD spectra of Con-T and Con-ET. The measurements reported here were carried out in 10 mM KF, pH 5, at 25°C, using a 1 mm path length cuvette. Each spectrum is an average of three scans measured with a 0.5 nm step size.

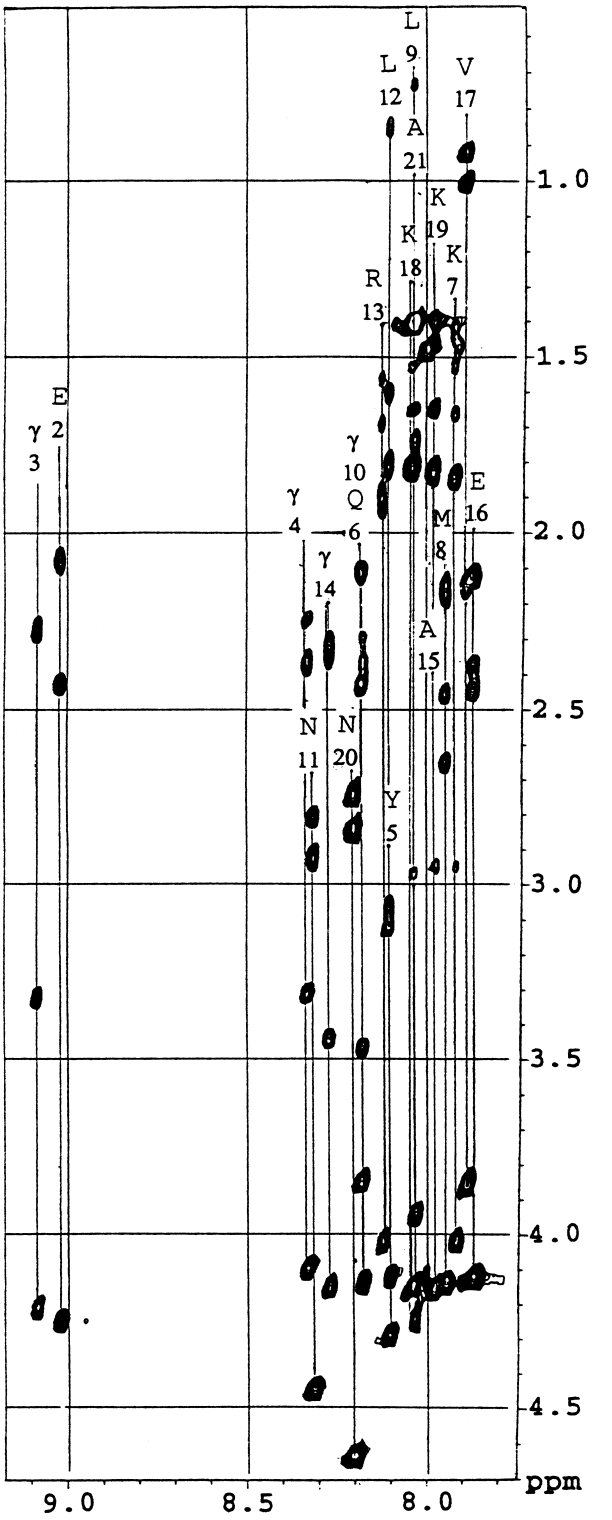

Fig. 2. Fingerprint region of the TOCSY spectrum of Con-T at 25°C. The mixing time is 90 ms. Each intraresidue spin system is connected with solid lines and labeled by standard one letter symbols for the amino acids (except γ for Gla) and the residue number in the sequence.

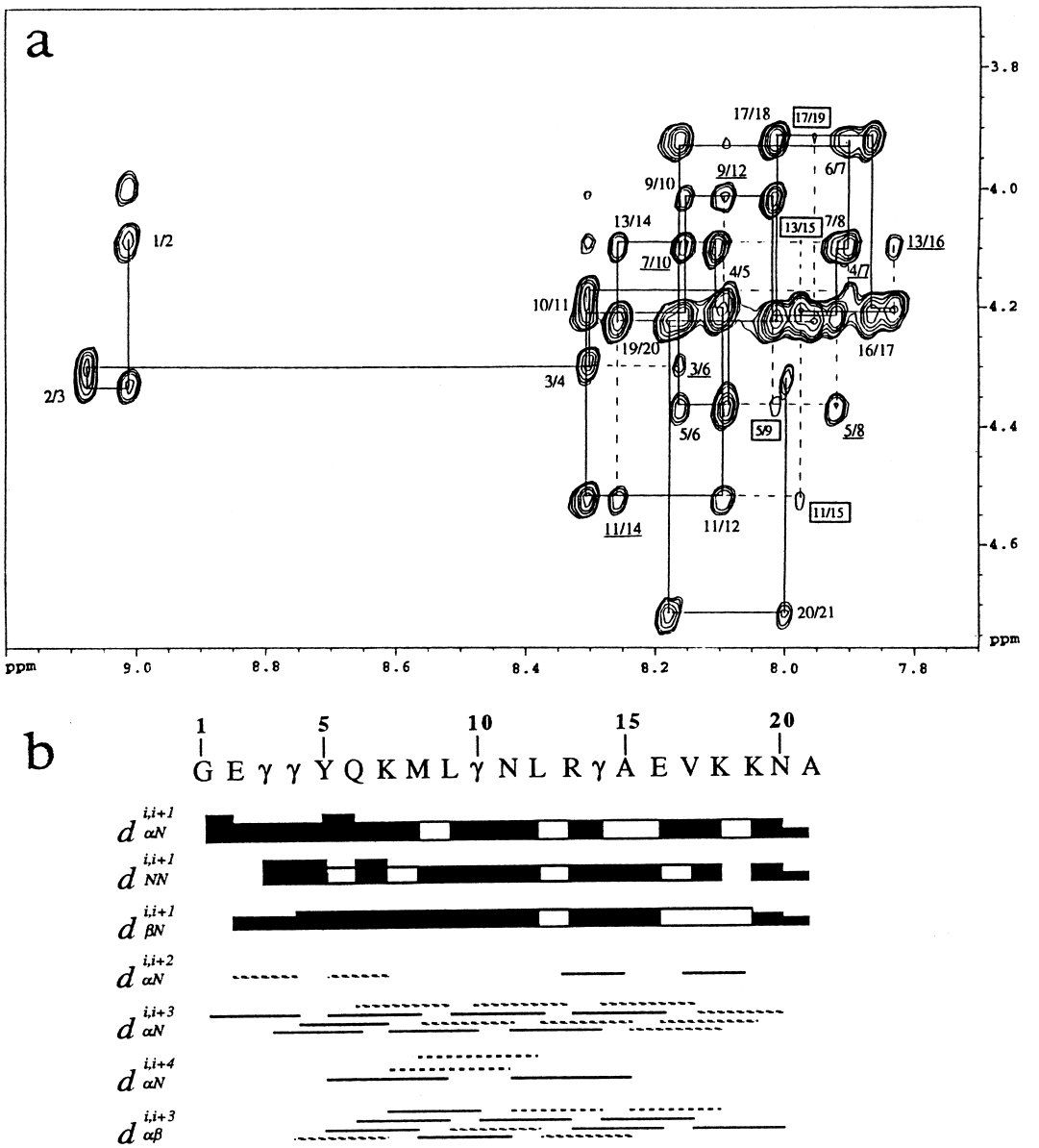

Fig. 3. a: CαH-amide region of the NOESY spectrum. The mixing time is 250 ms. A series of cross peaks between Cα proton and peptide NH are connected by solid line and indicated by the sequence numbers of the pair of adjacent residues. Dashed line connects those medium range NOEs; $d_{\alpha N}(i, i+3)$ (underlined), $d_{\alpha N}(i, i+2)$ and $d_{\alpha N}(i, i+4)$ (boxed). b: Schematic summary of NOE connectivities for Con-T. Filled squares and solid lines indicate unambiguous NOEs. Empty squares and dashed lines indicate possible NOEs where chemical shift degeneracy interferes with identification.

absence and presence of calcium ions has been monitored by CD at 222 nm (data not shown). The resulting straight line reveals that no aggregation occurred in the solution over the range examined [22]. We have also measured its apparent relative molecular mass (M_r) in sedimentation equilibrium experiments, using light absorption optical systems. A plot of the ln (absorbance) versus r^2 shows linear dependence with no apparent systematic residuals (data not shown), indicating that the peptide forms a unique species in solution with the molecular weight in the monomeric range. Con-T also exhibits a strong dependence on temperature; its helix content drops from about 95% at 4°C to 28% at 75°C. Thermal melting of this peptide does not yield a sigmoidal curve, which is expected for most dimers or multimers that exhibit cooperative behavior during thermal unfolding. This also supports that

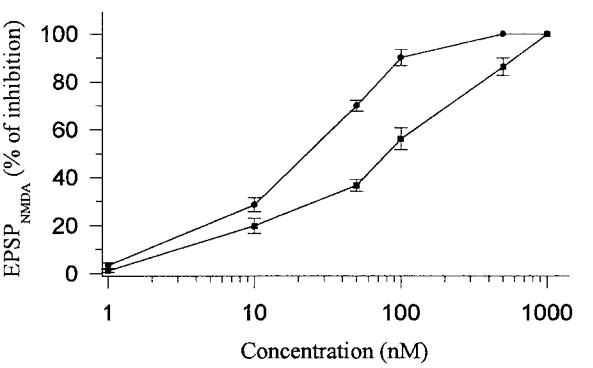

Fig. 4. Effects of Con-T and Con-ET on the pharmacological isolation of $EPSP_{NMDA}$ on the hippocampal CA1 neurons.

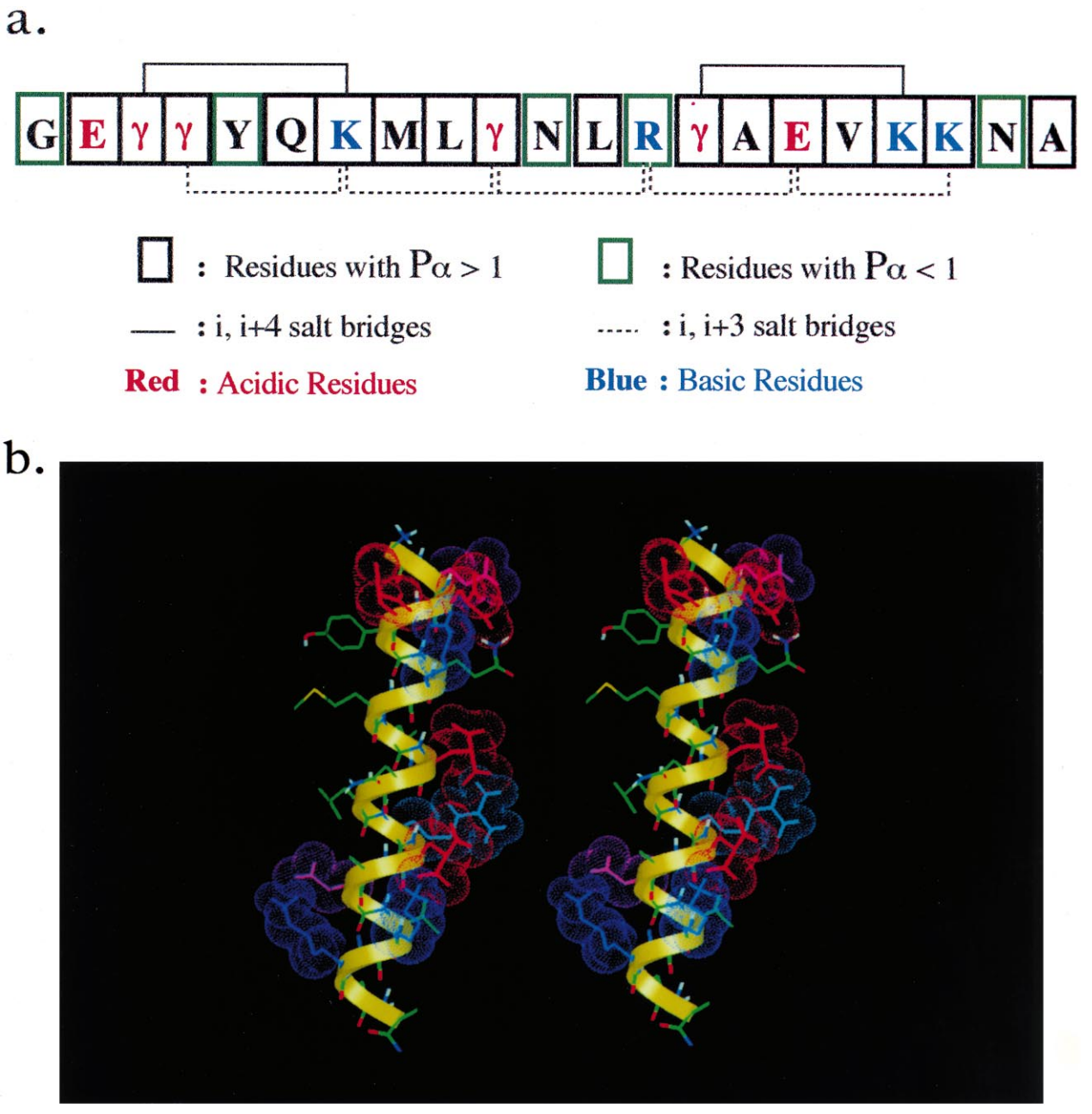

Fig. 5. a: Schematic illustration of the helix stabilization factors in conantokin-T. b: Stereo diagram showing the relative position of those charged side chains in a helical model of conantokin-T. The charged side chains that have potential to form salt-bridges or interacting with helix dipole are indicated by dotted van der Waals surfaces in different color; red, Gla; pink, Glu; dark blue, Lys; light blue, Arg. The model is generated on a IRIS workstation using the Insight II program (Biosym Technologies, Inc.).

helix formation in this peptide is not due to intermolecular association.

3.2. NMR studies on Con-T

To further characterize the helical conformation of Con-T, 2D NMR analysis has been carried out for this peptide. Assignments of the proton spectrum of Con-T were done by following the standard sequential procedure [23]. The fingerprint region of the TOCSY spectrum was used to identify the intraresidue spin systems (Fig. 2). The side chains of Gla residues are easily recognized in this region, since the Cγ proton of Gla shifts further down field (3.33–3.47 ppm) compared to the Cγ proton of Glu, due to the presence of an extra carboxyl group. The fingerprint region of the NOESY spectrum is shown in Fig. 3a. Continuous cross peaks between adjacent CαH and NH can be identified from the beginning of the chain to the end, which are characteristic of the α-helix [23]. The more stringent signatures of α-helix, such as medium-range $d_{\alpha N}(i, i+3)$ and $d_{\alpha N}(i, i+4)$ cross peaks, can also be identified in this region of NOESY spectrum. The presence of some weak $d_{\alpha N}(i, i+2)$ cross peaks may indicate an equilibrium between 3_{10} and α-helical forms. The other characteristic sequential NOEs, such as d_{NN}, $d_{\beta N}$ and $d_{\alpha\beta}(i, i+3)$, are observed as well (Fig. 3B). Compared to other isolated peptides, studied by NMR in aqueous solution [24,25], the NOE peaks observed in Con-T are clearer and thorough. All of

these sequential NOE connectivities confirm the presence of a stable helix of Con-T in aqueous solution.

3.3. The antagonism of Con-T and Con-ET on the NMDA receptor

The effect of Con-T and Con-ET on the excitatory synaptic transmission is studied in the CA1 region of rat hippocampal slices using intracellular recording techniques. We showed previously that superfusion of Con-T specifically and irreversibly decreased the pharmacologically isolated NMDA receptor-mediated excitatory postsynaptic potential ($EPSP_{NMDA}$) in a concentration-dependent manner [18]. This is due to an interaction of Con-T with the glutamate binding site and the polyamine recognition site of NMDA receptors. Here, we compare the effect of Con-T and its Glu analog on the NMDA receptor. Fig. 4 shows the percentage of inhibition at different concentrations of Con-T and Con-ET. The results indicate that Glu substitution decreases the inhibitory effect of Con-ET. The apparent IC_{50} of Con-T and Con-ET against $EPSP_{NMDA}$ is approximately 23 nM and 100 nM, respectively.

4. Discussion

Our CD and NMR studies show that Con-T adopts helical conformation even in the absence of calcium. The helix formation in Con-T in the absence of calcium ions must arise from factors other than chelation of Ca^{2+} by Gla residues. Although many peptides of this size are found to fold into α-helical conformation [26,27], the intrinsic helix propensity of amino acid per se is not the sole element for helix formation. Helix formation in short peptides frequently depends on the combination of several factors, including the side chain–side chain interactions, the helix dipole [28], salt-bridges [29], capping effects [23], and hydrophobic interactions in the case of coiled coils [30]. To our knowledge, the helicity of Con-T in aqueous solution is higher than most natural peptides of similar size. Con-T is a very good model for helix; its sequence carries several known elements for de novo design of helical structure in peptides [31]. These elements are illustrated in Fig. 5a. *First, the intrinsic helix propensity of this sequence is very high.* There are only five residues with a P_α value less than 1.00. The average P_α value for any window of four residues is greater than 1.00 and the overall average P_α value is about 1.20 if we use the P_α value of Glu for Gla [32]. *Second, the N-terminus of this peptide contains many acidic side chains, while the C-terminus has many basic ones.* Total charge of the first four residues at N terminus is −4 while at C terminus is +2. These charged groups can stabilize the helix through an interaction with helix dipole [28,33]. *Third, several salt-bridges can be formed.* Two i, i+4 salt bridges and five i, i+3 salt bridges can be potentially formed by combinations among these residues. Fig. 5b shows the relative positions of charged residues of Con-T in the helical conformation. The charged residues seem to form a 'salt-bridged network' on one side of helix. It has been shown that pairs of oppositely charged side chains at positions i and i ± 4 (or i ± 3) can stabilize helical conformation in proteins [34] and in model peptides [26,29]. In order to test this possibility in Con-T, we investigated the pH dependence of its CD spectra. The state of ionization of side chains controls their ability to pair. Thus, the structure of Con-T will be highly sensitive to pH if salt-bridges are important to the stability of the α-helix in this peptide. The bell-shaped profile obtained (data not shown) is a typical pattern of pH titration for peptides containing multiple salt-bridges [26]. Salt-bridges contribute considerably to helix formation in Con-T since each salt-bridge is estimated to contribute about 0.5 kcal/mol to the stability of helix in the isolated peptide model [29]. Thus, calcium binding by Gla is not a critical determinant for helix formation in Con-T; rather the side chain interactions mentioned above play important roles. Glu substitutions not only decrease the helical content of Con-T, but also diminish its inhibition of NMDA receptor. It was reported that α-helicity is not sufficient for the NMDA antagonism of Con-G [21]. The relationship between the diminution of function and the loss of helical conformation of Con-T by Glu replacements remains unclear at this stage. Further studies are necessary to unravel this correlation.

5. Note added in proof

An interesting paper describing the structures of Con-T and Con-G appeared after the submission of the manuscript. They indicate that the 3D structures of conantokins are composed of a dynamic mixture of 3_{10} and α-helix [Skjærbæk N, Nielsen KJ, Lewis RJ, Alewood P, Craik DJ (1997) J Biol Chem 272, 2291-2299].

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