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Covalent Structure and Some Pharmacological Features of Native and Cleaved α-KTx$_{12-1}$, a Four Disulfide-bridged Toxin from *Tityus serrulatus* Venom

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Abstract: A toxin with four disulfide bridges from *Tityus serrulatus* venom was able to compete with ^{125}I-kaliotoxin on rat brain synaptosomal preparations, with an IC_{50} of 46 nM. The obtained amino acid sequence and molecular mass are identical to the previously described butantoxin. Enzymatic cleavages in the native peptide followed by mass spectrometry peptide mapping analysis were used to determine the disulfide bridge pattern of α-KTx$_{12-1}$. Also, after the cleavage of the first six *N*-terminal residues, including the unusual disulfide bridge which forms an *N*-terminus ring, the potency of the cleaved peptide was found to decrease about 100 fold compared with the native protein.

Keywords: voltage-activated potassium channel; four disulfide-bridged toxin; *Tityus serrulatus*; MALDI-TOF analysis

INTRODUCTION

K^+ channels are widely distributed throughout the phylogenetic scale, with high levels of structural similarity, from prokaryotic to mammalian cells. They are also widespread in diverse tissues and cells, and are responsible for many regulatory processes, including the maintenance of the resting membrane potential, firing pattern, neurotransmitter release, hormone secretion and cell signalling [1,2].

Scorpion venoms contain several K^+ channel blockers, known as 'K^+ channel toxins'. These molecules are small (<45 amino acid residues) basic peptides, cross-linked by three or four disulfide bridges. In the past few years, the primary and three-dimensional structures of various K^+ channel toxins have been elucidated [3]. Despite differences in amino acid sequence, these peptides have a similar architecture, involving a cystine-stabilized motif (i.e. an α-helix linked to a two-stranded β-sheet by two disulfide bridges) [3–5]. Half-cystine residues are found in analogous positions in all known scorpion toxins with three disulfide bridges, resulting in identical S-S pairing (C1–C4; C2–C5; C3–C6) [3]. However, the presence of two Cys residues, in additional positions, may disrupt the pairing pattern, as has been reported for natural maurotoxin (MTx) [6] and shown for the synthetic one [7].

A four disulfide-bridged short toxin from *Tityus serrulatus* venom was isolated and named TsTx-IV

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[8]. TsTx-IV was described as a 41 residue long toxin that acts on Ca^{2+} -activated K^+ channels. More recently, the nuclear magnetic resonance (NMR) structure of a 40 residue long toxin, named butantoxin, was elucidated [9]. A unique difference, the Asn41 as the C-terminal residue, was found between TsTx-IV and butantoxin. However, the TsTx-IV sequence seemed to be wrongly published and both toxins are, in fact, the same [10]. According to the recently proposed unified nomenclature [3] this protein should be called α-KTx$_{12-1}$.

Kaliotoxin (KTx) binds with a picomolar affinity to receptors in rat brain P2 synaptosomal preparations. Recent studies have suggested that the main targets of KTX in rat brain are voltage-activated K^+ channels containing Kv1.1 and Kv1.3 subunits [11]. Thus, due to its very high affinity for these channels, ^{125}I-KTx is a valuable molecule for screening new Kv1.1 and Kv1.3 channel ligands using competitive binding assays [12]. These ligands will be useful tools for studying neurotransmission processes and for developing original therapeutic agents.

Searching for new voltage-activated K^+ channel ligands in *Tityus serrulatus* venom, using ^{125}I-KTx as a binding marker, we were able to describe a different biological activity for α-KTx$_{12-1}$. Peptide mapping analysis by MALDI-TOF of this toxin, after 'on plate' enzymatic cleavages confirmed the unique S-S pairing of this toxin.

MATERIALS AND METHODS

Venom Fractionation and Toxin Purification

The reagents used in all experiments were of analytical grade. *Tityus serrulatus* specimens were collected at Santa Bárbara, in the State of Minas Gerais, Brazil. The venom was first subjected to Sephadex G50 size exclusion chromatography as previously described [13]. Fraction III, which contains small peptides, was loaded (575 µg) on to a reverse-phase HPLC column (Merck 100 RP-18, 4 × 250 mm, Lichrospher, 5 µm), equilibrated with 0.1% TFA in water (solvent A). The gradient was achieved by eluting solvent B (0.1% TFA (v/v) in acetonitrile) using a ratio of 0.33% of B.min^{-1}. The flow rate was 1.0 ml/min and absorbance was measured at 230 nm and 280 nm. To prevent contamination, the fractions of interest were further purified, using a shallower gradient (ratio of 0.1% of solvent B min^{-1}).

Chemical Characterization

Amino acid analysis and native protein sequencing were carried out as previously described [14]. Briefly, protein samples were hydrolysed under vacuum in a Pico-Tag® workstation (Millipore/Water Associates®) with 6 M HCl at 110 °C for 20 or 70 h. Phenol (1%) was added before hydrolysis to improve the recovery of tyrosine residues. Native proteins were sequenced using an Applied Biosystems® 476A sequencer.

Mass Spectrometry

MALDI-TOF (matrix-assisted laser desorption ionization - time of flight) analysis was performed on a Voyager-DE™RP BioSpectrometer Workstation (Perseptive Biosystems) in the linear mode as already published [10]. The dried-drop method was used for sample preparation. Briefly, protein solution (0.5 µl of a 100.0 pmol/µl solution) was spotted on the target, followed by 0.5 µl of matrix CHCA (α-cyano-4-hydroxycinnamic acid) solution, and allowed to dry at room temperature. A 337 nm nitrogen laser was used to desorb the samples.

Enzymatic Digestion of α-KTx$_{12-1}$

The native protein (100 pmol) was incubated with 10% (w/w) endoproteinase Asp-N (Boehringer Mannheim) in digestion buffer (50 mM sodium phosphate buffer, pH 8.0), for 10 min at room temperature. The solution was then desalted using a ZipTip™ (Millipore) and immediately used for MALDI-TOF analysis. On-plate digestions were carried out directly on a MALDI-TOF plate by mixing 0.5–1.0 µl (50–100 pmol) of sample solution with 0.5–1.0 µl of enzyme solution (~10% w/w). The mixture was allowed to react for 10 min before matrix (CHCA) was added. The crystallized spots were then washed three times with TFA 0.1% to desalt samples prior to MALDI-TOF analysis. To prepare larger amounts of modified toxin, the digestion was achieved using 1 nmol of native toxin for 24 h at 37 °C. The products were separated by reverse-phase HPLC using a C18 column (see above for experimental conditions). To determine S-S pairing, a second and third set of cleavages were made using the cleaved α-KTx$_{12-1}$ (residues 7–40, 100 pmol). 'On plate' incubations were carried out with trypsin (10% w/w) and endoproteinase

Asp-N (10% w/w) and the fragments were analysed by MALDI-TOFMS.

Competition Assays

KTx was iodinated and competition assays carried out with rat brain synaptosomal fractions P2 as previously described [13-15]. Experiments were performed in triplicate.

RESULTS AND DISCUSSION

Chemical Characterization of Isolated Peptide

Fractions obtained by reverse-phase HPLC of fraction III from size-exclusion chromatography (Sephadex G50) of *Tityus serrulatus* venom [13] were screened in competition experiments using ^{125}I-KTx. The fraction with a retention time (RT) of 50.6 min

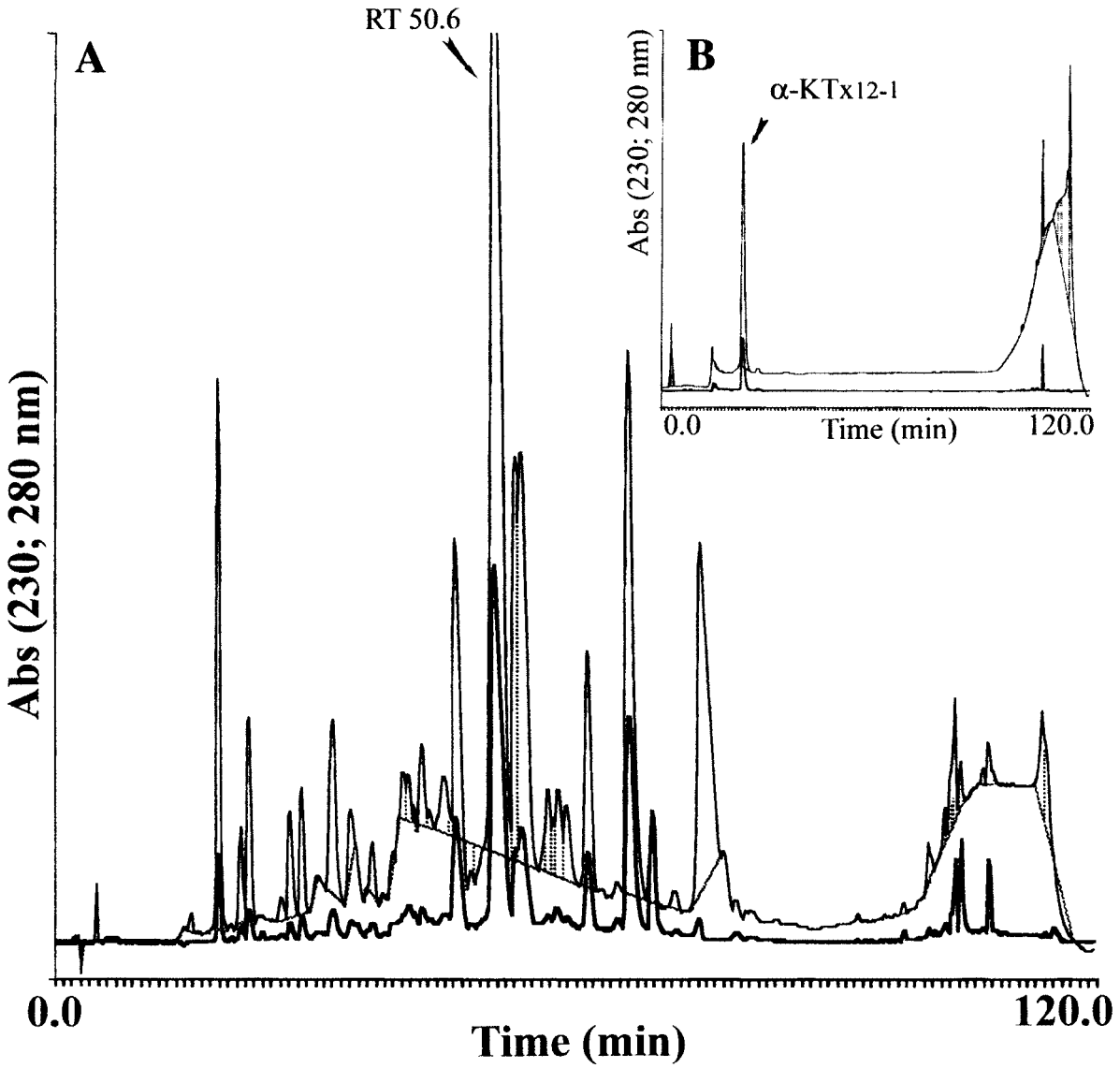
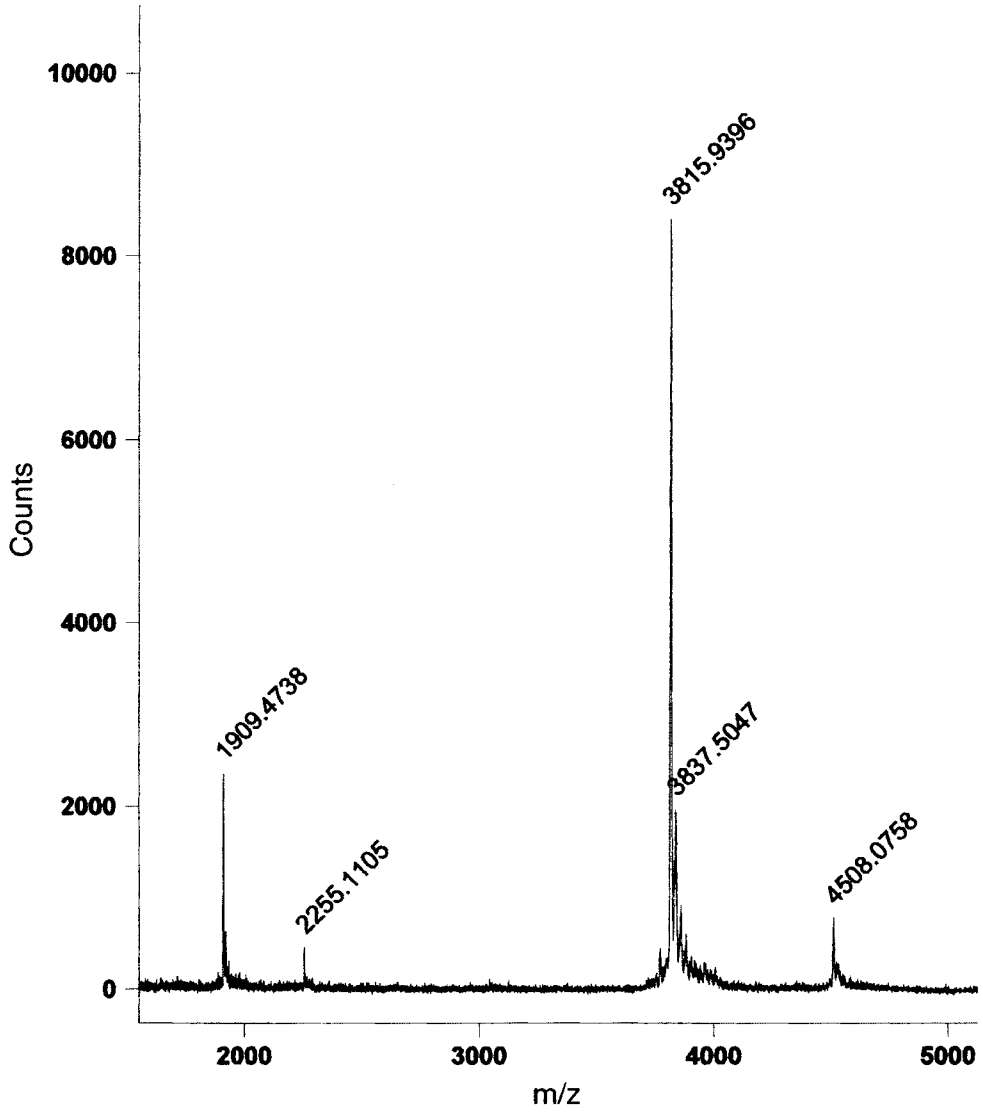


Figure 1 Purification of α-KTx$_{12-1}$ from *Tityus serrulatus* venom. (A) Fraction III (575 µg) from a size-exclusion chromatography step [13] was loaded on a C18 reverse-phase HPLC column and eluted by a linear gradient (gradient slope: 0.33%.min^{-1}B in A). Solvent A was 0.1% TFA and solvent B was acetonitrile+0.1% TFA (gradient 15% to 45% B in A for 90 min, followed by 45% to 100% B for 30 min). (B) Second chromatography step (shallower gradient; gradient slope: 0.1%. min^{-1}B in A; from 25% to 35% B in A for 90 min, followed by 35% to 100% B in A for 30 min) of the peak of interest obtained in A (RT = 50.6 min). Final α-KTx$_{12-1}$ is indicated in B (arrow). Flow rate was 1.0 ml. min^{-1} and elution was monitored at 230 (thin and upper line) and at 280 (thick and lower line) nm. Optical density unit full scale is 1.

A



B

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                    1         10        20        30        40
Native protein      WCSTCLDLACGASRECYDPCFKAFGRAHGKCMNNKCRCYT (4508.08 Da)

Endo Asp-N
fragments           WCSTCL (710.85 Da*)
                           DLACGASRECYDPCFKAFGRAHGKCMNNKCRCYT (3815.94 Da)
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Figure 2 MALDI-TOF analysis of enzymatic cleavage. (A) MALDI-TOF profile showing native peptide (4508.08 and 2255.11 as doubly charged molecule) and the cleaved protein (3815.94 and 1909.47 as doubly charged molecule). The expected mass of the cleaved peptide was 3815.41. One of the Na^+ adduct peaks is shown (3837.51). (B) Sequences of native protein and Asp-N fragments with their corresponding masses (*calculated). Enzymatic cleavage and MALDI-TOF analysis were carried out as described in Materials and Methods.

(Figure 1A) displaced ^{125}I-KTx and was further purified (Figure 1B), using a shallower gradient. Homogeneous protein with an experimentally determined molecular mass of 4508.08 Da (Figure 2A) was obtained.

Automated Edman degradation of native protein produced the following sequence: W **x**ST**x**LDLA**x**GASRE**x**YDP**x**FKAFGRAHGK**x**MNNK**x**R**x**Y. All blank cycles (character **x** in sequence) were assumed to be half-cystine residues. The calculated molecular mass of this sequence is 4406.13 Da. The difference between the calculated molecular mass and that determined by MALDI-TOF corresponds to the mass of an additional threonine (101.11 Da), which was also identified on the basis of amino acid composition (Table 1). The calculated mass of the complete sequence (i.e. with an extra Thr, probably at the *C*-terminus), is 4507.24. This amino acid sequence and molecular mass perfectly match with those observed for butantoxin [9].

TsTx-IV [8] has two additional half-cystine residues in positions 2 and 5, in the *N*-terminal region, and was found to be 41 residues long ended by Asn41. However, the molecular mass calculated from the published sequence (4621.34 Da) differs from those reported (4520 calculated and 4518 experimentally determined by MALDI-TOF) [8]. In our opinion, there is a mistake in the published TsTx-IV sequence and both TsTx-IV and butantoxin are in fact the same molecule. In a previous study, both on-line LC/ESMS and off-line LC/MALDI-TOFMS analysis of fraction III from *Tityus serrulatus* venom [10] revealed that our newly characterized toxin (RT = 50.6 min) coeluted with a minor molecular species of 4520.8 Da (not shown). This difference, i.e. 13 Da, could be explained by the presence of an isoform with an Asn40, instead Thr40, at the end.

A recent paper has evoked the necessity of a standard nomenclature for toxins acting on K^+ channels [3]. According to this unified nomenclature, both TsTx-IV and butantoxin would be called α-KTx$_{12-1}$.

Table 1 Amino-acid Composition of Native α-KTx$_{12-1}$

Amino acid	Analysed	Sequence
Ala (A)	3.94	4
Arg (R)	2.68	3
Asn (N)	Nd	2
Asp (D)	4.13	2
Cys (C)	5.9	8
Gln (Q)	Nd	0
Glu (E)	1.3	1
Gly (G)	3.8	3
His (H)	1.01	1
Ile (I)	0	0
Leu (L)	2.17	2
Lys (K)	3.12	3
Met (M)	0.84	1
Phe (F)	1.75	2
Pro (P)	1.12	1
Ser (S)	2.4	2
Thr (T)	1.79	2
Trp (W)	Nd	1
Tyr (Y)	1.57	2
Val (V)	0	0
Total residues		40
$MM_{(calc)}$	4507.24	
MALDI-TOF	4508.08	

Nd, non detectable in these experimental conditions.

Enzymatic Cleavage of α-KTx$_{12-1}$ and Peptide Mapping Analysis

After Asp-N proteolytic reaction, carried out with only 100 pmol of native toxin, the digestion product was desalted and analysed by MALDI-TOF (Figure 2A). A peptide with a molecular mass of 3815.94 Da was detected. This molecular mass corresponds perfectly to the expected mass of α-KTx$_{12-1}$ without the first six *N*-terminal residues (expected mass = 3815.41). A second Asp residue, within the protein core corresponding to amino acid residues 7–40, was not accessible to enzymatic cleavage (see Figure 2B). The addition of 18 Da (corresponding to a water molecule) was expected if cleavage occurred at Asp18. Instead, the MALDI-TOF profile (Figure 2A) showed Na^+ adduct peaks (+22 Da), as expected due to the use of sodium phosphate buffer as the digestion buffer.

Additional 'on plate' cleavage by trypsin resulted in three main molecular species as indicated in Figure 3C. A molecular species with an observed m/z of 1399.9 (mass spectra not shown) corresponded to peptide fragments 7–14 and 31–35 linked by one disulfide bridge. The two other main molecular species found on mass spectra (not shown) corresponded to peptide fragments 15–22 and 36–40, with observed m/z of 1646.91 and 1664.78. In fact, the difference between these two observed masses, i.e. 18 Da, was a missed cleavage site for trypsin, observed for Arg37 (as indicated in Figure 3C). Since the fragments 15–22 and

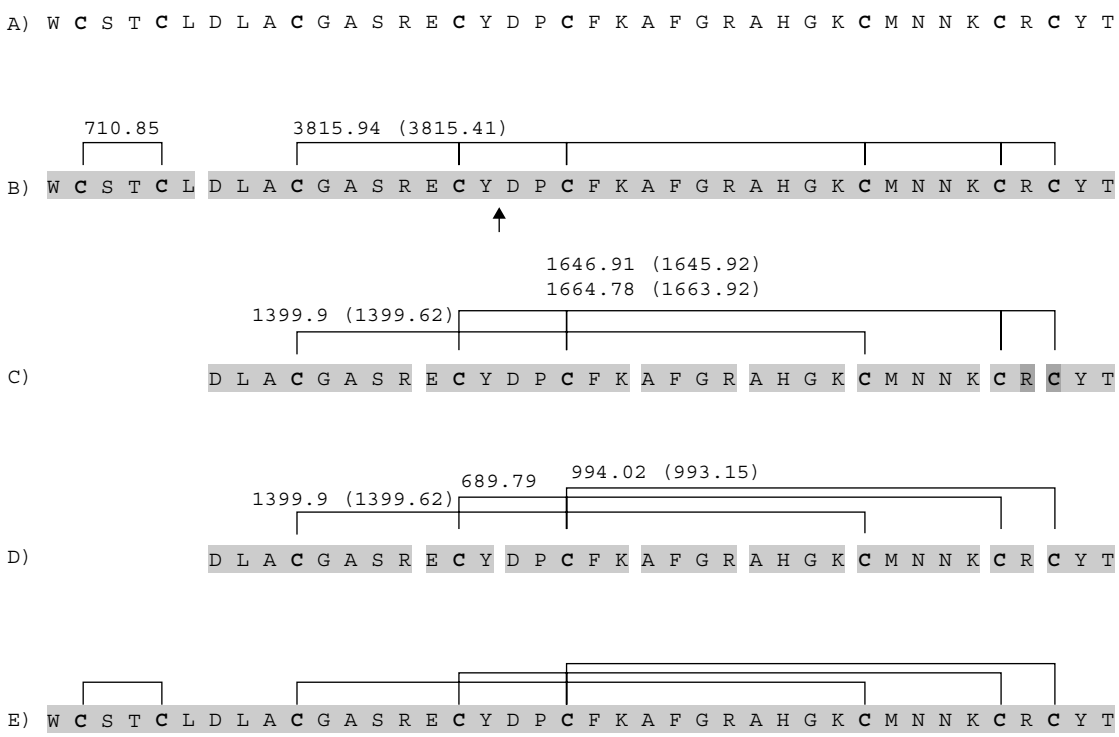

Figure 3 Schematic view of peptide mapping using 'on plate' digestions of native α-KTx$_{12-1}$ followed by MALDI-TOF analysis. Covalent structure of α-KTx$_{12-1}$. (A) Amino acid sequence; (B) Asp-N first cleavage fragments; (C) trypsin cleavage fragments; (D) trypsin + Asp-N cleavage fragments and (E) disulfide bridges pattern for α-KTx$_{12-1}$. Half-cystine residues are in bold letters. Blank spaces indicate cleavage sites. Arrows indicate missed cleavages and dark grey indicates both cleaved and uncleaved fragments, with 18 Da shifting in case of fragments retained by disulfide bonds. Observed molecular masses are shown along with the calculated molecular masses (into brackets). Disulfide bridges are drawn as black traces showing cysteine pairing and/or possibilities.

36–40 were still linked by two disulfide bridges, and since an Asp residue was still available in position 18 (between Cys16 and Cys20) a new endo Asp-N cleavage was performed. After the cleavage process, we were able to observe a molecular species with 994.02 m/z (not shown), which corresponded to 18–22 and 38–40 fragments linked by one disulfide bridge. The S-S pairing for Cys residues (pictured in Figure 3) chemically confirms the disulfide-bridge pattern described using NMR studies [9]. Thus, in spite of the presence of two additional cysteines in this toxin, their pairing maintains the classical one which is characteristic of scorpion toxins.

Binding Assays

Competition experiments showed that native α-KTx$_{12-1}$ inhibited the binding of ^{125}I-KTx to its receptor in rat brain synaptosomal preparations, with an IC$_{50}$ of 46 nM (Figure 4A). The absence of the six *N*-terminal residues reduced the affinity of the cleaved toxin for the KTx receptor by a factor of about 100 (Figure 4A), as shown by competition experiments with ^{125}I-KTx and the cleaved α-KTx$_{12-1}$. Both native and cleaved α-KTx$_{12-1}$ failed to compete with ^{125}I-apamin, a very specific ligand of low-conductance activated K^+ channels, in rat brain synaptosomal preparations (data not shown). The recently reported butantoxin [9], identical to α-KTx$_{12-1}$, inhibited T-cell proliferation and interleukin-2 production. The ability of α-KTx$_{12-1}$ to bind to the KTx receptor, as shown by ^{125}I-KTx competition experiments, suggests that the inhibition by butantoxin of T-cell proliferation is due to Kv1.3 blockade. Kv1.3 is the predominant type of Kv expressed by human, mouse and rat T-cells and is involved in T-cell activation and proliferation [16].

Much attention has been paid to putative elements in the structures of both the channel and the toxin, which have been shown to be critical

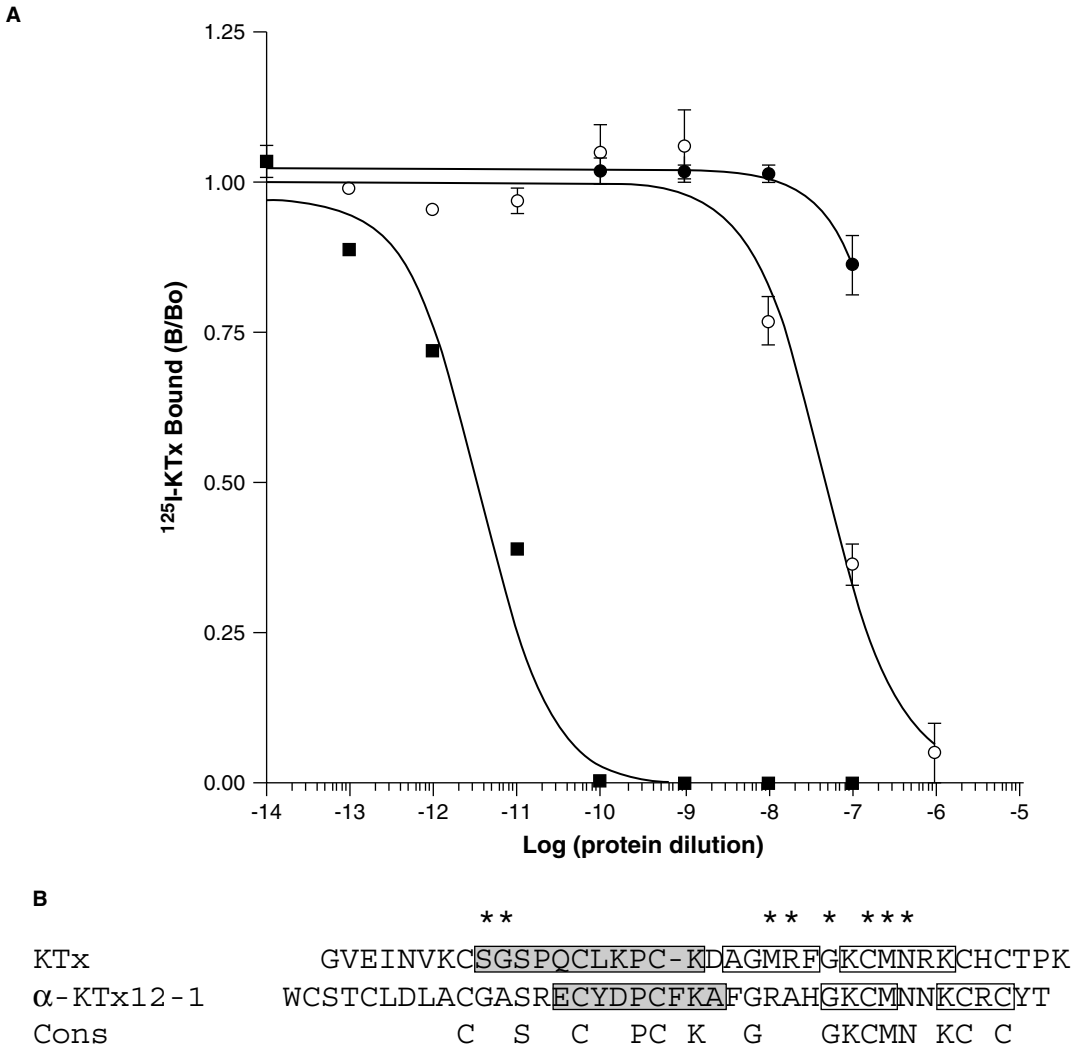

Figure 4 Competition for ^{125}I-KTx binding in rat brain synaptosomal P2 preparation and sequence comparison. (A) synaptosomal P2 membranes (30 μg/160 μl) and ^{125}I-KTx (40 pM) were incubated for 1 h at 25°C with each series of concentrations of the protein tested. The buffer was 25 mM Tris-HCl, 50 mM NaCl, 0.1% BSA, pH 7.4. Incubation was stopped by centrifugation (11 000 **g**, 3 min) and the pelleted membranes were washed twice with 1.0 ml of washing buffer. Radioactivity was measured. Bo is the binding of ^{125}I-KTx in the absence of competitor and B is binding in the presence of the indicated concentrations of native KTx (■), native α-KTx$_{12-1}$ (○) and cleaved α-KTx$_{12-1}$ (●). There was less than 20% nonspecific binding and all experiments were performed in triplicate. (B) Alignment of KTx and α-KTx$_{12-1}$ sequences. Some of most important residues in the KTx sequence for Kv1.3 binding are marked (*). Open boxes indicate β-sheet and grey boxes indicate α-helix structures for both toxins [23,9]. Cons: consensus.

to binding properties such as affinity and selectivity. Previous studies have attempted to describe these elements. For KTx-Kv1.3 binding, the interactions of KTx residues Gly10, Ser11, Arg24, Phe25, Lys27, Met29, Asn30 and Arg31 with various channel residues, have already been determined [1,12,17,18,19]. Most of these residues are in the β-sheet region, which forms the surface of interaction of KTx with the Kv1.3 channel. However, *N*-terminal modifications may also affect the binding of scorpion toxins to the Kv1.3 channel, as described previously for *N*-terminal extensions of KTx2 [20] and the acetylation and biotinylation of the *N*-terminal amino group of MgTx [21]. Some of the principal residues described as necessary for the KTX–Kv1.3 interaction are present in the α-KTx$_{12-1}$ sequence (i.e. Ser13, Lys30, Met32, Asn33; Figure 4B). The most important differences are His28 instead of Phe25

in KTx and Asn34 instead of Arg31 in KTx. These amino acid changes may be responsible for the lower affinity of this toxin for the KTx receptor, causing changes in the surface corresponding to the β-sheet.

The cleavage of the native toxin did not affect any of the principal residues analogous to those already described for the KTx–Kv1.3 interaction (Figures 2B and 4B). Due to greater exposure of the main residues involved in the receptor recognition, it might be supposed that the cleaved toxin would have a higher level of activity. On the contrary, our results suggest that the lack of the *N*-terminal portion reduced α-KTx$_{12-1}$ binding.

An aromatic residue at position 1 is unique to α-KTx$_{12-1}$ among K^+ channel toxins. Trp1 appears to be located far from the face of the toxin supposed to be in interaction with the channel [9]. However, due to its hydrophobic character in such an exposed position, Trp1 should not be ruled out totally as an important docking element in this kind of toxin. It was previously said that an aromatic side chain was frequently required to stabilize a complex toxin/receptor through an aromatic ring and confer high affinity binding to the interaction [22]. NMR determination of the structure of α-KTx$_{12-1}$ [9] (pdb code 1C55) shows that the *N*-terminal cycle motif is free from the protein core. This flexibility may render Trp1 very mobile and may provide the conditions required for this residue to play an allosteric role in toxin–channel interaction. Also, after cleavage, Asp7 lies in the first position and may contribute to changing the electrostatic surface potential of the molecule.

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