New Tricks of an Old Pattern

STRUCTURAL VERSATILITY OF SCORPION TOXINS WITH COMMON CYSTEINE SPACING

Received for publication, December 1, 2011, and in revised form, December 21, 2011 Published, JBC Papers in Press, January 10, 2012, DOI 10.1074/jbc.M111.329607

Alma Leticia Saucedo1,2, David Flores-Solis3, Ricardo C. Rodríguez de la Vega3,5, Belén Ramírez-Cordero1, Rogelio Hernández-López1,4, Patricia Cano-Sánchez3, Roxana Noriega Navarro3, Jesús García-Valdés1, Fredy Coronas-Valderrama2, Adolfo de Roode5, Luis G. Brieba5, Lourival Domingos Possani5, and Federico del Río-Portilla5

From the 1Instituto de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, México, D.F., 04510, México, the 2UMR 7138, Département Systématique et Evolution, Muséum National d'Histoire Naturelle, Paris 75005, France, the 3Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, the 4Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, México, D.F., 04510, México, the 5Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Apartado Postal 510-3, Cuernavaca México, the 6Centro de Patología Experimental y Aplicada, Facultad de Medicina, Universidad de Buenos Aires and Administración Nacional de Laboratorios e Institutos de Salud “Dr. Carlos G. Malbrán,” Ministerio de Salud, Av. Vélez Sarsfield 563, CP 1281, CABA, Buenos Aires, Argentina, and the 7Laboratorio de Genómica para la Biodiversidad, CINVESTAV Unidad Irapuato, 36821 Irapuato, Guanajuato, México

Background: Most scorpion venom peptides adopt a single structural scaffold around four strictly conserved cysteines.

Results: Two K+ channel-blocking peptides from Tityus venoms share this cysteine spacing but fold into a distinct cystine-stabilized helix-loop-helix scaffold.

Conclusion: These peptides define a new structural group of scorpion venom peptides.

Significance: Cysteine spacing does not dictate the three-dimensional fold of small disulfide-rich proteins.

Scorpion venoms are a rich source of K+ channel-blocking peptides. For the most part, they are structurally related small disulfide-rich proteins containing a conserved pattern of six cysteines that is assumed to dictate their common three-dimensional folding. In the conventional pattern, two disulfide bridges connect an α-helical segment to the C-terminal strand of a double- or triple-stranded β-sheet, conforming a cystine-stabilized α/β scaffold (CSα/β). Here we show that two K+ channel-blocking peptides from Tityus scorpions conserve the cysteine spacing of common scorpion venom peptides but display an unconventional disulfide pattern, accompanied by a complete rearrangement of the secondary structure topology into a CS helix-loop-helix fold. Sequence and structural comparisons of the peptides adopting this novel fold suggest that it would be a new elaboration of the widespread CSα/β scaffold, thus revealing an unexpected structural versatility of these small disulfide-rich proteins. Acknowledgment of such versatility is important to understand how venom structural complexity emerged on a limited number of molecular scaffolds.

Scorpion venoms are complex mixtures of biomolecules, products of millions of years of evolution (1, 2). Venoms contain, among others components, peptide toxins capable of interacting specifically with potassium, sodium, and calcium channels (3–5). These toxic peptides have contributed considerably to the understanding of the structure and functional mechanism of the ion channels (6). Some of them are considered good candidates for the design and development of new drugs (7), although the number of structural and functional analyses of these peptides is still limited (8).

Potassium channels blocker toxins (KTx)6 can be classified into four subfamilies accordingly to the accepted nomenclature: α, β, γ, and κ (9, 10). α-KTxs are peptides of 23–43 amino acids stabilized by three to four disulfide bonds, two of which are strictly conserved and link an α-helix and one strand of a β-sheet within the so-called cystine-stabilized α/β scaffold (CSα/β), the most common among scorpion toxins (10, 11). β-KTx toxins, known as “long chain,” are three disulfide-bridged peptides with ~60 amino acids that also contain the cysteine pattern of peptides adopting the CSα/β scaffold, although no structure is available yet (12). γ-KTxs are blockers of the ether-á-go-go-related gene family of K+ channels; they are 36–47 amino acids long with three or four disulfide bridges, although no structure is available yet (12).

The abbreviations used are: KTx, K+ channel toxin; Hx, hydrogen in α position; Hj, hydrogen in β position; HN, amide proton; RMSD, root mean square deviation; CS, cystine-stabilized.

6 The abbreviations used are: KTx, K+ channel toxin; Hx, hydrogen in α position; Hj, hydrogen in β position; HN, amide proton; RMSD, root mean square deviation; CS, cystine-stabilized.
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which also adopt the CSα/β scaffold (13). The last and smallest family, the κ-KTx family, displays purely helical structure stabilized by two disulfide bridges and fold into an α-hairpin fold known as CSα/α (cystine-stabilized helix-loop-helix) (11, 14–17). Peptides belonging to this family have been isolated from only two scorpion genera: Heterometrus (14, 15) and Opisthacanthus (16, 17). κ-KTx family is relatively poor blockers of K+ channels, despite presence of the typical functional dyad, Tyr and Lys, important for other activity of the toxin (3). It is still neither clear whether members of the κ-KTx family might all work through the same mechanism on potassium channels (16, 17), nor whether K+ channels are indeed their cognate receptors. In part this may be due to the lack of structural and functional characterization of peptides in this family.

This work describes the native isolation, recombinant production and structural characterization of a newly identified 28 amino acid K+ channel-blocking peptide from Tityus serrulatus venom, named κ-buthitoxin-Tt2b, κ-BUTX-Tt2b short, following the nomenclature proposal of King et al. (18). The sequence shows high identity with peptides classified within the α-KTx subfamily 20 (19), including the six cysteines of the sequence signature of the CSα/β scaffold. Unexpectedly, the peptide adopts a CSα/α fold in solution. The same overall structure was determined for a recombinant version of another peptide from Tityus serrulatus classified within the same subfamily (UniProt P86271, Ts16 in Ref. 46). Peptides of the family 20 were assumed to adopt the conventional CSα/β despite presence of the typical functional dyad, Tyr and Lys, amino acid K+duction and structural characterization of a newly identified 28 characterization of peptides in this family.

The following experimental procedures were performed.

**EXPERIMENTAL PROCEDURES**

**Purification and Sequence Determination of the Novel Peptide**

Venom from the scorpion *Tityus serrulatus* collected in the Province of Santa Fe, Argentina, was obtained by electrical stimulation and separated as earlier described (20). Only two steps of HPLC fractionation were necessary to obtain the pure peptide under study. An analytical C18 reverse phase column (Vydac, Hesperia) was used with a linear gradient from solvent A (water in 0.12% TFA, Sigma-Aldrich; all of the chemicals were purchased from this company, unless otherwise specified) to 60% solvent B (acetonitrile in 0.10% TFA), run for 60 min. The second separation used the same system with a gradient from 10 to 35% solvent B, run for 45 min. The amino acid sequence determination of the pure peptide was obtained using automatic Edman degradation with a Beckman LF 3000 protein sequencer, and MS measurements were obtained with an ion trap equipment from Finnigan LCQ<sup>(TM)</sup> as earlier described (20). The peptide was named κ-buthitoxin-Tt2b.

**Disulfide Bridge Determination**

Pure peptide (18 μg) was digested with tosylphenylalanylchloromethyl ketone-trypsin and endopeptidase V8 from *Staphylococcus aureus* (Roche Applied Science). Initially, 1 μg of trypsin was added in presence of an ammonium bicarbonate buffer (pH 8) and hydrolyzed for 5 h; then the endopeptidase V8 (1 μg) was added, and the reaction was incubated for 12 h at 36 °C. The peptides were separated using the same HPLC conditions used initially for the whole venom (see above) and sequenced.

**Design and Cloning of the Synthetic κ-BUTX-Tt2b Gene**

The κ-BUTX-Tt2b synthetic gene coding for the peptide was assembled based on the amino acid sequence obtained and optimized for *Escherichia coli* codon usage. The κ-BUTX-Tt2b gene (see supplemental data Fig. SM1) was cloned into the expression vector pET32a (Novagen). The resulting plasmid pET32-κ-BUTX-Tt2b was confirmed by DNA sequencing. The Ts16 synthetic gene used was based on the κ-BUTX-Tt2b gene (sequence available in supplemental data Fig. SM1).

**Protein Expression**

Recombinant-κ-BUTX-Tt2b protein was overexpressed in tuner *E. coli* cells (Novagen) transformed with the expression vector pET32-κ-BUTX-Tt2b. LB medium supplemented with ampicillin was used to grow cells at 37 °C. Protein expression was induced at an A<sub>600</sub> between 0.7–0.8 with isopropyl thio-β-galactopyranoside 0.5 mM. The cells were incubated for 6 h at 30 °C and harvested by centrifugation.

**Recombinant Protein Purification**

For κ-BUTX-Tt2b, the cell pellets were resuspended in lysis buffer (150 mM NaCl, 0.1 mg/ml lysisomize, 50 mM Tris/HCl, pH 8) and sonicated using (Misonix Sonicator 3000). The soluble fraction was separated by centrifugation at 32,000 × g for 30 min at 4 °C. Recombinant His<sub>6</sub>-tagged fusion toxin (20.3 kDa) was purified by metal-chelate affinity chromatography using a HiTrap Ni<sup>2+</sup> column (GE Healthcare) and eluted with two column volumes of elution buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 500 mM imidazole). Thrombin was used to release the κ-BUTX-Tt2b recombinant toxin from the N terminus thioredoxin fusion protein. Cleavage reaction was performed for 6 h at 18 °C according to the manufacturer’s instructions. A second round of isolation by metal-chelate affinity chromatography was required to remove κ-BUTX-Tt2b from cleavage reaction subproducts. Final κ-BUTX-Tt2b purification was performed with reverse phase HPLC in a Pro-Star Varian instrument equipped with a binary gradient solvent system on a Jupiter C18 column (250 mm × 4.6 mm; Phenomenex) using a linear gradient from 12 to 30% of water-acetonitrile run for 30 min (containing 0.05% TFA) at a flow rate of 1 ml/min.

**Protein Characterization**

**Mass Spectrometry**—MALDI-TOF MS on a Bruker Daltonics Microflex LT equipment was used to determine the molecular mass of the recombinant proteins. The data were acquired using the reflector operation mode acquiring 150 shots. The samples were prepared with α-cyano-4-hydroxycinnamic acid in a 1:10 ratio (21).

**Electrophysiological Measurements**—cRNAs for voltage-dependent potassium channels: Shaker-IR (Shaker channel with inactivation domain removed) and hKv1.2 subunits were tran-
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**Sample Preparation**—Native and recombinant κ-BUTX-Tt2b were prepared at 0.08 and 2.3 mM concentrations, respectively, by dissolving lyophilized protein in 95% H$_2$O, 5% D$_2$O (Cambridge Isotope Laboratories). The recombinant protein spectra were obtained at pH 3.5. No pH adjustment was done with the native sample. NMR data were recorded at 25 °C on an 800 MHz Varian spectrometer equipped with a HCN indirect detection probe. TOCSY spectra were recorded with isotropic mixing time of 75 and 20 ms. NOESY spectra were acquired using mixing times of 150 and 300 ms. The two-dimensional NMR spectra were conducted using a scheme of water suppression by gradient-tailored excitation for solvent suppression (22). All of the experiments were acquired using 2048 and 1024 complex points in direct and indirect dimensions, respectively. Ts16 recombinant protein (rTs16) spectra were recorded in a 500 MHz Varian instrument at 3.0 mM; experiments were obtained as before.

**Data Processing and Analysis**—NMRDraw and NMRpipe software were used for processing data (23). CARA1.5 software was used for NMR data analysis (24). Semi-automated assignment and distance geometry calculations were performed with CYANA 2.1 (25).

**Molecular Dynamics Refinement**

Structure refinement was performed using molecular dynamics AMBER 9 (26) suite with an explicit solvent model. Lowest CYANA target function values were used for further refinement. Molecular dynamics simulations and the energy minimizations were carried out with the topology and parameters of AMBER-99SB force field. Distance and dihedral angle constraints were used for AMBER calculations following the method proposed by Xia et al. (27). Geometric quality of structures was assessed using PROCHECK utility in the validation server of the Protein Data Bank.

**RESULTS**

κ-BUTX-Tt2b Isolation Sequence and Disulfide Determination—Fig. 1A shows HPLC separation of the soluble venom from *T. trivitattus*. The inset shows a second HPLC fractionation, after selecting only the component eluted at 24.7 min from the first run. The major component, labeled with an asterisk (see inset), was the peptide used for this study (~3.1% of the soluble venom). The amino acid sequence determination of this pure peptide gave an unequivocal sequence assignment up...
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**Shaker-IR**

**Kv1.2**

**Control**

**Toxin**

![Image](image-url)

**FIGURE 2. Effect of \( \kappa \)-BUTX-Tt2b toxin on Shaker-IR (left panel) and Kv1.2 channels (right panel).** Macroscopic K⁺ currents were recorded using two-electrode voltage-clamp technique in ND96 solution. Currents were elicited by depolarizing the membrane for 200 ms, from a holding potential of −60 mV to a pulse step of +60 mV. Pulse protocol was applied at 60 s each. Traces for control conditions are shown with a black line, and those in the presence of recombinant toxin are shown with a gray line (after 8 min of application at 200 \( \mu \)mol/liter). The current percentages of blockade for Shaker IR and Kv1.2 were 88.7 ± 9.9 and 74.9 ± 7.1 (mean ± S.E.), respectively. The inhibition of currents was not fully reversible (not shown).

The last amino acid, Arg in position 28, was inferred by mass spectrometry determination. The experimental mass determined for the native peptide was 3179.75 Da, and the theoretical mass expected for the first 27 residues directly determined was 3023.57, leaving a molecular mass of 156.18, which was identified as being that of arginine. This sequence was deposited in the UniProt Knowledgebase under the accession number B3A0L5. Sequence similarity searches revealed high identity with only two *Tityus* sp. venom peptides: Ts16 from *T. serrulatus* (P86271, 89%) and Tt28 from *T. trivittatus* (P0C183 (18), 65%). No disulfide pairing for any of these homologous peptides has been reported.

Fig. 1C shows the profile of HPLC separation after enzymatic hydrolysis. The peptide eluted at 23.0 min was compatible with a disulfide pairing between the first (Cys²) and fifth cysteines (Cys²⁴), C1 and C5. The automatic sequencer showed two amino acid residues for the first cycles: Gly and Asn. Position 2 was a blank, but Met in position 3 and Pro was in position 4. It implies that endopeptidase V8 cleaved the peptide bond at the Glu⁵, and trypsin cleaved Lys²² and Arg²⁵. The remaining peptides gave always more than two amino acid residues per cycle of the sequencer, because the core fragment of peptide after the enzymatic hydrolysis did not allow separating the other peptides containing the two remaining disulfide bridges. In this way, only one of the disulfide bridges of the toxin was directly determined.

**Fusion Protein Expression and Purification**—Most of the fusion protein was located in the soluble cellular lysate, and it was efficiently retained by the HiTrap Ni²⁺ column with purification yield of 36 mg/liter. Thrombin recognizes the sequence X₄X₅R/L—GSX₄X₅; therefore, the product of the hydrolysis reaction is the \( \kappa \)-BUTX-Tt2b sequence with additional glycine and serine at the N-terminal segment, \( \kappa \)-BUTX-Tt2b. Optimal cleavage conditions were obtained at 18 °C after 6 h of reaction. Finally, another isolation by metal-chelate affinity chromatography purification step was performed.

**Reverse Phase HPLC Purification and Protein Characterization**—Under chromatographic conditions selected, \( \kappa \)-BUTX-Tt2b elutes at 23.0 min with a MALDI-TOF mass of 3325.1 for [M+H]⁺, which is in good agreement with the theoretical mass of 3323.9 Da for an oxidized peptide forming three disulfide bridges, calculated with the ProtParam tool from ExPASy server (28). The purification procedures indicated above yielded 0.7 mg of \( \kappa \)-BUTX-Tt2b per liter in LB culture medium.

**Electrophysiological Measurements**—Electrophysiological assays were done on Shaker-IR and hKv1.2 channels. Fig. 2 shows the effect of \( \kappa \)-BUTX-Tt2b on both channels, using the experimental conditions described below. The protocol of test pulses was applied every 60 s to verify the current stability previous to the toxin addition. Only oocytes showing a minimum run-down were chosen for experimental assays. The control currents (black line) through Shaker-IR or hKv1.2 channels under whole cell recording are plotted. Fig. 2 shows representative traces for both channels only at +60 mV pulse. The addition of \( \kappa \)-BUTX-Tt2b (200 \( \mu \)mol/liter) blocked partially outward currents (gray line). Recombinant toxin did not change basically the original shape of currents, suggesting that \( \kappa \)-BUTX-Tt2b acts as a pore blocker, although, further experiments are needed to test this hypothesis. Current reduction was 88.7 ± 9.9 and 74.9 ± 7.1 \((n = 3)\) for Shaker-IR and Kv1.2, respectively.

**Nuclear Magnetic Resonance Analysis**—NMR experiments for the \( \kappa \)-BUTX-Tt2b native protein were obtained with poor signal to noise ratio at a very low concentration. The TOCSY experiment was obtained with sufficient sensitivity. However, the NOESY experiments displayed low sensitivity that made difficult to complete the sequential assignments. In contrast, NMR spectroscopic studies using higher concentration of \( \kappa \)-BUTX-Tt2b revealed well resolved resonances with good chemical shift dispersion between 6.3 and 10.3 ppm. A comparison of the chemical shifts between the amide region signals of the recombinant (black) and the native (red) toxins shows just slight differences mainly caused by different pH levels and concentrations used for the experiments (see Fig. 3). Aliphatic region is almost identical. A NOESY HN region spectrum shows the HN–HN correlations consistent with the presence of...
nal disulfide bridges. Conventional disulfide bridges and 0.78 Å for the nonconven-
tional disulfide bridges.

The RMSD against the reference structure gave 1.75 Å for the
highest energy structure for each set of calculations,

Table 1 shows the statistics and RMSD values of these three
calculations. After comparison of backbone for the residues
3–30 of the lowest energy structure for each set of calculations,
the RMSD against the reference structure gave 1.75 Å for the
conventional disulfide bridges and 0.78 Å for the nonconven-
tional disulfide bridges.

A total of 200 structures where generated with CYANA; only
20 structures (Fig. 4A) with the lowest target function are
shown. The proton shift index is in good agreement with the
secondary structure found (31). NOE correlations between
HN/HN, Hα/HN, and Hα/HA indicate that
the k-BUTX-Tt2b structure consists of an antiparallel helix-loop-
helix topology with a new fold pattern stabilized by three non-
conventional disulfide bonds between C1–C5, C2–C4, and
C3–C6. A loop of four residues, Cys11–Cys24, contains three disulfide
bridges Cys7–Cys20, and Cys11–Cys26. A loop of four residues, Cys11–
Cys24, connects the two helices.

The structure with the lowest energy is shown in Fig. 4C, and
its correspondent electrostatic potential surface diagram is

![Figure 3. NOESY spectra showing the aliphatic region of native and recombinant k-BUTX-Tt2b spectra obtained with 0.1 mM native (48 h of acquisition in red) and with 2.3 mM recombinant (in black). Differences are attributed to concentration and the glycine and serine in the N termini. The inset shows the correlations between Cys7 and Cys20 forming the cystine pair C2-C4 for the recombinant protein (Cys7 HB2–Cys20 HB2, Cys7 HB2–Cys20 HB3, Cys7 HB3–Cys20 HB2, and Cys7 HB3–Cys20 HB3 with distances found of 4.9, 4.0, 3.4, and 4.0 Å).](image-url)

| Table 1 | RMSD between 20 CYANA calculated structures for k-BUTX-Tt2b varying the disulfide bridge patterns |
|---------|--------------------------------------------------|
| k-BUTX-Tt2b | No DSB | cDSB | nDSB |
| Upper distance limits | | | |
| Total | 367  | 342 | 365 |
| Short range | 228 | 221 | 223 |
| Medium range | 64 | 55 | 64 |
| Long range | 75 | 66 | 78 |
| RMSD (3–30) | | | |
| Average backbone (Å) | 0.43 | 0.52 | 0.39 |
| Average heavy atom (Å) | 1.09 | 1.19 | 1.01 |
| Target function value (Å) | 0.0 | 0.08 | 0.01 |
shown in Fig. 4E. The rκ-BUTX-Tt2b atom coordinates and chemical shifts were deposited in the Protein Data Bank and Biological Magnetic Resonance Bank, with access codes 2LI3 and 17876, respectively.

Native versus Recombinant rκ-BUTX-Tt2b—Using the NMR assignments of rκ-BUTX-Tt2b as a reference, it was possible to identify all 28 spin systems for native rκ-BUTX-Tt2b. However, it was not possible to solve the tridimensional structure from the native sample because of the absence of a significant number of NOE signals. Chemical shift assignments were used to compare HN and aliphatic region between native and recombinant rκ-BUTX-Tt2b. Little difference in chemical shift was observed between native and recombinant peptides. Similar overlapped NOE peaks were used as spectroscopic evidence that both peptides have the same fold (Fig. 3). Furthermore, secondary structure prediction based on proton chemical shifts of native rκ-BUTX-Tt2b shows helical patterns as described for the recombinant protein.

rTs16 Three-dimensional Structure—The high identity between rκ-BUTX-Tt2b and Ts16 strongly suggests that the latter would also adopt a CSα/α structure. To verify this hypothesis, the rκ-BUTX-Tt2b gene was used as a template for Ts16
cloning. Following the experimental procedures reported above, it was possible to obtain the NMR solution structure of rTs16.

All 31 rTs16 spin systems were identified (chemical shift completeness of 96.8%). The disulfide bridges positions for rK-BUTX-Tt2b were determined based on the presence of Cys2–Cys24 and Cys7–Cys20 Hβ-Hβ NOE cross-peaks; neither Cys2–Cys20 nor Cys7–Cys24 showed any NOEY correlation between β protons.

Final CYANA calculations were performed as for rK-BUTX-Tt2b considering 809 NOE distance restraints. rTs16 peptide has helical conformations between Lys4–Gln10 and Lys14–His23 NOE correlations between HN/HN-ε, Hα/HN-ε, and Hα/HN-ε, and Hα/HN-ε indicate that Ts16 is mainly shaped by helical elements (see the supplemental data Fig. SM4). The rTs16 structure consists of an antiparallel helix-loop-helix topology with same fold pattern stabilized by three nonconventional disulfide bonds for scorpion toxins as for K-BUTX-Tt2b. A loop of three residues, Cys11–Gly13, is connecting the two helices. Ts16 toxin thus also belongs to the Csα/β scaffold (Fig. 4, B, D, and F), which shows the prevalence of the three disulfide bridged Csα/β peptides in different scorpion venoms. Atom coordinates and chemical shifts were deposited in the Protein Data Bank (2LKA) and the Biological Magnetic Resonance Bank (17987). Table 2 shows the statistics and RMSD values for Ts16.

### DISCUSSION

**New Structural Group of Scorpion Venom Peptides**—The venom of the Argentinean scorpion T. trivittatus is quite toxic, having caused human fatalities (32). Although the interest on studying this venom started several years ago (33), very little is known about its components structure and function. There are only two publications describing components of this venom, both dealing with peptides that affect K+ ion permeability (19, 20). The former publication describes a new subfamily of α-KTx toxins. The reported peptide was called Tt28, with the systematic name α-KTx20.1. It has 29 amino acid residues with a helical signature of the Csα/β scaffold in solution.

**TABLE 2**

|          | Ts16 | nDSBC |
|----------|------|-------|
| **RMSD (3–30)** |     |       |
| Average backbone (Å) | 0.35 |       |
| Average heavy atom (Å) | 1.00 |       |
| **Upper distance limits** |     |       |
| Total       | 421  |       |
| Short range | 251  |       |
| Medium range| 85   |       |
| Long range  | 85   |       |

The statistics and RMSD values for Ca(3–30) calculated structures for Ts16 using only NOE restraints considering the novel DSB (nDSB): C1–C5, C2–C4, and C3–C6 pairs. Here share large identity with Tt28 (Fig. 5), so they would also be accordingly classified as α-KTx 20 peptides; however, enzy-matic studies, NMR assignments, and molecular calculations with both peptides showed that they do not correspond to the typical Csα/β scaffold of most scorpion toxins known to date. Rather, both peptides adopt a Csα/β scaffold in solution.

All α Variation of Csα/β Scaffold—The Csα/β fold is very uncommon among scorpion venom peptides; all of the described examples are highly similar and have been collectively named κ-KTx (9, 11, 14–17). Disulfide pairing of κ-KTx, C1–C4, and C2–C3 is different from the one assigned to κ-BUTX-Tt2b and Ts16, C1–C5, C2–C4, and C3–C6; thus it is not possible to align the sequences based on Tygat criteria (9), which establish that toxins must be aligned based on their cysteine position. Likewise, structure-based alignments of κ-BUTX-Tt2b and Ts16 with κ-KTx produce rather poor matches, thus indicating that their structure define a novel structural fold of scorpion venom peptides. The founding member of this group, although so far unnoticed (see below), would be Tt28 (19).

Database searches with κ-BUTX-Tt2b and Ts16 sequences retrieved only Tt28 as a significant match. It was nonetheless possible to find other Csα/β peptides, mainly α-KTx, by remote homology searching within three rounds of PSI-BLAST against SwissProt database (34). The six cysteines could be satisfactorily aligned (Fig. 5). Similarly, Ts16 and Tt28 were retrieved within three PSI-BLAST rounds starting with typical α-KTx. Their low sequence similarity with α-KTx prevents an unambiguous homology assignment; thus further evidence, such as conserved gene structure, is needed to resolve whether or not there is an evolutionary link between these Csα/β peptides and the typical α-KTx.

Structural comparison with TopMatch (35) revealed fairly good overlaps for two regions of κ-BUTX-Tt2b and Ts16 with the α-helix and second β-strand of several α-KTx retrieved during PSI-BLAST searches. Fig. 6A shows the structural alignment of κ-BUTX-Tt2b (green) and a chimeric peptide based on two α-KTx subfamily 6 peptides (Protein Data Bank code 1wpd [36] in gray); the structural alignment of main chain atoms over 15 residues has a RMSD of 1.8 Å. Detailed analysis of this structural superposition unveiled a tight overlap, 1.4 Å over the main chain atoms, between the four cysteines defining the sequence signature of the Csα/β scaffold in both structures (Cys7, Cys11, Cys24, and Cys26 in κ-BUTX-Tt2b and Cys9, Cys13, Cys29, and Cys31 for the chimera). Although we favor the noncanonical disulfide pattern for Ts16 on the basis of NOE cross-peaks, structure calculations with rTs16 suggest that it could form the typical Csα/β disulfide pattern, while maintaining the Csα/β scaffold. Moreover, it has been previously shown that another scorpion venom peptide, maurotoxin, could accommodate two different disulfide patterns without major changes in its Csα/β three-dimensional structure (37). It thus seems that the same cysteine pattern could accommodate more than one disulfide connectivity, as previously shown for the cyclic peptide kalata B1 (38).

From the structural comparison it is also evident that the main difference between κ-BUTX-Tt2b and Ts16 with respect to Csα/β peptides is the second α-helix of the newly described...
structures. It occupies the place of the first β-strand of CSα/β peptides; therefore the CSα/α version of the CSα/β scaffold where the second β strand is conserved as an extended conformation of the C-terminal residues. A thought-provoking possibility is the existence of a structural switch between CSα/α and CSα/β conformations. Indeed, two chromatographic peaks were consistently found during the reverse phase chromatography of the recombinant peptides described here; each peak presents the same molecular mass in MALDI-TOF results (data not shown). Such structural transitions have been previously found in a number of proteins that undergo major shifts in secondary structure (39). Further characterization ought to be done to test this hypothesis.

Hint about Convergent Molecular Determinants for K⁺ Channel Blockade—Like many other peptides targeting K⁺ channels, most scorpion α-KTx blocks ion conduction through a common pharmacophore composed by a pore-plugging lysine and an aromatic residue located ~7 Å apart, which has been dubbed as the “functional dyad” (11, 40). Neither the peptides described here nor the homologous Ts28 present the most common variant of the functional dyad among α-KTx (see dots above and below the alignment in Fig. 5). However, structural superposition showed that the functional dyad residues of charybdotoxin (ChTX), Lys27 and Tyr36 (Protein Data Bank code 2crd) (41), overlap with the nonhomologous residues Lys22 and Tyr19 of α-BUTX-Tt2b (Fig. 6B), two residues conserved in Ts16 and Ts28. Thus, although mutagenesis studies would be needed to corroborate this hypothesis, it might be possible that convergent evolution has lead to the emergence of a novel variant of the functional dyad.

Note on Toxin Nomenclature and Classification—Here we followed the proposal of King et al. (18) about naming of novel toxins. Their classification scheme has the advantage of being systematic, although it intentionally let out the information about three-dimensional folding of the toxins. These authors stressed the fact that the knowledge about the three-dimensional scaffolds recruited into venoms is still “rudimentary” (18); the unexpected folding of peptides described here reinforces their cautionary remark. The newly described peptide was named α-BUTX-Tt2b, acknowledging its activity against K⁺ channels (κ), the taxonomic classification of the original source (buthitoxin-Tt), and the fact that it is the second example of the second type of K⁺ channel blockers to be found in this species (2b, the first one would be Ts28).

Alternatively we could have followed the “KTx” classification scheme for scorpion toxins active on K⁺ channels proposed by an international panel of experts (9). This nomenclature has the virtues of recapitulating what is known about the phylogeny of many scorpion toxins and of mirroring to a certain extent their pharmacology (10). With the exception of α-KTx, all other scorpion KTx are supposed to fold according to the CSα/β scaffold, a conjecture largely supported by all of the structures known until now and the strict conservation of the cysteine pattern (11). Following this scheme, the amino acid sequence of the novel peptide described here would lead to classify it as another member of the α-KTx 20 subfamily, along with Ts28 (19) and Ts16. However, it was only until the α-BUTX-Tt2b
structure was obtained that it was possible to realize that a new structural group of scorpion venom peptides has been found, a result corroborated by the solution structure of Ts16.

Assuming that Ts28 also folds into a CSα/α structure, the three similar peptides could be transferred to the only other group of scorpion venom peptides, which adopt the same structural pattern, the κ-KTx subfamily. However, we hesitate to do so because the helix-loop-helix topology of κ-BUTX-Tt2b and Ts16 appear to be an elaboration of the most common CSα/β scaffold, rather than a variation of the κ-KTx α-hairpin. Such structural elaboration based on different cysteine patterns has been previously proposed to explain the emergence of the inhibitory cystine knot motif over an “ancestral” disulfide directed β-hairpin (42).

Concluding Remarks—The three-dimensional structures reported here highlight the structural versatility that could be attained by peptides sharing a common cysteine pattern, thus revealing a new trick of this old pattern. Further research would show whether or not they are unique examples of their kind, but providing that animal venoms are arsenal-like mixtures of bioactive compounds, among which a small number of disulfide rich scaffolds are highly prevalent (11, 43), we expect that other CSα/α peptides will be found in the future, thus helping to clarify their biological function in the venom and their relationships with other venom components.

Acknowledgments—We acknowledge the technical assistance given to the project by Dr. Fernando Zamudio Zuñiga, Carmen Marquez, and Eréndira García. We are grateful to Professors E. Wanke (Università di Milano-Bicocca) and L. Toro (David Geffen School of Medicine at UCLA) for Kv1.2 and Shaker-IR clones, respectively.

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