Eur. J. Biochem. **268,** 5407–5413 (2001) © FEBS 2001

Isolation and characterization of a novel type of neurotoxic peptide from the venom of the South African scorpion *Parabuthus transvaalicus* (Buthidae)

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The venom of the South African scorpion *Parabuthus transvaalicus* was characterized using a combination of mass spectrometry and RP-HPLC separation and bioassays. The crude venom was initially separated into 10 fractions. A novel, moderately toxic but very high abundance peptide (birtoxin) of 58 amino-acid residues was isolated, identified and characterized. Each purification step was followed by bioassays and mass spectroscopy. First a C_4 RP-HPLC column was used, then a C_{18} RP Microbore column purification resulted in $> 95\%$ purity in the case of birtoxin from a starting material of 230 μg of crude venom. About 12–14% of the D_{214} absorbance of the total venom as observed after the first chromatography step was composed of birtoxin. This peptide was lethal to mice at low microgram quantities and it induced serious symptoms including tremors, which lasted up to 24 h post injection, at submicrogram amounts. At least seven other fractions that showed different activities including one fraction with specificity against blowfly larvae were identified. Identification of potent components is an important step in designing and obtaining effective antivenom. Antibodies raised against the critical toxic components have the potential to block the toxic effects and reduce the pain associated with the scorpion envenomation. The discovery of birtoxin, a bioactive long chain neurotoxin peptide with only three disulfide bridges, offers new insight into understanding the role of conserved disulfide bridges with respect to scorpion toxin structure and function.

Keywords: *Parabuthus transvaalicus*; scorpion; toxin; disulfide; birtoxin.

Scorpion venoms are a rich source of neurotoxic peptides with diverse modes of action. Within the complex mixture of those venoms studied, peptides have been found to possess the majority of the biological effect towards the target organisms. Numerous peptides toxic against mammals and insects have been identified. The potent peptides are usually low in abundance [1]. *P. transvaalicus* is a large (up to 150 mm), South African scorpion species from the family Buthidae and considered to be medically important [2]. The victims of envenomation by *P. transvaalicus* suffer from neurotoxic effects and prolonged pain lasting from 1 day in minor cases up to 1 week in severe cases. Symptoms include abnormal reflexes, bladder symptoms, dysphagia, sweating and hypersalivation [2].

Venom of *P. transvaalicus* can be considered a more 'simple' venom compared to other scorpion venoms as it contains less than 100 major peptides [3]. The venom can be characterized by defining the individual components of the system (identification of peptide toxins), analysis of the structure of the components (primary, secondary and tertiary structure determination), analysis of the function of each component (determination of the mode of action), analysis of the relationships between these components (synergism) and the target sites or the environment (binding sites and kinetics). The knowledge of a phenomenon depends on the way in which it is observed. Here we have limited our variables to neurotoxic peptides possessing activity against mice or insects.

Recently inhibition of T-type voltage-gated calcium channels by a novel toxin, kurtoxin, from the venom of *P. transvaalicus* has been reported [4]. Furthermore the venoms of three *Parabuthus* species have been compared and partially characterized previously [5], primarily by gel filtration chromatography. In this study we are reporting a more detailed characterization of the venom of *P. transvaalicus*, including the determination of seven different toxic fractions with molecular masses determined by a combination of MALDI and ESI-TOF techniques. The low potency of venom against insects and high potency against mammals was an unexpected result that led to the identification, isolation and characterization of a novel, moderately toxic but very high abundance peptide, birtoxin.

Birtoxin is 58 residues long, close to the 60- to 70-residue range of the 'long chain neurotoxin' peptide family [3,6–8]. However, this family of peptides are known to contain eight cysteine residues. The second family of scorpion toxins is the 'short chain neurotoxins' which are 30- to 40-residue peptides with six or eight cysteine residues. Birtoxin, having six cysteines bridges the gap between these two distinct groups, perhaps as an evolutionary link. We have also examined the 3D NMR structure of closely related toxin peptides from *C. exilicauda*, *C. sculpturatus* and *C. noxius* [9–11] and overlaid our primary sequence onto these structures. The results indicate a novel structural alternative

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(Received 11 May 2001, revised 23 August 2001, accepted 23 August 2001)

to the fourth disulfide bridge that is currently unique for birtoxin.

MATERIALS AND METHODS

Sample preparation

Scorpion venom was obtained from captive scorpions by passing a small electric shock through the telson to contract the muscles. The venom was collected in a cryo tube as a pooled sample from three milkings at the South African Vaccine Producers Ltd facilities, dried and sent to the University of California Davis (UC Davis). Dried venom was resuspended in sterile water at 10 mg·mL^{-1} by vigorous vortexing and filtered through a 0.45-μm filter.

HPLC purification

Filtered venom sample was injected into a Vydac (Hesperia, CA, USA) RP C_4 Analytical HPLC column (4.6 mm internal diameter × 25 mm length) connected to a Hewlett-Packard HP1100 system coupled to a diode array detector and a computer running the Chemstation® software. A gradient was formed with the following conditions; 5–65% solvent A in 60 min, 65% solvent A for another 20 min for a total of 80 min at a flow rate of 600 μL per minute (solvent A, 95% acetonitrile, 5% water, 0.1% trifluoroacetic acid; solvent B, 95% water, 5% acetonitrile, 0.1% trifluoroacetic acid). Elution was monitored by following the UV trace at 214 and 280 nm. Fractions were collected manually into tubes pretreated with BSA (1 mg·mL^{-1}) and washed with 1 : 1 (v/v) acetonitrile/water, 1 : 1 (v/v) methanol/water and water, respectively. Biologically active fractions were further separated using a Michrome Magic 2002 Microbore HPLC system equipped with a RP C_{18} column and an online 5 μ peptide trap (Michrome BioResources, Inc. Auburn, CA, USA) with a linear gradient from 5 to 70% solvent A in 23 min at a flow rate of 50 μL·min^{-1}. Fractions were collected manually into BSA pretreated tubes by following the UV trace.

Bioassays

Male Swiss-Webster mice were purchased from Charles River Inc., and housed at the Animal Housing Facility, UC Davis. Blowfly (*Sarcophaga* spp.), crickets (*Acheta domesticus*) and anole lizards (*Anoles caroliensis*) were purchased from Carolina Biologicals (Burlington, NC, USA), cotton bollworms (*Heliothis virescens*) were obtained from USDA/ARS (Stoneville, MI, USA) and reared on artificial diet. Collected fractions were tested on mice by intracerebroventricular injections and by injecting last instar blowfly larvae. Third instar cotton bollworms and adult common house crickets were also used in screening for insecticidal components. Briefly fractions were concentrated to dryness using a Heto Speed Vac (ATR, Inc. Emeryville, CA, USA). Dried samples were resuspended in 10 μL of 20 mM ammonium acetate buffer with 1 μg·μL^{-1} BSA and incubated overnight at 4 °C to reduce variability in toxicity before injection to the test animals. Mice were anesthetized using ethyl ether and intracerebroventricular injections of peptide solutions were executed immediately. Control animals injected with BSA in buffer did not show any symptoms when recovering from anesthesia. All symptoms were observed and recorded up to 24 h post injection. All experiments were carried out in accordance with the guidelines laid down by the National Institutes of Health in the USA regarding the care and use of experimental animals.

Mass spectroscopy

Mass spectra of crude venom, separated fractions and isolated peptide were analyzed off-line in a Biflex III (Bruker Daltonics, Bremen, Germany) MALDI-TOF instrument in positive ion mode. The instrument was equipped with a nitrogen laser operating at an output of 337.1 nm with a pulse width of 3 ns, and a repetition rate of 6 Hz. Experiments were conducted in reflector mode with an acceleration potential of 19.3 kV, a reflector potential of 20.0 kV, a time base of 2–4 ns, and a delay of 10–30 ms operated at $R < 3000$ (full-width half maximum definition). The output signal of the detector was digitized at a sampling rate of 500 MHz per channel using a 1 GHz Lecroy digitizer. A camera mounted on a microscope facilitated inspecting the sample crystallization and selecting the largest crystals for analysis. External calibration was performed using angiotensin II (1046.53 Da, monoisotopic), somatostatin 28 (3147.47 Da, monoisotopic), and human recombinant insulin (5808.6 Da, average) from Sigma. For analysis, matrix solutions consisting of sinapinic acid, 3,5-dimethoxy-4-hydroxycinnaminic acid, or α-cyano-4-hydroxycinnamic acid, were mixed in a 1 : 1 (v/v) ratio with samples, spotted on the target and allowed to dry. MASSLYNX (Micromass UK Limited, Manchester, UK) software was used for data processing and analysis.

Edman sequencing

Purity following HPLC was evaluated by MALDI-TOF. For amino-acid sequence determination, the cysteine residues of the peptide were reduced and carboxymethylated by incubating in 6 M guanidine hydrochloride, 0.1 M Tris/HCl (pH 8.3), 1 mM EDTA and 20 mM dithiothreitol for 1 h at 37 °C. Iodoacetic acid was then added to a final concentration of 50 mM and incubated for an additional 1 hour at 37 °C in the dark. Finally 300 pm of peptide was subjected to automated Edman sequencing for 60 cycles using a Hewlett-Packard HP GS1000 Sequence Analyzer at the Molecular Structure Facility at UC Davis.

Peptide quantification and amino-acid analysis

Amino-acid analysis was conducted by the Molecular Structure Facility at UC Davis with standard methods using a Beckman 6300 Na citrate-based amino acid analyzer. Peptide quantification was accomplished after determination of the molecular mass, primary sequence and amino-acid analysis. The extinction coefficient for birtoxin was calculated according to Gill *et al.* [12] using the following formula;

$$\Sigma_{280} = 5690 \cdot n_{trp} + 1280 \cdot n_{tyr} + 120 \cdot n_{ss} \quad (1)$$

where n_{trp}, n_{tyr} and n_{ss} represent the number of tryptophan residues, tyrosine residues and disulfide bonds, respectively. The extinction coefficient for birtoxin was calculated as

13730 $M^{-1}\cdot cm^{-1}$. Σ_{280} for birtoxin was confirmed experimentally by utilizing the A_{280} and concentration of birtoxin determined by amino-acid analysis according to Beer–Lambert law. Absorbance at 280 nm was determined using a Biorad SmartSpec 3000 Spectrophotometer using a quartz cuvette.

Structural analysis

NMR structures for peptide toxins from *C. exilicauda*, *C. sculpturatus* and *C. noxius* were downloaded from the Expasy server (http://www.expasy.ch) in pdb format. The amino-acid sequence of birtoxin was aligned and the backbone atoms were overlaid on the NMR resolved 3D structures using the magic fit and improve fit functions of the Swiss PDB VIEWER sequentially. Peptide tools from the Expasy web site was used for mass calculation of sequenced peptide. Sequence homologies were determined using the CLUSTALW program at the EMBL server (http://www2.ebi.ac.uk/clustalw). Sequence alignments were visualized using the ESPRIPT tool at the Expasy server.

RESULTS

Characterization of venom

We initially determined the mass profile of the crude venom using MALDI-TOF. Mass spectroscopy of the crude venom resulted in detection of 72 components within the mass range of 750–7500 Da (Table 1; J. Lango, B. Inceoglu and B. D. Hammock, unpublished results). Optimization of the HPLC gradient conditions resulted in consistent separation of the venom into 10 fractions (Fig. 1). Soluble crude venom and each of the fractions were tested for activity against mice, three insect species, and anole lizards (Table 1). As shown in Table 1 fractions P0, P1, PreP3, P3, P4, P5 and P6 had activity against mice and fractions P2 and P3 had activity against blowfly larvae. Fraction P2 was specific for blowfly only. Interestingly, the crude venom had limited toxicity against insects. The material between the above peaks was collected and assayed, however, it showed little biological activity. The LD_{99} for common house cricket and bollworm were well above 48 μg per insect for the crude venom. In constrast, the venom was quite potent against mice, with an LD_{99} of 4.8 μg crude venom per mouse of 20 g with intracerebroventricular injection. Venom was at least five times less toxic by subcutenaous injection and 50 times less toxic when injected intraperitoneally. The crude venom did not show noticeable activity towards adult anole lizards by subcutenaous injection at even high doses (100 μg venom per lizard of 10 g) except for slowing their motion temporarily for about 3 min.

Purification and characterization of birtoxin

Fraction P4 (Fig. 1) from the C_4 column gave severe symptoms when injected to mice. The injected mice were first stunned then started to tremble, the tremors severity increased with time and lasted up to 24 h. Also the paws were contracted and the body took the shape of hunchback with frequent whole body jerks. The symptoms did not initiate immediately after the injection was completed but increased in intensity for 30 min after a 10-min lag. Control animals injected with BSA in buffer rapidly recovered within three minutes. From the UV absorbance of the C_4 column profile, birtoxin was estimated to constitute 12–14% of the crude venom. We further purified the fraction P4 from the first column by injecting into a C_{18} Microbore column. Toxicity was only seen in the UV dense fraction collected from the microbore run. This second step resulted in more than 97% purity as determined by MALDI-TOF (Fig. 2). This fraction was also injected to mice and biological activity was confirmed. Injection of 1 μg of pure birtoxin induced severe neurotoxic symptoms in mice up to 24 h but was not lethal. However 2 μg of pure peptide was lethal. The peptide was then reduced, carboxymethylated and the amino-acid sequence was determined as described above.

Table 1. Biological activity of the C4 fractions of the venom of *Parabuthus transvaalicus* and the major molecular masses detected from each fraction. HPLC fractions from the C4 column were tested for bioactivity in mice and insects. Relative toxicity is denoted as: –, nontoxic; +, toxic; ++, moderately toxic; and ++++, highly toxic. Molecular masses from each fraction which are above 2000 Da are also given. The birtoxin mass is in bold. IC, intracerebroventricular.

Fraction	Mouse (IC injection)	Larvae		Molecular ion masses detected $(M + H)^+$
		Blow fly	*H. virescens*	
P0	+	–	–	None detected above 2000 Da
P1	+	–	–	None detected above 2000 Da
P2	–	+++	–	3492, 3640, 3769, 3912, 4092[a], 4297, 4512,
PreP3	++	–	–	Not determined
P3	+++	+++	–	5757, 6544[a], 6615[a], 6635, 7221, 7215, 7261
P4	++	–	–	2437, 5258, 5441, 5648, 6057, 6356, 6526, **6543**[a], 6574
P5	+++	–	–	5543, 5752, 6160, 6647[a],6854, 7225, 7303
P6	++	–	–	6603[a]
P7	+++	–	–	None detected above 2000 Da
P8	+	–	–	Not determined
Venom	LD_{99} is 4.8 μg crude venom/20 g mouse	LD_{99} for blowfly is 1.2 μg venom/100 mg larvae		

[a] Most abundant species.

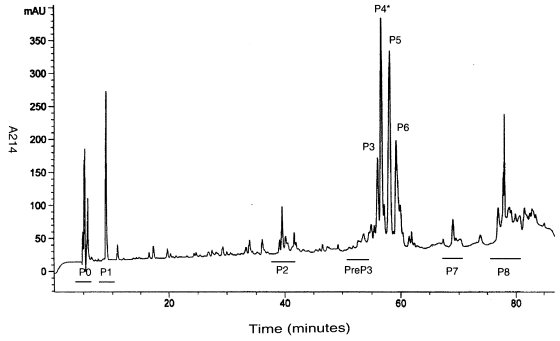

Fig. 1. Separation of crude venom by RP-HPLC using a C_4 column. The separation was conducted with a shallow gradient as explained in Materials and methods section. Individual peaks are collected, dried and used for mass determination and bioassays. In the case of P3, P4, P5 and P6 the apparent peaks were collected separately. In other fractions bars show the region of the chromatogram that were combined for assay. Birtoxin was predominantly in fraction P4.

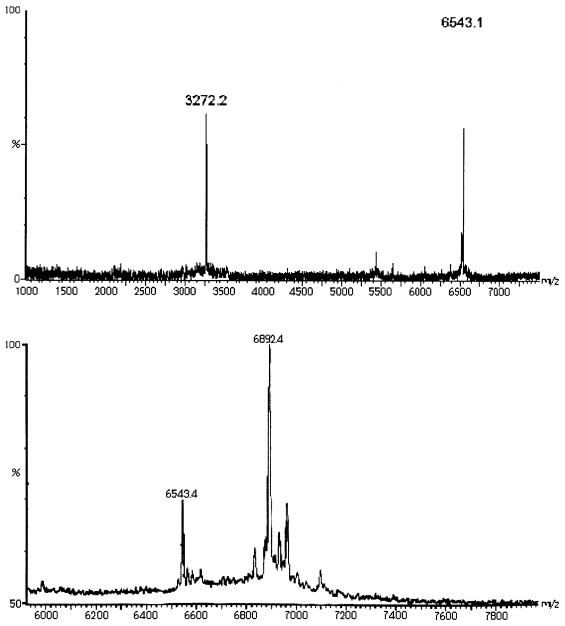

Fig. 2. Mass spectra of native and reduced and carboxymethylated birtoxin. Molecular masses were determined by MALDI-TOF. Native birtoxin (A) and modified birtoxin (B) were both purified using a C_{18} column on the Microbore HPLC. After reduction and carboxymethylation modified birtoxin was re-purified using the same column. Native birtoxin was used as an internal standart for the mass determination of the modified birtoxin. The observed mass (6892.4) for the modified birtoxin was in agreement with the expected mass of six cysteine residues being carboxymethylated.

The average molecular mass $(M + H)^+$ for native birtoxin is determined to be 6543.6 Da using MALDI-TOF. Birtoxin has 58 amino-acid residues. Amino-acid sequencing resulted in 57 amino acids giving a calculated average mass of 6455.2 Da. Serine 57 was not detected by Edman degradation due to the low conversion efficiency and the low amount of peptide left on the column, but valine 58 was detected clearly. Amino-acid analysis revealed the presence of an extra serine, which was assigned to position 57. The calculated and expected mass were in agreement after assigning the 57th amino acid as a serine residue. The presence of six cysteine residues was confirmed by measuring the molecular mass of reduced and carboxymethylated birtoxin (Fig. 2B). Homology searching revealed over 40 matches of scorpion toxins with identity ranging from 22% to 57%. The best of these matches were selected for multiple sequence alignment (Fig. 3). Birtoxin shows significant identity to the previously discovered sodium channel blocker toxins from the scorpions *C. exilicauda*, *Centruroides sulcatus, C. sculpturatus* and *C. noxius*.

Structural analysis

The avilability of NMR structures for similar peptides has prompted us to examine these structures and compare birtoxin to these structures [9–11]. The backbone overlay of birtoxin on CeNV1 structure (for definition of toxin abbreviations, see Fig. 3) (Fig. 4A) resulted in a good agreement between the two backbones (calculated rmsd of 0.01 Å between the two aligned backbones). Thus, we were able to visualize the positions of the four disulfides in the CeNV1 toxin with respect to the three disulfides in birtoxin. The aligned structure revealed that both toxins have a conserved core with three disulfide bridges (Fig. 4B). In the CeNV1, cysteines 16–41, 25–46 and 29–48 are disulfide bridged and form the core disulfides whereas in birtoxin all disulfide bridges are at the core. CeNV1 has a fourth disulfide bridge between Cys12 and Cys65 (Fig. 4A). This fourth disulfide is, like the other three, very well conserved among the long chain neurotoxin peptide toxin family (Fig. 3). Birtoxin does not possess an equivalent for Cys12 and does not possess the 9–13 residues at the C-terminus of CeNV1, CsN-3 and CnN2. The NMR structure reveals that the fourth disulfide wraps the N- and C-termini from the outside of the molecule (Fig. 4). Thus we named this the 'wrapper' disulfide. The absence of two cysteine residues in birtoxin corresponding to Cys12 and Cys65 in CeNV1 and other long chain neurotoxins indicates the absence of the fourth disulfide. Therefore we concluded that birtoxin has a novel structural organization and an alternative system for keeping the polypeptide chain in a particular three dimensional confirmation that retains biological activity without the fourth disulfide bridge.

DISCUSSION

Isolation of peptide toxins from scorpions have two major applications. As summarized in the introduction identified potent peptides can be employed in the design and production of superior anti-venom. Also peptide toxins from scorpions are probes for identifying distinct types of ion channels and important tools for understanding their physiology [13,14]. One interesting feature of the venom of *P. transvaalicus* is its specialization towards mammalian activity, only one out of 10 initial fractions showed insect specificity. This might well be because of the larger size of this scorpion, which makes it a more visible prey to mammalian predators such as bats. Moreover the lack of strong insecticidal components can also be explained by the larger size of this scorpion because it does not require a toxin to subdue an insect prey. A further interesting finding from our bioassays on anole lizards indicate that the venom

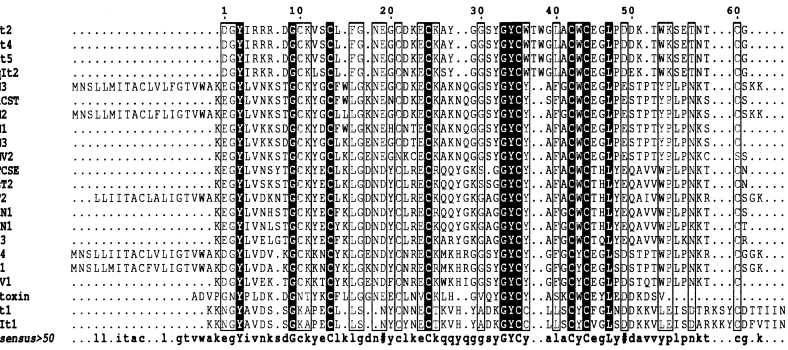

Fig. 3. Multiple alignment of known scorpion toxins with birtoxin. Primary sequence of birtoxin was aligned with known scorpion toxins using CLUSTALW program and visualized using the ESPRIPT tool. Toxin abbreviations used are as follows: BaIT2, *Buthacus arenicola* insect toxin 2; BoIT4/5, *Buthus occitanus* insect toxin 4/5; LqqIT2, *Leiurus quinquestiatus quinquestriatus* insect toxin 2; CnN2/3, *Centruroides noxius* neurotoxin 2/3 precursor; CllCST, *Centruroides limpidus* crustacean specific toxin; CsN1/3, *Centruroides sculpturatus* neurotoxin 1/3; CsNV2, *Centruroides sculpturatus* toxin variant 2; CsTCSE, *Centruroides noxius* toxin CSE M1; CssT2, *Centruroides suffusus suffusus* toxin 2, CnT1/2/4, *Centruroides noxius* toxin 1/2/4 precursor CltN1, *Centruroides limpidus tecomanus* neurotoxin 1; CllN1, *Centruroides limpidus limpidus* neurotoxin 1; CnT3, *Centruroides noxius* toxin 3; CsNV1, *Centruroides sculpturatus* neurotoxin variant 1; AaIT, *Androctonus australis* insect toxin 1; LqqIT1, *Leiurus quinquestriatus quinquestriatus* insect toxin 1.

of *P. transvaalicus* does not possess components that would target reptilian ion channels. Ion channels from reptiles have not been fully characterized, and our finding suggests that they may have different pharmacological properties compared to either mammalian or insect ion channels. The venom of *P. transvaalicus* proved to have less than 100 major peptides. Furthermore we were able to detect at least seven different toxic activities within the crude venom. In-depth characterization of each of the activities present in the venom may reveal a structurally unique family of long chain neurotoxin peptides with three disulfide bridges.

The primary sequence of many peptide toxins from numerous scorpions are known but few of the structures have been resolved and we know even less of how the particular structure affects the activity [9–11]. Mutating the suspected amino-acid residues in the toxin sequence has been the method of choice over chemical modification of functional groups of amino acids for understanding the structure–activity relationships [15–18]. However, identification of naturally occurring variants or peptides belonging to the same family with some differences in amino-acid sequence offers an alternative to mutagenesis for investigating function [19]. In this study for the first time, a biologically active long chain neurotoxin-like peptide possessing only six cysteine residues was isolated and identified. Primary structure and secondary structure prediction imply a similar general structure to known scorpion peptide toxins with the cysteine-stabilized α-helical motif, which involves a Cys–X–X–X–Cys stretch of the α helix bonded through two disulfide bridges to a Cys–X–Cys triplet in a β strand belonging to an antiparallel β sheet [20] despite the absence of the fourth disulfide bridge. As confirmed by X-ray crystal structures and NMR studies, all known toxins in the long chain neurotoxin family have four disulfide bridges. Three of these constitute the core of the structure, two disulfides bonding the α helix to the antiparallel β sheet and a third disulfide links the β sheet to an extended segment preceding the helix [21,22]. Typically the fourth disulfide cross links the first and the last cysteine residues. Due to its position we have named this disulfide bridge the 'wrapper' disulfide bridge. As virtually all known peptide toxins in this family posses the wrapper disulfide holding the molecule together, one can hypothesize that the wrapper disulfide must be important for the peptide functionality. Interestingly, the absence of the fourth disulfide in birtoxin may not contradict this argument because as seen in Fig. 4 the N- and C-termini of birtoxin might be in close proximity, which would allow multiple hydrogen bonding between the N- and the C-terminal residues. We speculate that the interaction between the N- and the C-termini domains might in turn be adequate for holding the confirmation of birtoxin in a manner similar to other peptide toxins with four disulfide bridges. As shown in Fig. 1, birtoxin is a very abundant peptide, possibly the major peptide species in the venom. One hypothesis is that the absence of the fourth disulfide bridge reduces the complexity of folding into the active conformation. Therefore birtoxin can be expressed in higher quantities relative to four disulfide bridged peptide toxins. This hypothesis is yet to be tested experimentally. The biological activity of most neurotoxic peptides have been attributed to the C-terminal domain [23]. The primary structure of birtoxin reveals that this toxin is slightly shorter than other long chain neurotoxins (Fig. 4) not possessing the amino-acid residues that are suggested to be involved either in spatial arrangement of the molecule or in the binding of the molecule to the target ionic channels. Another interesting feature at the N-terminus of birtoxin is the presence of two proline residues separated by three amino acids, which might cause the N-termini to have a double turn (Fig. 4). This possible double turn motif to our knowledge has not been observed at the N-termini of scorpion toxins previously. The experimental determination of the three dimensional structure of birtoxin may clarify how this toxin retains its 3D conformation and will improve our understanding of the structure and function of peptide toxins. Finally, it is interesting to note that birtoxin shows the closest resemblance to neurotoxins from the New World species. The genus *Parabuthus* is distributed only in Africa. The similarity between birtoxin and toxins from American scorpions contributes to the hypothesis that scorpion toxins originate from a common ancestor [24]. The most similar toxin to birtoxin is Cse-VIII, to which it is 54% identical.

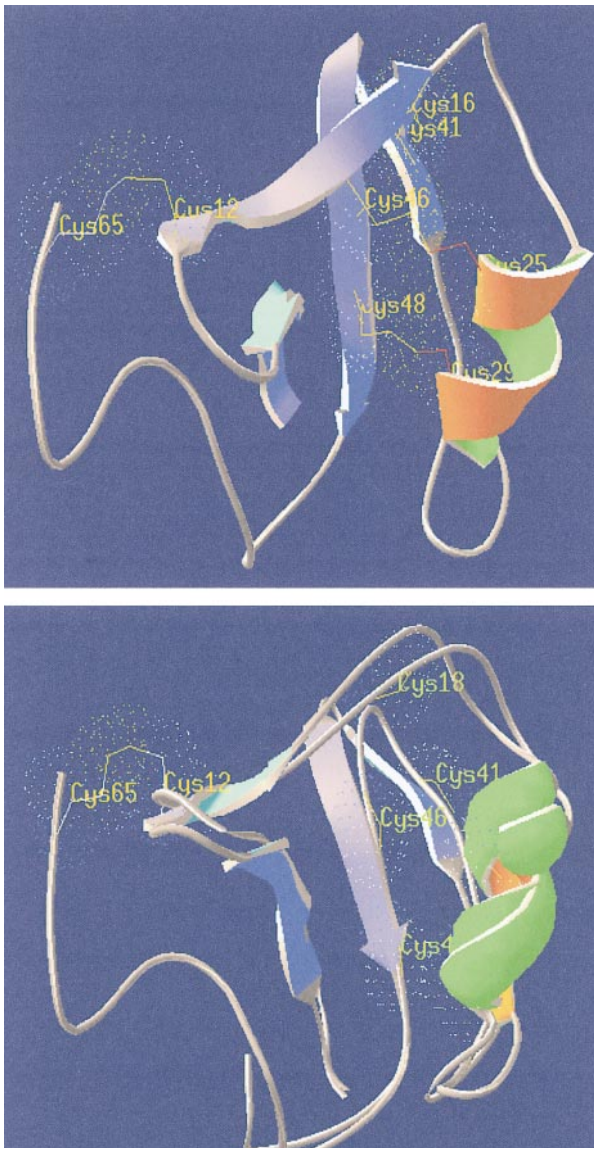

Fig. 4. Molecular modeling of birtoxin. The pdb file for CeNV1 was downloaded from the structural database and visualized using the SwissPdb VIEWER. In the upper panel, the NMR resolved structure of CeNV1 is seen. The disulfide bridge between residues cys12 and cys65 is the 'wrapper' disulfide. In the lower panel the primary sequence of birtoxin was overlaid an fitted on to the NMR structure. The disulfide bridge between Cys12–Cys65 in CeNV1 is absent in birtoxin.

This level of divergence also implies that birtoxin belongs to a new family of long chain scorpion toxins with three disulfide bridges.

ACKNOWLEDGEMENTS

On March 27, 2000 Takuya Abe and Shigeru Nakano of Kyoto University and Gary Polis of University of California at Davis died in the Sea of Cortez. This paper is dedicated to these excellent ecologists and scorpion biologists. A. B. Inceoglu is supported by a scholarship from Ankara University, Ankara, Turkey. We thank Dr S. G. Kamita and all Hammock Laboratory members for their support and discussions. We also extend special thanks to Dr Isaac Pessah for the critical review and support during the preparation of this manuscript. This work was supported by USDA Grant 97-35302-09919 and NIEHS Grant RO1ES02710. Davis is a NIEHS Health Science Research Center P30 ES0-5707. Mass spectra were run by the Superfund Analytical Laboratory supported by the NIEHS Superfund Basic Research Program P42ES04699.

REFERENCES

1. Nakagawa, Y., Lee, Y.M., Lehmberg, E., Herrmann, R., Herrmann, R., Moskowitz, H., Jones, A.D. & Hammock, B.D. (1997) Anti-insect toxin 5 (AaIT5) from *Androctonus australis*. *Eur. J. Biochem.* **246**, 496–501.
2. Bergman, N.J. (1997) Clinical description of *Parabuthus transvaalicus* scorpionism in Zimbabwe. *Toxicon* **35**, 759–771.
3. Possani, L.D., Becerrill, B., Delepierre, M. & Tytgat, J. (1999) Scorpion toxins specific for Na^+-channels. *Eur. J. Biochem.* **264**, 287–300.
4. Chuang, R.S.I., Jaffe, H., Cribbs, L., Perez-Reyes, E.J. & Swartz, K.J. (1998) Inhibition of T-type voltage-gated calcium channels by a new scorpion toxin. *Nat. Neurosci.* **1**, 668–674.
5. Debont, T., Swerts, A., Van der Walt, J.J., Müller, G.J., Verdonck, F., Daenens, P. & Tytgat, J. (1998) Comparison and characterization of the venoms of three *Parabuthus* scorpion species occurring in Southern Africa. *Toxicon* **36**, 341–352.
6. Lebreton, F., Delepierre, M., Ramirez, A.N., Balderas, C. & Possani, L.D. (1994) Primary and NMR three-dimensional structure determination of a novel crustacean toxin from the venom of the scorpion *Centruroides limpidus limpidus* Karsch. *Biochemistry* **33**, 11135–11149.
7. Syed, A.A., Stoeva, S., Schütz, J., Kayed, R., Abassi, A., Zaidi, Z.H. & Voelter, W. (1998) Purification and primary structure of low molecular mass peptides from scorpion (*Buthus sindicus*) venom. *Comp Biochem. Physiol.* **121**, 323–332.
8. Gordon, D., Savarin, P., Gurevitz, M. & Zinn-Justin, S. (1998) Functional anatomy of scorpion toxins affecting sodium channels. *J. Toxicol. Tox Rev.* **17**, 131–159.
9. Lee, W., Jablonsky, M.J., Watt, D.D. & Krishna, N.R. (1994) Proton nuclear magnetic resonance and distance geometry/simulated annealing studies on the variant-1 neurotoxin from the New World scorpion *Centruroides sculpturatus* Ewing. *Biochemistry* **33**, 2468–2475.
10. Jablonsky, M.J., Watt, D.D. & Krishna, N.R. (1995) Solution structure of an Old World-like neurotoxin from the venom of the New World scorpion *Centruroides sculpturatus* Ewing. *J. Mol Biol.* **248**, 449–458.
11. Pintar, A., Possani, L.D. & Delepierre, M. (1999) Solution structure of toxin 2 from *Centruroides noxius* Hoffmann, a beta scorpion neurotoxin acting on sodium channels. *J. Mol Biol.* **287**, 359–367.
12. Gill, S.C. & Von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem.* **182**, 319–326.
13. Becerril, B., Marangoni, S. & Possani, L.D. (1997) Toxins and genes isolated from scorpions of the genus *Tityus*. *Toxicon* **35**, 821–835.
14. Froy, O., Zilberberg, N., Chejanovsky, N., Anglister, J., Loret, E., Shaanan, B., Gordon, D. & Gurevitz, M. (2000) Scorpion neurotoxins: structure/function relationships and application in agriculture. *Pest Manag. Sci.* **56**, 472–474.
15. Rochat, H., Bernard, P. & Couraud, F. (1979) Scorpion toxins: Chemistry and mode of action. In *Advances in Cytopharmacology* (Ceccarelli, B. & Clementi, F., eds), pp. 325–334. Raven Press, New York.
16. Zilberberg, N., Froy, O., Loret, E., Cestele, S., Arad, D., Gordon, D. & Gurevitz, M. (1997) Identification of structural elements of a scorpion alpha neurotoxin important for receptor site recognition. *J. Biol. Chem* **272**, 14810–14816.
17. Froy, O., Zilberberg, N., Gordon, D., Turkov, M., Gilles, N., Stankiewicz, M., Pelhate, M., Loret, E., Oren, D.A., Shaanan, B. &

Gurevitz, M. (1999) The putative bioactive surface of insect-selective scorpion excitatory neurotoxins. *J. Biol. Chem* **274**, 5769–5776.

18. Martinez, F., Munoz-Garay, C., Gurrola, G., Darszon, A., Possani, L.D. & Becerril, B. (1998) Site directed mutants of noxiustoxin reveal specific interactions with potassium channels. *FEBS Lett.* **429**, 381–384.
19. Wang, X., Smith, R., Fletcher, J.I., Wilson, H., Wood, C.J., Howden, M.E.H. & King, G.F. (1999) Structure–function studies of ω-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. *Eur. J. Biochem.* **264**, 488–494.
20. Kobayashi, Y., Takashima, H., Tamaoki, H., Kyogoku, Y., Lambert, P., Kuroda, H., Chino, N., Watanabe, T.X., Kimura, T., Sakakibara, S. & Moroder, L. (1991) The cysteine-stabilized alpha-helix: a common structural motif of ion-channel blocking neurotoxic peptides. *Biopolymers* **31**, 1213–1220.
21. Drakopoulou, E., Vizzavona, J., Neyton, J., Aniort, V., Bouet, F., Virelizier, H., Ménez, A. & Vita, C. (1998) Consequence of the removal of evolutionary conserved disulfide bridges on the structure and function of charybdotoxin and evidence that particular cysteine spacings govern specific disulfide bond formation. *Biochemistry* **37**, 1292–1301.
22. Carlier, E., Avdonin, V., Geib, S., Fajloun, Z., Kharrat, R., Rochat, H., Sabatier, J.M., Hoshi, T. & De Waard, M. (2000) Effect of maurotoxin, a four disulfide-bridged toxin from the chactoid scorpion *Scorpio maurus*, on Shaker K^+ channels. *J. Peptide Res.* **55**, 419–427.
23. Martin, M.F., Vargas, O. & Rochat, H. (1989) Importance of the C-terminal amino acid residues on scorpion toxins activity. In *Second Forum on Peptides* (Aubry, A.,Marraud, M. &Vitoux, B., eds), Vol. 174, pp. 483–486. Colloque INSERM/John Libbey Eurotext Ltd, London, UK.
24. Froy, O., Sagiv, T., Poreh, M., Urbach, D., Zilberberg, N. & Gurevitz, M. (1999) Dynamic diversification from a putative common ancestor of scorpion toxins affecting sodium, potassium, and chloride channels. *J. Mol Evol* **48**, 187–196.