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A depressant insect-selective toxin analog from the venom of the scorpion *Leiurus quinquestriatus hebraeus*

Purification and structure/function characterization

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The scorpion venom-derived excitatory and depressant insect-selective polypeptide neurotoxins modify sodium conductance in insect neuronal membranes and differ greatly in their primary structures and symptoms induced in blow fly larvae. We report here the purification and characterization of a new insect selective toxin, $LqhIT_5$. $LqhIT_5$ is more similar to the excitatory toxins in its mode of action and the depressant toxins in its primary structure. This toxin is a single polypeptide composed of 61 amino acids that are cross linked by four disulfide bonds. When $LqhIT_5$ is injected into blow fly larvae, a fast contraction paralysis occurs without depressant activity. No mammalian toxicity was detected by subcutaneous or intracranial injections of this toxin into mice. Sequence comparison of $LqhIT_5$ and known depressant toxins shows a high degree of similarity among the amino acids located on the C-terminus of the toxins. However, there are some clear differences in the amino acids located close to the N-terminus of the toxins. By the aid of homology modeling, we demonstrated that these amino acids have the same orientation in the tertiary structure of the molecule and are exposed to the environment. The change in the mode of action of $LqhIT_5$ (no depressant activity) by substitutions of a few amino acids located on a specific exposed area of the toxin shed a new light on the structure/function relationship of scorpion toxins. These results caution that similarity in the mechanism of action of scorpion toxins does not always follow from an overall similarity in sequence.

Keywords: scorpion toxins; depressant insect toxin; mode of action.

Scorpion venoms contain a number of polypeptide toxins that specifically block or alter gating properties of ion channels. Among those toxins are the small polypeptides (30–40 amino acids) with three or four disulfide bridges which mainly affect K^+ channels [1, 2] and the large polypeptides (60–70 amino acids) with four disulfide bridges that mainly affect sodium channels [3–5]. The latter group of toxins can be subdivided into mammalian toxins [4, 6] which are responsible for human envenomation, and the insect-selective toxins which are shown to modify the sodium conductance exclusively in insect neuronal membranes [7, 8]. The insect-selective toxins are subdivided into two categories which can easily be distinguished according to their effect on blow fly larvae and their amino acid sequences (Table 1). One category of the insect-selective toxins include the depressant toxins which, so far, are composed of 61 amino acids and induce a slow progressive onset of flaccid paralysis preceded by a short transient phase of contractility [5]. The other category of the insect-selective toxins is the excitatory toxins which, so far, are composed of 70 amino acids and induce an immediate reversible fast contraction paralysis upon injection [3]. There is a high sequence similarity among the toxins in each category, however, the similarity between categories is much lower [5]. Recently, new depressant toxin analogs (analogous in sequence but not in activity) named $BotIT_2$ and $AaIT_5$ were purified from the venoms of the scorpions *Buthus occitanus tunetanus* and *Androctonus australis* Hector, respectively. $BotIT_2$ was shown to have high sequence similarity to the depressant insect toxins. However, an excitatory activity in insects and some mammalian toxicity were also reported [9]. $AaIT_5$ also showed high sequence similarity to the depressant toxins but did not show mammalian toxicity upon injection into mice. No depressant or excitatory activity was reported for $AaIT_5$ when injected

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Abbreviations. AaIT, excitatory insect toxin derived from the venom of the scorpion *Androctonus australis*; $AaIT_5$, insect toxin derived from the venom of the scorpion *Androctonus australis*; $BaIT_2$, a depressant insect-selective toxin derived from the venom of the scorpion *Buthacus arenicola*; $BotIT_2$, toxin derived from the venom of the scorpion *Buthus occitanus tunetanus*; CPU, dose that causes a contractile paralysis to 50% of the animals; FPU, dose that causes a flaccid paralysis to 50% of the animals; LqhαIT, α insect toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; $LqhIT_2$, depressant insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; $LqhIT_5$, excitatory insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; LqhIV, toxin derived from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; $LqqIT_1$, excitatory insect-selective toxin derived from the venom of the scorpion *Leuirus quinquestriatus quinquestriatus*; $LqqIT_2$, depressant insect-selective toxin derived from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus*; LqqIII, LqqIV, α mammalian toxins derived from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus*.

Note. The novel amino acid sequence mentioned in this paper has been deposited with the Protein Identification Resource and European Bioinformatics Institute (EBI) data bases and are available under accession numbers A59006 and P81240, respectively.

into larvae of the blow fly. Moreover, this toxin showed high toxicity to larvae of *Lepidoptera* but very low toxicity to blow fly larvae [10].

We report here the purification and characterization of a new excitatory insect-selective toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus* ($LqhIT_5$). This toxin is similar to the excitatory toxins in its action and similar to the depressant toxins in its primary structure. $LqhIT_5$ is 61 amino acids and has high sequence similarity to the known depressant insect-selective toxins but does not show the depressant activity of flaccid paralysis. In the present study, the importance of the change in the mode of action without changing the selectivity of the toxin towards insects is discussed.

EXPERIMENTAL PROCEDURES

Venom. Venom of the scorpion *Leiurus quinquestriatus hebraeus* was obtained from Sigma Chemical Co.

Test animals. Larvae of the blow fly *Sarcophaga falculata* were bred in our laboratory as previously described [11]. Swiss Webster mice (three weeks of age) were obtained from Charles River and were housed according to approved animal care protocols.

Bioassays. For the data reported in this study, the toxicity of $LqhIT_5$ was determined on the material following microbore HPLC purification and its purity evaluated by other analytical methods including capillary electrophoresis, isoelctric focusing, mass spectrometry and a single amino acid in the first cycle of amino acid sequencing. Three replicates of 25−40 blow fly (*S. falculata*) larvae each were injected with each of the toxins. The dose causing contractile paralysis in 50% of the animals (CPU) was determined (using the method developed by Reed and Muench [12]) as that needed for a fast contraction paralysis 1-min post-injection of the toxin into an abdominal intersegmental membrane, and the dose causing a flaccid paralysis in 50% of the animals (FPU) was determined as that needed for a progressive flaccid paralysis 5-min post-injection. The symptoms induced by depressant insect-selective toxin derived from the venom of *L. quinquestriatus quinquestriatus* ($LqhIT_2$) and excitatory insect toxin derived from the venom of *Androctonus australis* (AaIT) were used for comparison with a standard depressant and standard excitatory toxin, respectively. The toxicity towards mice was evaluated 24 hours after subcutaneous or intracranial injection of the toxin. Mice were monitored for symptoms throughout the 24-hour period.

Column chromatography. Lyophilized venom (50 mg) of *L. quinquestriatus hebraeus* was homogenized in 2 ml 10 mM ammonium acetate, pH 6.4, and the insoluble material removed by centrifugation at 27000 *g* for 20 min. The supernatant was collected and the pellet was resuspended in 2 ml 10 mM ammonium acetate, pH 6.4, homogenized and centrifuged again. This extraction was repeated four times to maximize the yield of protein extracted from the venom. The supernatant from all the extractions was loaded on a low-pressure cation-exchange column (10 ml), using CM-52 cellulose (Whatman) equilibrated in 10 mM ammonium acetate, pH 6.4, at a flow rate of 10 ml/hour. The fraction that did not bind to the cation-exchange column was further fractionated by HPLC (Perkin Elmer Series 410 pump) reverse-phase chromatography using a Vydac C_4 column (4.6 mm×250 mm). The column was equilibrated in 5% acetonitrile containing 0.1% trifluoroacetic acid (buffer A), and the fraction was loaded onto the column and eluted with a linear gradient of 0−60% buffer B for 70 min at a flow rate of 0.6 ml/min. Buffer B was 95% acetonitrile containing 0.1% trifluoroacetic acid. The fractions were monitored at 214 nm on a Perkin Elmer diode array detector which was controlled by Perkin Elmer Omega-4 software. The final step of purification was carried out using a Reliasil C_{18} reverse-phase column on a microbore HPLC (Michrom Bioresources Inc.). The column was equilibrated in buffer A. The toxic fraction (as determined by injection into blow fly larvae) that was obtained by the reverse-phase C_4 chromatography was loaded onto the C_{18} column and eluted in a linear gradient of 0−60% buffer B over 60 min at a flow rate of 50 µl/min. Absorbency was monitored at 214 nm and peaks were collected accordingly.

Purity determination. Following two HPLC steps, the homogeneity and purity of the toxin was evaluated by several techniques including free solution capillary electrophoresis (Applied Biosystems model 270A). The 75-cm uncoated fused-silica capillary was equilibrated for 4 min with 20 mM sodium citrate, pH 2.9, and the toxin (0.2 mg/ml) was loaded using vacuum for 2 s. The running buffer was 20 mM sodium citrate, pH 2.9, the electric potential was 20 kV and the absorbance was monitored at 214 nm. Peak areas were integrated. The purity of the toxin was also evaluated by analytical isoelectric focusing (IEF) utilizing cast polyacrylamide gels (Ampholine PAGplate pH 3.5−9.5; Pharmacia Biotech) and pI markers in the range 3.5−9.3 (Pharmacia Biotech). Proteins were visualized by staining with Coomassie brilliant blue G-250 [13].

Sequence determination. $LqhIT_5$ was reduced and alkylated by incubation in 6 M guanidine hydrochloride, 0.1 M Tris/HCl, pH 8.3, containing 1 mM EDTA and 20 mM dithiothreitol for 1 hour at 37°C. Iodoacetic acid was added to a final concentration of 50 mM and incubated for an additional 1 hour at 37°C in the dark. The N-terminal sequence of the reduced and alkylated toxin was determined using a HP GS1000 sequence analyzer by automated Edman degradation (in the Protein Structure Lab at U. C. Davis, USA).

Mass spectrometry. The molecular mass of $LqhIT_5$ was determined using electrospray mass spectrometry on a VG/Fisons Quattro-BQ mass spectrometer (VG Biotech). An Isco µLC-500 syringe pump delivered the mobile phase (acetonitrile/water, 50:50, by vol.) at 5 µl/min. Solutions of purified peptides were analyzed by direct flow injection using an injection volume of 10 µl. Spectra were obtained in positive-ion mode using a capillary voltage of + 3.5 kV, a cone voltage of 50 V and a source temperature of 65°C. Spectra were scanned over the range 500−1500 *m/z* at a rate of 20 s/scan; 20 scans were combined using the VG MCA acquisition mode. Molecular masses were determined using the maximum entropy deconvolution algorithm (MaxEnt) to transform the *m/z* range 650−1500 to give a true mass scale spectrum. Mass calibration was performed using horse heart myoglobin (Sigma Chemical Co.).

Homology modeling. The coordinates for the three-dimensional structure of depressant insect-selective toxin derived from the venom of *L. quinquestriatus hebraeus* ($LqhIT_2$) were obtained from Swiss-Model Protein Modeling Server [14−16]. The amino acid sequence of $LqhIT_2$ was submitted and the coordinates of the structure of the amino acids 4−60 were obtained as a PDB file. The structure of this molecule was visualized and plotted using the software written by Roger Sayle, RasMol Molecular PDB Visualization Software.

Protein determination. The amount of protein was determined using the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard.

RESULTS

Purification of $LqhIT_5$. The crude venom (50 mg protein) of *L. quinquestriatus hebraeus* was loaded onto a 10-ml cation-

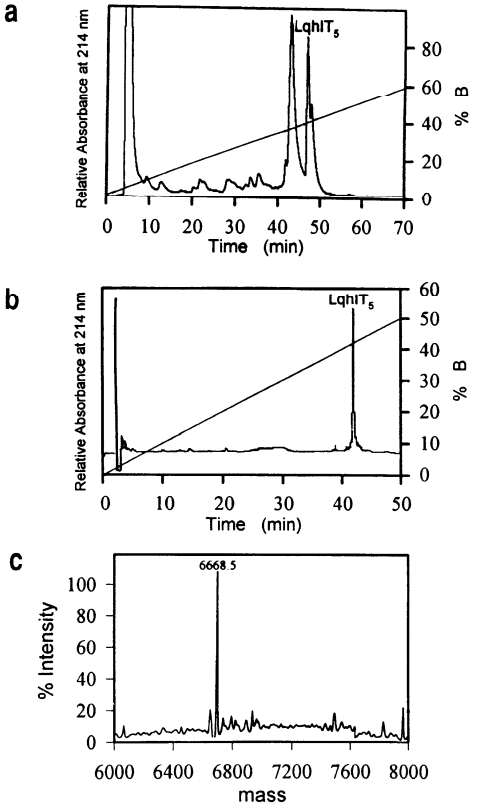

Fig. 1. Purification and mass determination of LqhIT$_5$. The purification of LqhIT$_5$ was carried out as follows. The non-binding fraction from the ion-exchange column (1.4 mg protein) was further purified by a reverse-phase C_4 column (Vydac) using HPLC (a) as described in the Experimental Procedures. The big peak that eluted from the column before LqhIT$_5$ was identified as the depressant insect toxin LqhIT$_2$ by several analytical methods such as identification with specific antibodies, electrospray ionization mass spectrometry corresponded to a mass of 6576 Da, IEF resulted in a pI value of 6.7, comobility with pure LqhIT$_2$ on reverse-phase HPLC on a Vydac C_4 column as well as symptomology when injected to blow fly larvae (data not shown). The last step of purification of LqhIT$_5$ was carried out on a microbore HPLC (Michrom Bioresources Inc.) utilizing a reverse-phase C_{18} column (b) as described in the Experimental Procedures. The molecular mass of LqhIT$_5$ (c) was determined on a VG/Fisons Quattro-BQ mass spectrometer (VG Biotech) using the maximum entropy deconvolution algorithm (MaxEnt) to transform the range *m/z* 650–1500 to give a true mass scale spectrum. Mass calibration was performed using horse heart myoglobin (Sigma Chemical Co.).

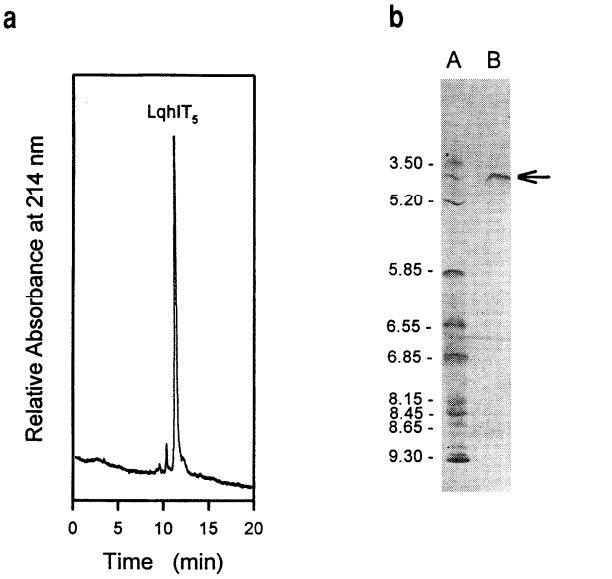

Fig. 2. Purity determination of LqhIT$_5$. The purity of LqhIT$_5$ was evaluated by free-solution capillary electrophoresis and by isoelectric focusing. Capillary electrophoresis (a). LqhIT$_5$ (0.2 mg/ml) was loaded onto a free-solution capillary column equilibrated in 20 mM sodium citrate, pH 2.9 by vacuum for 2 s. The sample was eluted from the capillary using an electric force of 20 kV and absorbance was monitored at 214 nm (a). Isoelectric focusing (b). Proteins were focused on a cast polyacrylamide gels (Ampholine PAGplate pH 3.5–9.5; Pharmacia Biotech Uppsala, Sweden). Broad-range pI markers (Pharmacia) (A) and LqhIT$_5$ (B) were loaded onto the gel and were visualized by staining with Coomassie brilliant blue G-250 [13]. The calculated pI value of LqhIT$_5$ corresponded to 3.75.

exchange column (CM-52 cellulose) and the proteins were eluted from the column. Absorbance was monitored at 280 nm and peaks collected. The toxic fraction which did not bind to the column (2 mg protein) was loaded directly on a Vydac C_4 reverse-phase HPLC column. Absorbance was monitored at 214 nm and peaks were collected accordingly (Fig. 1a). The fraction designated as LqhIT$_5$ (0.25 mg protein) was further purified on a Reliasil C_{18} column using a microbore HPLC (Fig. 1b). The single peak accounted for >97% of the absorbing material from the column and no other peak accounted for >1% of the total absorbance detected. The purity and homogeneity of LqhIT$_5$ was further evaluated using a free-solution capillary electrophoresis system which resulted in a major peak accounting for >95% of the absorbing material and showing no other significant peaks (Fig. 2a), and analytical IEF resulted in a single band (Fig. 2b). A balance study indicated that the amount of purified LqhIT$_5$ corresponded to 0.5% of the proteins in the crude venom of the scorpion. The toxicity of LqhIT$_5$ was tested by injection into blow fly larvae and mice. The amino acid sequence of LqhIT$_5$ was determined. Only a single amino acid was detected during the first cycle of Edman degradation.

Primary structure determination. The amino acid sequence of LqhIT$_5$ (Tables 1 and 2) was determined in two steps. The N-terminus (60 residues) of the reduced and alkylated toxin was determined using an HP G1000 sequence analyzer. The determination of glycine at the C-terminus was carried out utilizing electrospray ionization mass spectrometry and sequence comparison to known toxins. The major peak in the MaxEnt-transformed electrospray mass spectrum of purified LqhIT$_5$ corresponded to a molecular mass of 6668.5 Da (Fig. 1c). The result showed good agreement with the molecular mass calculated from the amino acid sequencing (6677.11 Da theoretical) assuming that four disulfide bridges are present in the native form of the peptide as it occurs in other scorpion toxins with a similar mass [17], and a glycine at the C-terminus of the toxin. This assumption is supported by the 8-Da difference between the expected mass from sequence analysis (including the glycine residue and eight thiol groups) and the mass obtained from the electrospray ionization mass spectrometry of the native form of LqhIT$_5$ (with four disulfide bonds). It also is supported by the difference between the expected pI (4.39) from the amino acid composition (using Prosis analysis) and the observed pI (3.75) on analytical IEF (Fig. 2b) as anticipated from four disulfide bonds.

Toxicity of LqhIT$_5$. LqhIT$_5$ did not show any toxicity or noticeable symptoms when injected either subcutaneously (9 μg/20 g

Table 1. Comparison of scorpion toxin amino acid sequences. Amino acids thought to confer depressant symptoms are shown in bold. $BotIT_5$, depressant insect-selective toxin derived from the venom of *Buthus occitanus tunetanus*.

Toxin		Amino acid position							
		1	10	20	30	40	50	60	70
Depressant toxins	$LqhIT_2$	DGYIKR**R**DGC K**VACLIGNEG** CD**K**ECKA**Y**GG SYGYCW**T**WGL ACWCEGLPDD K**T**WKSETNTC G							
	$LqqIT_2$	DGYIRK**R**DGC K**LSCLFGNEG** CN**K**LCKS**Y**GG SYGYCW**T**WGL ACWCEGLPDE K**T**WKSETNTC G							
	$BaIT_2$	DGYIRR**R**DGC K**VSCLFGNEG** CD**K**ECKA**Y**GG SYGYCW**T**WGL ACWCEGLPDD K**T**WKSETNTC G							
	$BotIT_5$	DGYIRK**R**DGC K**VSCLFGNEG** CD**K**ECKA**Y**GG SYGYCW**T**WGL ACWCEGLPDD K**T**WKSETNTC G							
Depressant toxin analogs	$BotIT_2$	DGYIKGYKGC KITCVINDDY CDTECKAEGG TYGYCWKWGL ACWCEDLPED KRWKPETNTC							
	$AaIT_5$	DGYIKRHDGC KVTCLINDNY CDTECKREGG SYGYCYSVGF ACWCEGLPDD KAWKSETNTC D							
	$LqhIT_5$	DGYIRGGDGC KVSCVIDHVF CDNECKAAGG SYGYCWGWGL ACWCEGLPAD REWKYETNTC G							
Excitatory toxins	AaIT	KKNGYAVDSSG KAPECLLSNY CNNQCTKVHYA DKGYCCL-L SCYCFGLNDDK KVLEISDTRKSYCDT TIIN							
	$LqqIT_1$	KKNGYAVDSSG KAPECLLSNY CYNECTKVHYA DKGYCCL-L SCYCVGLSDDK KVLEISDARKKYCDF VTIN							

Table 2. Similarity of scorpion toxins. The similarity was calculated using the computer software Hibio Prosis™ (Hitachi Software Engineering Co. Ltd) which compares identity between pairs of amino acids. $BotIT_5$, depressant insect-selective toxin derived from the venom of *B. occitanus tunetanus*.

Toxin	Similarity of								
	$LqhIT_2$	$LqqIT_2$	$BaIT_2$	$BotIt_5$	$LqhIT_5$	$BotIT_2$	$AaIT_5$	AaIT	$LqqIT_1$
	%								
$LqhIT_2$	100	85	95	95	74	69	75	36	34
$LqqIT_2$		100	90	91	66	60	60	35	32
$BaIT_2$			100	98	72	66	72	35	34
$BotIT_5$				100	72	66	73	35	34
$LqhIT_5$					100	69	66	31	32
$BotIT_2$						100	72	30	32
$AaIT_5$							100	34	34
AaIT								100	87
$LqqIT_1$									100

Table 3. Toxicity towards larvae of blow fly (*S. falculata*). Data for $LqhIT_2$ are from [5].

Toxin	CPU	FPU
	ng/100 ml larvae	
AaIT	2.5	–[a]
$LqhIT_2$	50	50
$LqhIT_5$	7.0	–[a]

[a] Flaccid paralysis was not detected when a high doses of 0.5 mg toxin or serial dilutions down to 1 ng were injected into 100 mg larvae.

mouse) or intracranially (4 μg/20 g mouse) into mice. $LqhIT_5$ had an excitatory effect with high toxicity when injected into blow fly larvae (7 ng/100 mg larvae). This toxicity is comparable to the toxicity of the excitatory insect toxin AaIT (Table 3) and, unlike the depressant toxin $LqhIT_2$, $LqhIT_5$ did not cause a flaccid paralysis when injected into blow fly larvae (Table 3) 5 min post injection.

DISCUSSION

The present study reports the purification and characterization of a new insect-selective excitatory toxin. This toxin ($LqhIT_5$), derived from the venom of the Israeli yellow scorpion *L. quinqestriatus hebraeus,* showed a high sequence similarity to the known depressant toxins (75% identity to $LqhIT_2$). However, unlike the depressant insect-selective toxins, $LqhIT_5$ elicited classical excitatory paralysis when injected into insects with no flaccid paralysis detected upon its injection. $LqhIT_5$ is an acidic protein with pI value of 3.75, that is highly potent toward insects (7 ng/100 mg larvae of blow fly) but shows no toxicity when injected into mice (4 μg/20 g mouse by intracranial injection).

Previous studies revealed that insect-selective toxins can be divided into two categories, the excitatory and the depressant toxins. Those toxins were shown to share a high degree of sequence similarity within each category (i.e. excitatory toxins are 80–90% identical and depressant toxins are 80–90% identical). However, a low amount of similarity exists (30–35% identity) between the excitatory and the depressant toxins. Recently, Nakagawa et al. [10] characterized a depressant toxin analog by sequence ($AaIT_5$) with no depressant or excitatory activity and a very low toxicity toward larvae of blow fly. In addition, Borchani et al. [9] reported purification of a new toxin from the venom of *Buthus occitanus tunetanus* ($BotIT_2$). The amino acid sequence of this toxin shows high similarity to the amino acid sequence of the depressant toxins. However, due to the fact that this toxin demonstrated an excitatory activity in electrophysiological studies and mammalian toxicity upon injection into mice, $BotIT_2$ was not characterized as an insect selective toxin. $BotIT_2$ as well as the depressant insect toxins were shown to affect the sodium channels in electrophysiological studies [5, 8, 9].

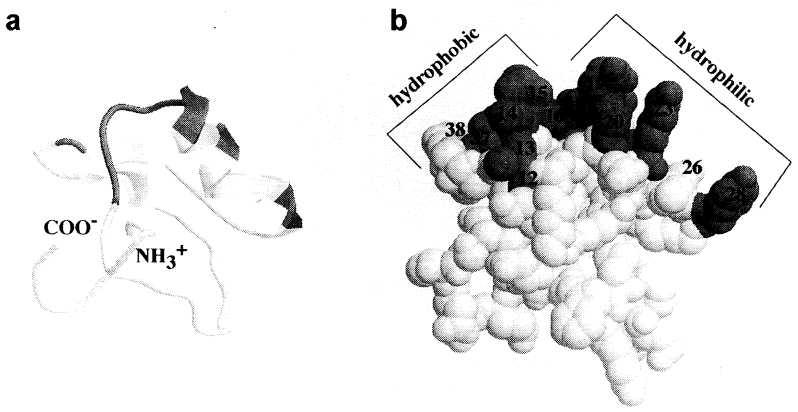

Fig. 3. Three-dimensional structure of the amino acids located at positions 4–60 of LqhIT$_2$. The amino acid sequence of the toxin LqhIT$_2$ was submitted to the Swiss-Model Protein Modeling Server and the coordinates for the three-dimensional structure were obtained. The molecule was visualized using the RasMol program and is displayed in the same orientation of the molecule in (a) and (b). (a) Carbon trace of the molecule which demonstrates the basic scaffold that is composed of an α helix, two β strands (plotted as arrows) and the loops that connect them. This basic scaffold of the molecule is common to all scorpion toxins, even those with different activities (see Discussion). Therefore, this tertiary structure of LqhIT$_2$ is similar to the tertiary structure of the depressant toxins analog LqhIT$_5$ which has 75% similarity to LqhIT$_2$ (Table 1). The N-terminus and C-terminus of the peptide (a) are indicated by NH_3^+ and COO^-, respectively. (b) Predicted orientation of the amino acids that distinguish between the depressant toxins (such as LqhIT$_2$) and the depressant toxin analogs (such as LqhIT$_5$). These amino acids are thought to be important to the depressant activity of the flaccid paralysis of the toxin (in dark gray and numbered corresponding to the sequence of the toxin in Table 1, bold) are exposed to a certain orientation of the environment and might affect a specific location on the insect sodium channel causing the flaccid paralysis effect.

The binding of the long chain scorpion toxins to the sodium channels was shown to involve multi-site attachments. The insect-selective toxins bind in close proximity on the sodium channels [18] and the depressant toxins are able to displace the excitatory toxins from their binding sites [19]. However, the depressant toxins do not displace the α insect toxins from their target sites on the sodium channels [20]. Interestingly, analysis of the three-dimensional structure of scorpion toxins affecting different target sites and having diverse mode of action (AaH_{II} [21], Var3 [22], AaIT [23], charybdotoxin [24], PO5-NH_2 [25] and chlorotoxin [26]) reveal that they share the same basic scaffolding in the molecular structure. The toxins are compact molecules with a defined core consisting of an α helix, three anti-parallel β strands, and several loops connecting them. Scorpion toxins with very high sequence identity (95%) and structure similarities can show a different insect/mammalian toxicity ratio. For example, α insect toxin derived from the venom of *L. quinquestriatus hebraeus* (LqhαIT) [27] differs in three amino acids from α mammalian toxins derived from the venom of *L. quinquestriatus quinquestriatus* (LqqIII) [28] and is a more potent insect toxin than the latter. In addition, the toxin derived from the venom of *L. quinquestriatus quinquestriatus* (LqhIV) differs in two amino acids from LqqIV [29] and has a higher insect toxicity than LqqIV (Herrmann, R., unpublished results).

Sequence comparison of depressant and depressant-analog scorpion toxins shows a high degree of similarity among the amino acids located at the C-terminus of the toxins. However, there are some clear differences with those amino acids thought to confer depressant symptoms (Tables 1 and 2). These amino acids include the arginine residue at position 7, lysine at position 23, tyrosine at position 28, thyreonine at position 37, threonine at position 52 and a sequence of amino acids that are facing the same orientation in the tertiary structure of the molecule and include the amino acids located at residues 12−20, the lysine residue at position 23, the tyrosine residue at position 28 and the threonine residue in positions 37. These residues are different in the depressant analog group of toxins. A homology model, based on the solution NMR structures of Var3 [22] and Var1 [30], of a typical depressant toxin (LqhIT$_2$) has a variable region at residues 12-20 and several other key amino acids though to result in flaccid paralysis (Fig. 3 a and b). Moreover, these amino acids demonstrate a unique hydrophobicity pattern in the tertiary structure of the molecule with a strict discrepancy between a highly hydrophobic region (amino acids 12−17, 37 and Trp38) and a highly hydrophilic region (amino acids 18, 19, 23, 28 and the lysine residue at position 26). The mammalian toxicity of BotIT$_2$ can be related to the lysine residued located in positions 8 and 37 and the arginine residue at position 53, which differ from all other depressant and depressant-analog insect toxins.

The results of this study and the structural information described above suggest that the core structure of the scorpion toxin molecules that include the α helix and the three β strands serve as a scaffold to anchor specific amino acids at the correct orientation in three-dimensional space (Fig. 3). These amino acids and not the length of the chain appear to determine the insect or mammalian selectivities and whether the mode of action of the toxin will be excitatory or depressant. Our results with toxins from *L. quinquestriatus* caution that minor changes in specific regions of the toxin can lead to major changes in pharmacology so that groups of toxins based on length or apparent identity in sequence may not necessarily reflect the biological effects of the toxins.

Insect-selective toxins are used as pharmacological tools for the characterization of the insect sodium channel and as potency enhancing factors when cloned into baculoviruses (as insect-selective biocontrol agents). LqhIT$_5$ could serve as a tool for the clarification and identification of structures responsible for the animal group selectivity and the mode of action of scorpion toxins. The comparison of changes of the three-dimensional structure of the depressant and depressant-analog toxins could explain the selectivity of the toxins (when compared to BotIT$_2$) and the depressant or excitatory mode of action of the toxins. The identification of structures or sequences responsible for in-

sect selectivity may lead to the rational design of highly selective synthetic insecticides.

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REFERENCES

1. Carbone, E., Wanke, E., Prestipino, G., Possani, L. D. & Maelicke, A. (1982) Selective blockage of voltage-dependent K^+ channels by a novel scorpion toxin, *Nature 296*, 90–91.
2. Miller, C., Moczydlowski, E., Latorre, R. & Philips, M. (1985) Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle, *Nature 313,* 316–318.
3. Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietschoten, J. & Rochat, H. (1982) Covalent structure of the insect toxin of the North African scorpion *Androctonus australis* Hector, *Int. J. Pept. Prot. Res. 20*, 320–330.
4. Rochat, H., Bernard, P. & Couraud, F. (1979) Scorpion toxins: chemistry and mode of action, *Adv. Cytopharmacol. 3*, 325–334.
5. Zlotkin, E., Eitan, M., Bindokas, V. P., Adams, M. E., Moyer, M., Burkhart, W. & Fowler, E. (1991) Functional duality and structural uniqueness of depressant insect-selective neurotoxins, *Biochemistry 30*, 4814–4821.
6. Couraud, F. & Jover, E. (1984) *Mechanism of action of scorpion toxins*, vol. 2, Marcel Dekker, New York.
7. Pelhate, M. & Zlotkin, E. (1982) Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on isolated giant axons of the cockroach (Periplaneta americana), *J. Exp. Biol. 97*, 67–77.
8. Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F. & Rochat, H. (1985) An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site, *Arch. Biochem. Biophys. 240*, 877–887.
9. Borchani, L., Mansuelle, P., Stankiewicz, M., Grolleau, F., Cestele, S., Karoui, H., Lapied, B., Rochat, H., Pelhate, M. & El Ayeb, M. (1996) A new scorpion venom toxin paralytic to insects that affects Na^+ channel activation. Purification, structure, antigenicity and mode of action, *Eur. J. Biochem. 241*, 525–532.
10. Nakagawa, Y., Lee, Y., Lehmberg, E., Herrmann, R., Herrmann, R., Moskowitz, H., Jones, A. D. & Hammock, B. D. (1997) New anti-insect toxin (AaIT5) from *Androctonus australis, Eur. J. Biochem. 246,* 496–501.
11. Zlotkin, E., Fraenkel, G., Miranda, F. & Lissitzky, S. (1971) The effect of scorpion venom on blowfly larvae – a new method for the evaluation of scorpion venoms potency, *Toxicon 9*, 1–8.
12. Reed, L. J. & Muench, S. (1938) A simple method of estimating fifty percent end point, *Am. J. Hyg. 27*, 493–497.
13. Righetti, P. G. & Chillemi, F. (1978) Isoelectricfocusing of peptides, *J. Chromatogr. 157*, 243–251.
14. Peitsch, M. C. (1995) Protein modeling by E-Mail, *Biotechnology 13*, 723–723.
15. Peitsch, M. C. (1996) ProMod and Swiss-Model: Internet-based for automated comparative protein modelling, *Biochem. Soc. Trans. 24*, 274–279.
16. Peitsch, M. C. & Jongeneel, C. V. (1993) A 3-D model for the Cd40 ligand predicts that it is a compact trimer similar to the tumor necrosis factors, *Int. Immunol. 5*, 233–238.
17. Possani, L. D. (1984) Structure of scorpion toxins, in *Handbook of natural toxins* (Tu, A., ed.) pp. 513–550, M. Dekker, New York.
18. Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W. A. & Zlotkin, E. (1992) Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies, *Biochemistry 31*, 7622–7628.
19. Moskowitz, H., Herrmann, R., Zlotkin, E. & Gordon, D. (1994) Variability among insect sodium channels revealed by selective neurotoxins, *Insect. Biochem. Molec. Biol. 24*, 13–19.
20. Gordon, D. & Zlotkin, E. (1993) Binding of an alpha scorpion toxin to insect sodium channels is not dependent on membrane potential, *FEBS Lett. 315*, 125–128.
21. Housset, D., Habersetzer-Rochat, C., Astier, J. P. & Fontecilla-Camps, J. C. (1994) Crystal structure of toxin II from the scorpion *Androctonus australis* Hector refined at 1.3 A resolution, *J. Mol. Biol. 238*, 88–103.
22. Zhao, B., Carson, M., Ealick, S. E. & Bugg, C. E. (1992)) Structure of scorpion toxin Variant-3 at 1.2 A resolution, *J. Mol. Biol. 227*, 239–252.
23. Darbon, H., Weber, C. & Braun, W. (1991) Two-dimensional 1H nuclear magnetic resonance study of AaH IT, an anti-insect toxin from the scorpion *Androctonus australis* Hector. Sequential resonance assignments and folding of the polypeptide chain, *Biochemistry 30*, 1836–1845.
24. Bontems, F., Roumestand, C., Gilquin, B., Menez, A. & Toma, F. (1991) Refined structure of Charybdotoxin: common motifs in scorpion toxins and insect defensins, *Science 254*, 1521–1523.
25. Meunier, S., Bernassau, J. M., Sabatier, J. M., Martin-Eauclaire, M. F., Van Rietschoten, J., Cambillau, C. & Darbon, H. (1993) Solution structure of P05-NH2, a scorpion toxin analog with high affinity for the apamin-sensitive potassium channel, *Biochemistry 32*, 11969–11976.
26. Lippens, G., Najib, J., Wodak, S. J. & Tartar, A. (1995) NMR sequential assignments and solution structure of Chlorotoxin, a small scorpion toxin that blocks chloride channels *Biochemistry 34*, 13–21.
27. Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M. & Zlotkin, E. (1990) A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation – purification, primary structure, and mode of action, *Biochemistry 29*, 5941–5947.
28. Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M. F., Rochat, H. & Miranda, F. (1993) Characterization of toxin III of the scorpion *Leiurus quinquestriatus quinquestriatus*: a new type of alpha-toxin highly toxic both to mammals and insects, *Natural Toxins 1*, 308–312.
29. Kopeyan, C., Martinez, G. & Rochat, H. (1985) Primary structure of toxin IV of *Leiurus quinqestriatus quinqestriatus*. Characterization of a new group of scorpion toxins, *FEBS Lett. 181*, 211–217.
30. Lee, W., Jablonsky, M. J., Watt, D. & Krishna, N. R. (1994) Proton Nuclear Magnetic Resonance geometry/simulated annealing studies on the Variant-1 neurotoxin from the new world scorpion *Centruroides sculpturatus Ewing*. *Biochemistry 33*, 2468–2475.
31. Cestele, S., Kopeyan, C., Oughideni, R., Mansuelle, P., Granier, C. & Rochat, H. (1997) Biochemical and pharmacological characterization of a depressant insect toxin from the venom of the scorpion *Buthacus arenicola, Eur. J. Biochem. 243*, 93–99.
32. Borchani, L., Stankiewicz, M., Kopeyan, C., Mansuelle, P., Kharrat, R., Cestele, S., Karoui, H., Rochat, H., Pelhate, M. & Ayeb, M. E. (1997) Purification, structure and activity of three insect toxins from *Buthus occitanus tunetanus* venom, *Toxicon 35*, 365–382.
33. Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Bahraoui, E. M., Rochat, H. & Granier, C. (1990) Primary structure of scorpion anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus, FEBS Lett. 261*, 423–426.